

Luminal decoration of blood vessels by activated perivascular mast cells in allergic rhinitis

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Abstract

Background: In allergic diseases, like in rhinitis, antigen challenge induces rapid degranulation of tissue resident mast cells and subsequent recruitment of leukocytes in response to soluble immunomodulators. The fate of mast cell-derived, membrane associated factors in inflamed tissue remained however unresolved.

Methods: Components of the mast cell granular membrane, including the unique marker CD63^{var}, were examined by FACS and by confocal laser scanning microscopy in cell culture and in diseased human tissue.

Results: We discovered that selected mast cell membrane components appeared on the surface of distinct bystander cells. Acceptor cells did not acquire these molecules simply by uptake of soluble material or in the form of exosomes. Instead, physically stable cell-to-cell contact was required for transfer, in which a Notch2-Jagged1 interaction played a decisive role. This process is activation-dependent, unidirectional, and involves a unique membrane topology. Endothelial cells were particularly efficient acceptors. In organotypic 3D *in vitro* cultures we found that transferred mast cell molecules traversed an endothelial monolayer, and reappeared focally compacted on its distal surface, away from the actual contact zone. Moreover, we observed that such mast cell-derived membrane patches decorate microcapillaries in the nasal mucosa of allergic rhinitis patients.

Conclusion: Direct membrane transfer from perivascular mast cells into nearby blood vessels constitutes a novel mechanism to modulate endothelial surface features with apparent significance in allergic diseases.

Key finding

Mast cells transfer selected granular membrane patches onto neighboring cells and cross-decorate the luminal surface of endothelium in allergic rhinitis patients.

Introduction

Mast cells are specialized secretory immune cells and a major source of active substances, which can attract and co-stimulate other immune cells, promote tissue remodeling and vasodilatation at infectious sites, mediate pain and itching sensation, and contribute to the primary defense line against pathogens and parasites at mucosal and epithelial interfaces (1). Mast cell cargo remains encapsulated within specialized secretory granules until the cell gets activated by external stimuli. The most potent natural degranulation trig-

ger is antigen-induced cross-linking of IgE molecules that are themselves bound by the high-affinity FcεRI receptor on the surface of the mast cell. This interaction launches an intracellular signaling cascade, which in a Ca²⁺-dependent manner induces massive reorganization of plasma and granular membranes (2–4). For the granular cargo, there are apparently multiple ways to reach their destination. The classic paradigm is that as a consequence of plasma/granular membrane fusion, the granular content is released into the immediate surroundings of the mast cells and exerts local effects. As a possible alternative, also the secretion of entire intact mast cell granuli has been reported (5, 6). These vesicle-like particles can utilize the lymphatic system for long-distance travel and may even reach remotely located lymph nodes (7). We herein describe the discovery of a hitherto unrecognized additional mode of mast cell signal transfer.

By characterizing molecules involved in degranulation-related changes on the mast cell surface, we have recently identified a novel isoform of CD63 (8). This glycosylation variant, termed CD63*var*, is highly specific for human mast cells but is absent from other cells, even if they express CD63. Moreover, the epitope is naturally buried within resting granuli and becomes surface exposed only with degranulation. Antibodies specific for this structural hallmark, like NIBR63/1, thus provide excellent mast cells degranulation markers. CD63 itself belongs to the tetraspanin superfamily (TM4SF) that comprises various members in different species (32 in mammals, 35 or more in drosophila, 21 in worm) (9). These proteins are not well established as ligands or receptors on their own, but rather direct the assembly of associated membrane proteins into functionally important complexes, so-called tetraspanin-enriched microdomains (TEM) (10). Tetraspanins were shown to be required for successful mammalian fertilization, regulate neuronal-astrocyte interactions in brain, facilitate neuromuscular synapse formation in *Drosophila* embryos, and co-regulate integrin-dependent cell migration by strengthening adhesion (11). CD63 in particular is known to associate with integrins, syntenin-1, and membrane metalloproteases and to promote lysosomal targeting of synaptotagmin VII (12, 13). Cell adhesion, cell motility, and phagocytosis/endocytosis are thus influenced by CD63 (14), yet an explicit intercellular contact via CD63 has not been described.

In following the fate of CD63*var* in postdegranulation human mast cells, we now discovered that mast cells can directly transfer patches of their granular membrane, characterized by the CD63*var* protein and associated TEM components, onto the surface of selected bystander cells. Transferred molecules traverse the acceptor cell and reappear focally compacted preferentially on its distal surface, away from the actual contact zone. Exemplifying the *in vivo* relevance of this finding, we observed perivascular mast cells to actively alter the luminal surface of nearby blood vessels in nasal mucosa derived from allergic rhinitis patients. We thus suggest that this new signaling process enables individual mast cells to communicate a local antigen challenge to circulating blood cells across an endothelial barrier.

Methods

Cell culture

Human mast cells were differentiated from cord blood-derived progenitor cells (15–17). In brief, stem cells were isolated from heparinized human cord blood by gradient centrifugation and magnetic enrichment for CD133-positive cells (CD133 Micro Bead kit; Miltenyi Biotech, #130-050-801, Bergisch Gladbach, Germany). Cells were propagated at 37°C and 7.5% CO₂ in serum-free expansion medium (Stem Cell Technologies, #09650, Vancouver, BC, Canada) supplemented with antibiotics and a cytokine cocktail consisting of 100 ng/ml rhSCF, 100 ng/ml rhIL-6, and 30 ng/ml rhIL-3 (PeproTech EC Ltd., London, UK) for 3 weeks. Finally, cultures were shifted to RPMI1640 medium supplemented with

10% FBS, antibiotics, 100 ng/ml SCF, and 100 ng/ml IL-6 for at least another 6 weeks before degranulation experiments were performed. Human dermal microvascular endothelial cells (HDMEC; passage <10) were seeded onto BD cell culture inserts of 0.3-, 1.0-, 3.0-, and 8.0-μm pore size and propagated to confluence in endothelial cell growth medium with supplements (PromoCell GmbH, Heidelberg, Germany). Further cell types investigated in epitope transfer experiments and/or used for RNA expression profiling are the commercially available B-cell lymphomas BJAB, LA350, Ri-1, U266, proprietary EBV-transformed B-LCLs AB4, and CBF4:2; T-cell leukemia lines CEM, HPB-ALL, HUT78, Jurkat, MOLT4, and Peer; NK92 natural killer cells; erythroleukemia cell lines HEL, K562, and TF-1; and pro-monocytic U937 cells.

Epitope transfer experiments

To distinguish donor from acceptor populations, either mast cells or the respective acceptor lines were labeled with CFSE. Cells were mixed at a cellular ratio of 1 : 1 and incubated for 1.5 h at 37°C and 5% CO₂ to allow cell contacts to form. In case of filter-based assays, endothelial cells were propagated to confluence and 10⁶ mast cells sedimented onto the opposing side of the membrane. Degranulation was induced either with 25 ng/ml PMA and 500 ng/ml ionomycin, or alternatively, mast cells had been coated with 5 μg/ml IgE antibody B11 and 25 ng/ml IL-4 over night, and degranulation was induced by the addition of 5 μg/ml cross-linking antibody Le27. Samples were then shifted to 4°C, stained with the indicated antibodies, and analyzed on a BD FACSCalibur workstation or by fluorescence microscopy.

Microscopy

Co-cultures of mast cell and U937-eGFP were stained with antibody NIBR63/1, mounted in Mowiol (Plüss-Stauffer), and analyzed on an Axiovert 200M microscope (Carl Zeiss AG, Oberkochen, Germany). Filter membranes carrying monolayer endothelial cells on one side and mast cells on the other were fixed (4% formalin), stained with indicated antibodies, and analyzed on a Leica SP5 LSM (Leica Microsystems GmbH, Wetzlar, Germany). Mucosal tissue was formalin-fixed and stained with hematoxylin and anti human β-tryptase (Dako Cytomation, M7052 & K0670, Glostrup, Denmark), or cryo-preserved and stained with DAPI, anti CD31 (BD, 558068), and Alexa546-conjugated NIBR63/1 (Zenon; Invitrogen, Carlsbad, CA, USA). Data were recorded on an inverted confocal laser scanning microscope (LSM710; Zeiss) and 3D image reconstruction performed in Imaris.

Molecular biology

Total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany), and gene expression patterns determined using Affymetrix HG-U133plus2 chips. MAS5-transformed expression values were normalized to 150 and converted to log10. Full-length Jagged-1 cDNA was retrieved from the I.

M.A.G.E collection and subcloned into an expression vector. Linearized DNA was transfected using FuGene6 transfection reagent (F. Hoffman-La Roche AG, Basel, Switzerland), and positive lines were established by multiple rounds of cell sorting.

Results

Mast cells transfer granular membrane components onto selected acceptor cells

Human mast cell degranulation can be monitored by surface appearance of a characteristic glycosylation variant of tetraspanin CD63 that is naturally buried within the granuli of resting mast cells (8). The formation of this isoform, termed CD63*var*, involves complex posttranslational modifications. To identify factors involved in the biosynthesis of this structural hallmark, we asked whether fractions of mast cells (whole cells, isolated granuli, or fractionated lysates) would introduce the CD63*var* epitope onto cell lines that are normally not stained by antibody NIBR63/1, that is if mast cells could switch CD63*var*(–) into CD63*var*(+) cells. Promonocytic U937 cells, for example, express CD63 protein on their surface, but are not marked by the NIBR63/1 antibody (Fig. 1A). When incubated in the presence of degranulating mast cells, however, the CD63*var* epitope appeared on the U937 cells, because they were stained by the NIBR63/1 mAb (Fig. 1A, lower right panel; and Fig. S1). Appearance of CD63*var* on U937 cells strictly required mast cell activation and was not seen when mast cells and U937 cells were co-cultivated without degranulation stimulus. Noteworthy, both FcεRI-dependent and chemical stimuli using PMA/ionomycin were able to induce signal transfer (Fig. S2); the latter was used throughout the experiments because of its stronger, more uniform effects. Also, only in stimulated co-cultures was an additional population noticed, which was highly positive for antibody NIBR63/1 and CFSE (Fig. 1A, lower right panel; marked 'Ag'). The double positive staining and a prominent shift in the FSC-H/SSC-H scatter clearly defined this population as aggregates formed between mast cells and U937 cells. Time courses of aggregate formation and CD63*var* transfer tightly correlated (Fig. S1).

These experiments allowed two possible interpretations: first, the CD63 protein already expressed by the U937 cells was transformed into a conformation recognized by antibody NIBR63/1, or second, the CD63*var* protein itself was transferred from mast cells onto the co-cultivated U937 (acceptor) cells. To differentiate between both options, we incubated U937 cells in the presence of various mast cell-derived preparations (Fig. 1B). Neither exposure to conditioned mast cell medium nor incubation in a cell-free supernatant of degranulated mast cells containing entire mast cell exosomes (5,6) did render U937 cells positive for CD63*var*. Only co-cultivation with active mast cells turned U937 cells positive for antibody NIBR63/1, suggesting that intercellular protein exchange has taken place that required intimate cell-to-cell contact.

Epitope transfer from activated mast cells is cargo specific, unidirectional and involves a unique membrane topology

We then wanted to know whether only selected mast cell factors are exchanged, or whether a mass transfer of mast cell components takes place. Acceptor cells were thus stained with antibodies directed against various molecules expressed by human mast cells (Fig. 1C). The granular markers CD63 and CD107a were both strongly transferred to the acceptor cells. Among the tetraspanin proteins, CD9 and CD81 were also transferred, while CD151 was clearly excluded from translocation. Notably, in mast cells, CD9 and CD81 are dominantly granular proteins (18), whereas CD151 is constitutively expressed on the plasma membrane (19, 20). As CD63, CD9, and CD81 co-localize to tetraspanin-enriched microdomains (TEMs), one may speculate that not only CD63*var*, but rather entire granular membrane patches, demarcated by TEMs, are transferred on to the respective acceptor cell. In contrast, translocation of other constitutive plasma membrane components, like CD45, CD117, and CD203, was not seen. Thus, protein transfer clearly prefers granular membrane factors, whereas components constitutively residing in plasma membrane are discriminated against.

We followed CD63*var* protein translocation also by confocal microscopy (Fig. 1D). Even before degranulation had been induced, contacts between mast cells and U937-eGFP cells were readily noticed (Fig. 1D, left panels). Expression of the CD63*var* epitope was restricted to cytosolic granuli within the mast cells. In contrast, once cultures were activated, the formation of an extensive contact zone was noticed in which donor and acceptor surfaces seemingly merged (Fig. 1D, right panel, arrows). Similar rearrangements in the contact zone, including the protuberance of mast cell pseudopodia, had been observed between mast cells and endothelium in early electronmicroscopic studies (21). At the same time, the CD63*var* signal redistributed from granuli onto the surface of degranulated mast cells. More importantly, focal patches of CD63*var* signal appeared in the acceptor cells. The CD63*var* signal however did not disperse within the acceptor cells or randomly distributes over their surface, but rather persisted in clearly defined, distinct foci located to the opposite side of the acceptor cell (Fig. 1D, circles). Thus, granular membrane patches, carrying the CD63 variant protein with them, appear to traverse acceptor cells and are then displayed on their distal surface. This material flow is unidirectional, from donor mast cells into the acceptor U937 cells, as no retrograde exchange of membrane components or GFP signal was seen (Fig. S3).

Reciprocal expression of Jagged-1/Notch-2 protein is a molecular determinant of epitope transfer

Collectively, these findings suggested that mast cells can be active donors in a special form of intercellular molecular transfer resembling the formation of an immunological synapse and the transfer of plasma membrane in lymphocytes (trogocytosis) (22). Convincing examples of trogocytosis however are restricted to the transfer of loaded MHC molecules,

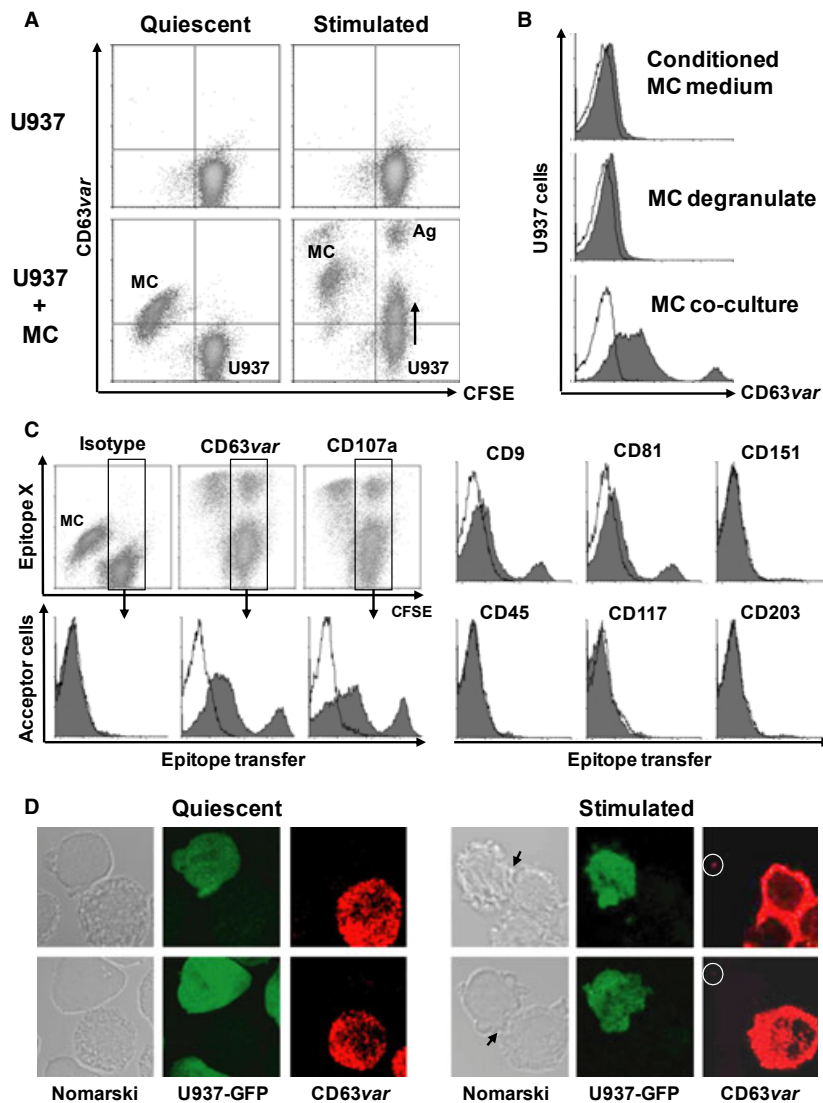


Figure 1 Epitope transfer from activated mast cells onto neighboring acceptor cells. (A) Activation-dependent epitope transfer from degranulating human mast cells onto U937 acceptor cells ($n > 20$). U937 cells were incubated with or without human mast cells (MC), and either left untreated (quiescent, 1.5 h incubation in mock medium) or stimulated with 25 ng/ml PMA and 500 ng/ml ionomycin for 1.5 h at 37°C to induce mast cell degranulation. Staining with antibody N1BR63/1 documents activity-dependent cell aggregation (Ag) and transfer of CD63var epitopes onto U937 cells (arrow). (B) Conditioned MC medium or cleared MC degranulation supernatant alone does not induce CD63var epitopes on U937 cells ($n = 4$). Quiescent U937 cells (open graph), U937 cells treated with conditioned MC medium, cleared MC degranulation sup, or co-cultivated with PMA/

ionomycin stimulated mast cells for 1.5 h (gray histograms, top to bottom). (C) Dot plot analyses of stimulated co-cultures and histograms of the indicated gating quadrants. Antibodies document selectivity of epitope transfer and were used in at least two independent transfer experiments ($n \geq 2$). Stimulated co-cultures (1.5 h of PMA/ionomycin treatment, gray histograms), quiescent co-cultures (1.5 h in medium only, open graph). (D) Confocal laser scanning microscopy of co-cultivated MCs, stained with CD63var mAb (N1BR63/1, red), and GFP-labeled U937 cells (green). Upon stimulation with PMA and ionomycin, cell contacts intensify (arrows) and focal patches of CD63var signal appear on the distal surface of the acceptor cell (circles). Micrographs exemplify representative cell-cell interactions as observed in two independent microscopic sessions.

either in a T-cell receptor (TCR)-dependent manner (23) or by uncharacterized mechanisms between antigen-presenting cells (24). As in our systems no TCR was present, but cell-cell contact was clearly needed, we set out to identify alternative receptor-ligand pairs critical for epitope transfer. We

first examined the performance of a broad panel of cell lines as acceptors in the transfer assay (Fig. 2A). Several patterns were observed: (i) contradictory to a putative TCR-mediated trogocytosis, T-cell lines, like CEM, HPB-ALL, Jurkat, MOLT4, Peer, and NK92 natural killer cells, were poor ac-

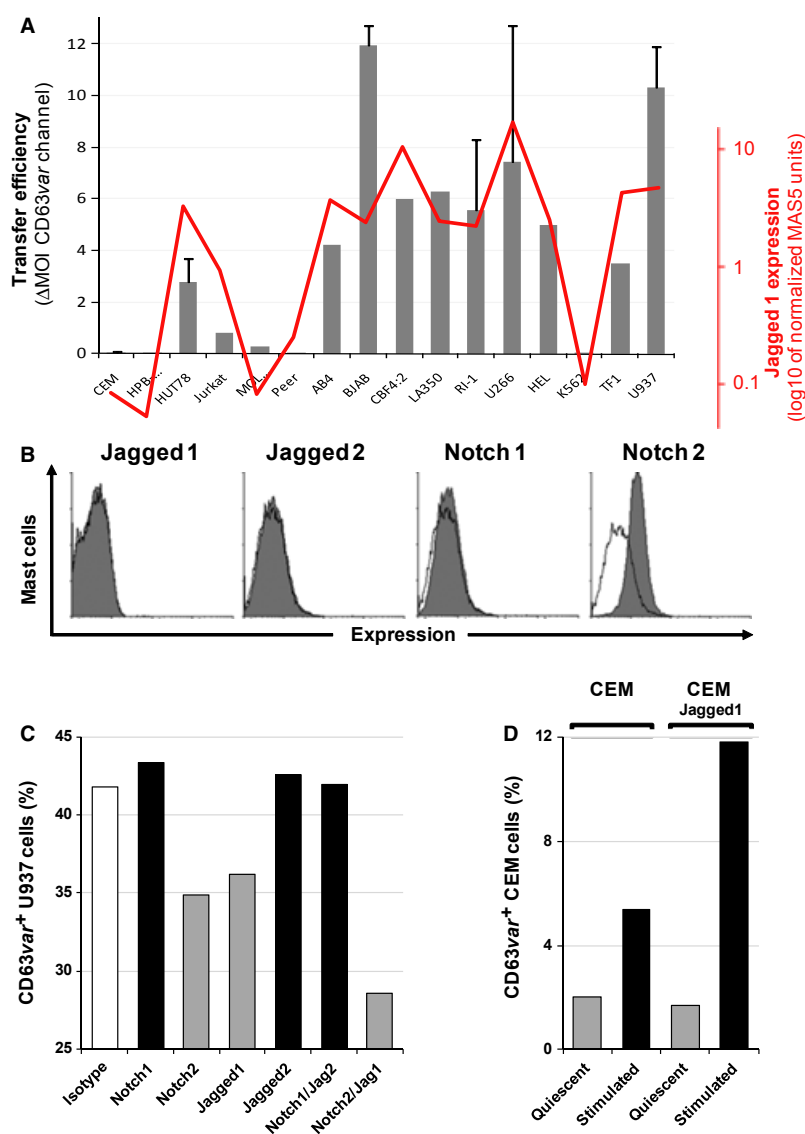


Figure 2 Reciprocal expression of Jagged1 and Notch2 is a molecular determinant of epitope transfer. (A) Comparative analysis of cell lines as acceptors in mast cell contact protein transfer. Bars depict the mean fluorescence intensity shift of stimulated (1.5 h in the presence of mast cells treated with 25 ng/ml PMA and 500 ng/ml ionomycin) over nonstimulated (1.5 h in medium with quiescent mast cells) acceptor cells (left y-axis, $n \geq 2$). Relative mRNA expression of Jagged1 is given as red overlay (log10 of MAS5 normalized Affymetrix data, right y-axis). (B) Surface expression of selected Notch/Notch ligand pairs on human mast cells (gray histograms; isotype controls shown as open graphs). Surface staining was confirmed for three independent mast cell donors ($n = 3$). (C)

Epitope transfer from PMA/ionomycin-treated human mast cells onto U937 cells in the presence of indicated blocking antibodies (5 μ g/ml each, $n = 2$). From left to right: isotype (mIgG1, BD 557273), anti-Notch1 (GenTex 24883), anti-Notch2 (BioLegend 348304), anti-Jagged1 (R&D MAB1277), anti-Jagged2 (R&D FAB1726P), or combinations of these. Transfer efficiency is expressed as % CD63var recipient cells. (D) Wild-type (left) or Jagged1 expressing CEM cells (right) were co-cultivated in the presence of quiescent (gray) or PMA/ionomycin-treated (black) mast cells. Jagged1 expression increases CD63var signal transfer. Depicted are average transfer rates as observed for two independently cloned, transient Jagged1 transfectants.

ceptors; (ii) B cells on the other hand (e.g. AB4, BJAB, CBF4:2, LA350, and Ri-1) were in general rather good acceptors; (iii) cells of erythroid and myeloid lineage gave mixed results. We hypothesized that the presence or absence of only a few surface molecules determined whether an

individual cell could or could not serve as acceptor of mast cell-derived epitope transfer. Cell lines were thus subjected to mRNA expression analysis, and a hit list of candidate genes was derived for which the transfer efficacy and relative gene expression correlated best. This comparative analysis revealed

a significant correlation between Jagged1 (JAG1) mRNA expression and transfer competence (Fig. 2A). On the other hand, Notch2, a ligand for JAG1, is expressed on the surface of human mast cells (25, 26), while other members of the Notch and Jagged families are absent (Fig. 2B). Blocking antibodies directed against either one of the two molecules impaired the efficacy of CD63^{var} epitope transfer onto recipient U937 cells. This effect increased when a combination of anti-Notch2 and anti-Jagged1 antibodies was applied (Fig. 2C). Conversely, transfection of JAG1 into the otherwise nonacceptor CEM line considerably enhanced epitope transfer (Fig. 2D). Accordingly, reciprocal expression of Notch2 on the mast cell side and Jagged1 on the acceptor

cell side is a functional determinant of epitope transfer, although the fine mechanisms of how these factors regulate membrane translocation remain to be determined.

Organotypic 3D cultures document epitope transfer across monolayer endothelium

Because mast cells frequently co-localize with the abluminal side of blood vessels *in vivo*, we explored whether endothelial cells may serve as acceptor candidates in our transfer experiments. Noteworthy, expression and function of Jagged1 was confirmed for various human endothelial cell lines, explicitly in HDMEC cells (27), and basal-to-apical transfer of soluble

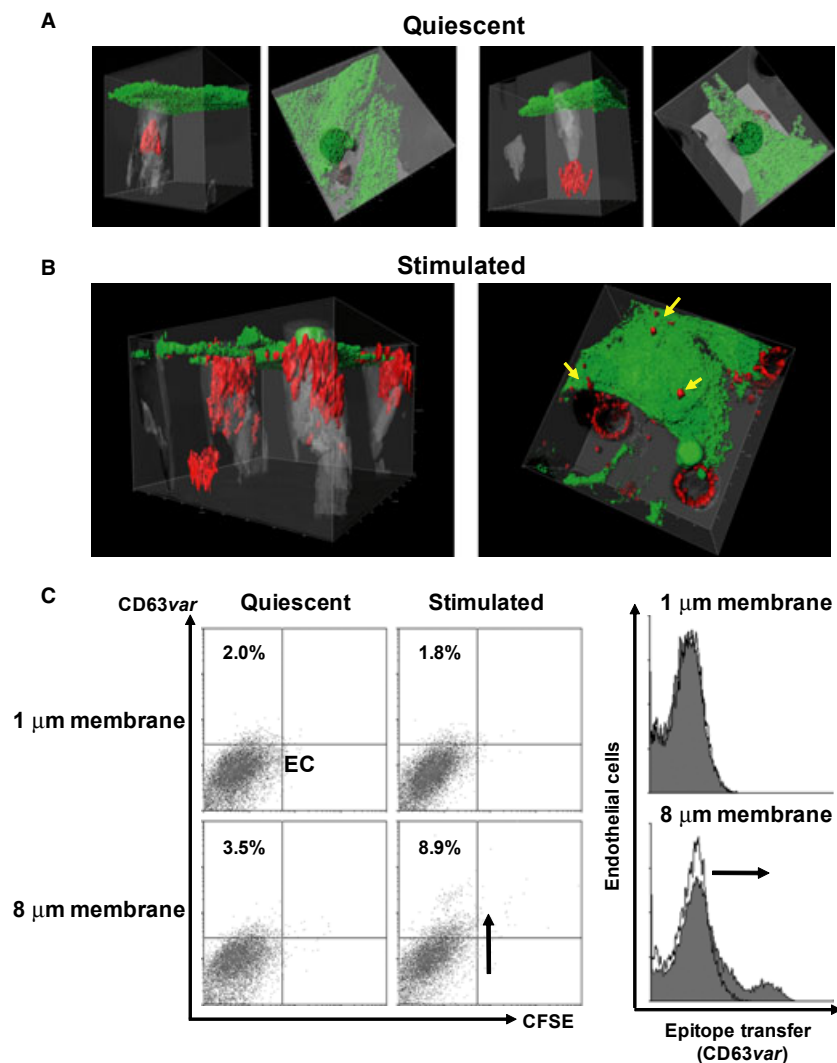


Figure 3 An organotypic model of epitope transfer. (A and B) Human dermal endothelial cells (HDMEC) were cultured on filter membranes, mast cells sedimented onto the opposite (basal surface), and transfer of CD63^{var} epitopes followed by immunostaining ($n \geq 3$). Image reconstitutions illustrate activation-dependent fluorescence signal transfer onto endothelial cells in juxtaposition of membrane pores. CFSE-labeled endothelial cells (green), CD63^{var}

signal (red), translocated membrane foci (arrows). Co-cultures without (A), and with PMA/ionomycin activation (B) shown. (C) FACS analysis of CD63^{var} epitope transfer onto endothelial cells through filter membranes of different pore sizes (1 μm top; 8 μm bottom) ($n = 3$). Quiescent co-cultures (1.5 h in buffer, left) and stimulated co-cultures (1.5 h in the presence of PMA and ionomycin, right) are shown; arrows indicate CD63^{var} signal transfer.

mast cell-derived mediators (transcytosis) had been reported in these cells (28). To mimic the relative physiologic orientation of an endothelium with underlying mast cells, co-cultures separated by membranes were set up (Fig. 3). Endothelial cells were seeded on filter membranes of different pore sizes and propagated to confluence. Subsequently, mast cells were sedimented onto the opposite, basal side of the filter. Image reconstitutions of corresponding z-stack sections clearly demonstrate that quiescent mast cells more frequently located to the peripheral, lower end of the pore channel or did not penetrate the filter membrane at all (Fig. 3A). In contrast, activated mast cells migrated through filter pores to directly contact the endothelial cell layer (Fig. 3B). Focal patches of CD63 ν ar signal were detected on the distal, apical side of the endothel in stimulated co-cultures only (Fig. 3B, right panel, arrows), whereas no such translocation was noticed in the controls. Remarkably, epitope transfer was restricted to the surrounding of membrane pores, where mast cells and endothelial cells could interact. Efficient translocation of CD63 ν ar epitopes was only seen with a pore size of $\geq 8 \mu\text{m}$, and transfer was effectively abolished with a pore diameter of $1 \mu\text{m}$ and below (Fig. 3C). While the $1\text{-}\mu\text{m}$ pore size precludes co-localization of mast cells and endothelial cells, transport of fluids and small particulate materials like exosomes would still be possible. The apparent lack of protein transfer under these conditions also argues against a mere secretion–reuptake process and once again emphasized the importance of direct cell-to-cell contact for epitope transfer.

Luminal decoration of blood vessels by activated perivascular mast cells in allergic rhinitis

Rapid accumulation of mast cells in excess of the tissue-resident population and recruitment of other immune cell types is a hallmark of allergic rhinitis (29) (Fig. 4A). Moreover, in nasal mucosal tissue samples from diseased patients, we frequently detected degranulating mast cells not only at exposed mucosal sites, but also in the vicinity of small blood vessels (Fig. 4B). Frozen sections of nasal mucosa from three donors with allergic rhinitis were thus stained with anti-CD31 to identify endothelial cells and with anti-CD63 ν ar to trace the fate of mast cell products (Fig. 4C). Confocal microscopy of these samples confirmed the presence of degranulating, immunoreactive mast cells in perivascular spaces, positioned close to the abluminal side of microcapillaries. More importantly, and similar to our organotypic co-cultures, the CD63 ν ar protein was also detected on bystander endothelial cells, where it was displayed particularly on the luminal side of the vessel (Fig. 4C). Thus in congruence with our experimental postulate, the transfer process is in place *in vivo*, especially under chronic inflammatory conditions, like in the nasal mucosa of allergic rhinitis patients.

Discussion

Many cells in the body use vesicles to store and concentrate molecules that are intended to be used for intercellular

communication or for effector functions, like synaptic vesicles in nerve termini, melanosomes in melanocytes, or granuli in leukocytes and platelets. In most of these cells, the vesicles fuse to the cell membrane upon stimulation, and their content is released into the immediate surrounding. A few other cell types, like dendritic cells or mast cells, can also discharge exosomes, which are complete, membrane-covered vesicles (30, 31). These exosomes can carry the whole repertoire of the vesicular cargo, including proteins and various RNA species (32), and are able to travel long distances with the help of the lymphatic system (7).

We now propose another, novel way of how vesicular cargo can reach neighboring cells: upon triggering, granular membrane factors directly traverse from the activated mast cell onto an acceptor cell. We first detected epitope transfer from activated mast cells onto neighboring cells by following the localization of CD63 ν ar, which is a specific molecular marker of human mast cell granuli. The CD63 protein has been linked to phagocytosis/endocytosis in the past and is known to influence the recruitment of vesicular components, like neutrophil elastase and synaptotagmin VII (33–35). Moreover, antibodies directed against CD63 significantly impair the degranulation of rat basophiles and of human mast cells *in vitro* (8, 36). CD63 belongs to the family of tetraspanins (TM4SF proteins) that are organizers of specialized membrane patches (tetraspanin-enriched microdomains, TEMs). Other selected TM4SF member proteins, like CD9 and CD81, which are also part of TEMs, were likewise transferred to acceptor cells. Interestingly, a physical association and functional interdependence particularly of CD9, CD63, and CD81 has been reported, which affects the surface presentation of associated glycoproteins, like HLA-DR and integrins (37). Indeed, glycoprotein Lamp-1 (CD107a), a heavily glycosylated molecule most frequently associated with (post) lysosomal compartments, has been identified as yet another reliable marker of epitope transfer in our experiments. Thus, one may speculate that not only CD63 ν ar, but rather entire granular membrane patches, demarked by TEMs, are passed on to the respective acceptor cell.

Importantly, the new transport mode described here differs from conventional granular secretion, as it requires an intimate cellular coupling of donor and acceptor cells. Several lines of evidence support the necessity of direct cell-to-cell contacts: (A) the presence of a (transiently) aggregated population in transfer experiments; (B) parallel kinetics of aggregate formation and epitope transfer; (C) morphologic changes in the contact zone of donor and acceptor cells; (D) an exclusion size of approximately $8 \mu\text{m}$ for epitope transfer through filter membranes. Moreover, transferred epitopes were repeatedly found to localize to the opposing surface of the acceptor cell, instead of a preferentially proximal fusion in case of diffusion-based uptake. All these findings argue that the proposed direct channeling mechanism is distinct from a secretion–reuptake process. Surface markers of the acceptor population (i.e. CD19, CD20, and other B-cell markers for example) were not found to shift onto stimulated donor mast cells. Similarly, when U937 acceptor cells had been labeled with cytosolic eGFP, only insignificant amounts

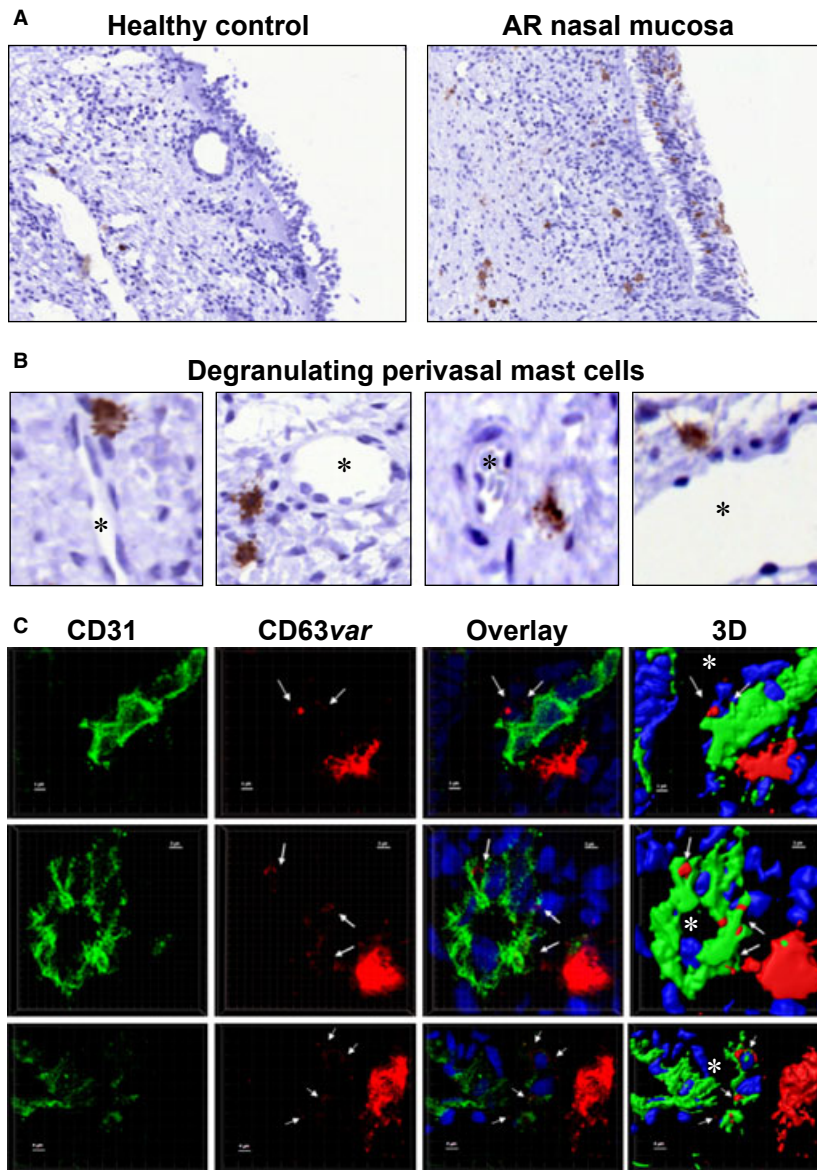


Figure 4 Epitope transfer in human disease. (A) Histological overview images of nasal mucosa illustrating tissue accumulation of mast cells (b-tryptase, brown, on hematoxylin background staining) in allergic rhinitis patient (AR, right) and healthy control (left); preparation representative for $n = 6$ donors. (B) Activated mast cells co-localize with peripheral blood vessels in AR nasal mucosa.

Asterisks indicate capillary lumen. (C) Confocal laser scanning microscopy of AR nasal mucosa stained with DAPI (nuclei, blue), anti CD31 (endothelial cells, green), and anti CD63var (red). Arrows indicate fluorescence signal transfer from degranulating mast cells into the lumen of microcapillaries across the juxtapositioned endothelial cells.

of this probe traversed into mast cells. The fact that essentially no retrograde material flow was detected, not even of soluble cytosolic protein, implies that although mast cell and acceptor cell physically interact, the cells do not actually fuse. Thus, the translocation of mast cell epitopes appears to be selective and essentially unidirectional.

While the CD63var molecule may initiate, coordinate, or at least influence the association of mast cells with their acceptors, on its own CD63 clearly is insufficient to maintain this

adhesion. This association, strong enough to withstand the sheering forces imposed onto cells during FACS separation, is more likely to involve a whole cascade of specialized adhesion and interaction molecules, like it was shown for CD44 and CD51 in mast cell–muscle cell interactions (38). We have identified Jagged-1 (JAG1) as one critical component of this mast cell–target cell interaction. JAG1 appeared as an attractive candidate for a number of reasons: (A) JAG1 was selectively expressed on a wide variety of acceptor cell lines, whereas it

was absent on cells on which transfer was unsuccessful; (B) its cognate receptor, Notch2, is expressed on mature human mast cells; in fact, expression of Notch2 is largely restricted to myeloid cells in adult humans; (C) upon their association, both of them undergo proteolytic processing, get actively internalized, and induce downstream signaling (39). Noteworthy, a Notch2/JAG1 interaction is particularly important in organ development, as lack of either JAG1 or Notch2 causes Alagille syndrome (40, 41). However, the fine mechanism of how Notch2 or JAG1 regulates the fate of vesicular components has still to be elucidated.

As a result of the direct transfer, we have repeatedly detected mast cell-derived molecules exactly on the opposing distal surface of an attached acceptor cell. This observation suggested that a directional translocation of selected granular factors could be utilized by mast cells to modify distant surfaces of barrier-forming cells. Baso-lateral-to-apical translocation of individual chemokines through the cytosol of endothelial cells (transcytosis) has been reported before (28), and as a result, MIP-1 β (CCL-4) is displayed on the luminal face of endothelial venules in inflamed lymph nodes (42). Also mast cells were observed to play a role in promoting endothelial permeability (43). However, an activation-dependent physical association of mast cells and endothelium had not been reported before, and in particular, no selective, direct passage of transmembrane molecules from mast cell onto the distal surface of neighboring endothelial cells has been described.

Based on our novel findings, we thus postulate that mast cells actively contact endothelial cells in an inflammatory context to directly translocate granular components onto the luminal side of a blood vessel. The cargo, which comprises CD63 ^{var} , CD9, CD81, CD107a, and very likely further associated granular factors, traverses the endothelial cells in a unidirectional fashion and is finally displayed on their luminal cell surface. Thus in an inflammatory context, a stimulated mast cell may directly modulate its neighboring endothelium and for example may influence leukocyte recruitment toward sites of antigen challenge.

In conclusion, we describe details of a novel intercellular communication mechanism, wherein granulated cells can select distinguished acceptor cells via receptor–ligand interactions and then transfer a set of their own molecules onto them. We demonstrated this concept using highly granulated mast cells, which use their Notch2 receptor to identify appropriate Jagged1 expressing recipient cells and then directly deliver part of their granular cargo. While here we demonstrate the forward transfer of membrane components, one can imagine that as part of the same process also other macromolecules, like mRNA or miRNAs, are directionally deliv-

ered and exert their regulatory function (5, 44). This way mast cells can control, modify, or even reprogram selected bystander cells and may fundamentally reshape their microenvironment in case of an allergic challenge. As mast cells frequently co-localize with blood vessels *in situ*, we propose that via this transcytotic channeling, mast cells instantly modulate the membrane composition of neighboring endothelium. Providing first evidence for such a mechanism in human disease, distinct patches of mast cell-derived cargo, delineated by inflammation marker CD63 ^{var} , were observed on the luminal side of blood vessel in allergic rhinitis patients. Accordingly, we discovered a new signaling process that enables the individual mast cell to convey an inflammatory signature across neighboring endothelium, and this way communicates a local antigen challenge to circulating blood cells.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Cell-to-cell contact is a functional prerequisite of epitope transfer.

Figure S2. Epitope transfer in response to different activation stimuli.

Figure S3. Epitope transfer is essentially uni-directional.

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Supplemental Information

Luminal decoration of blood vessels by activated perivascular mast cells in allergic rhinitis

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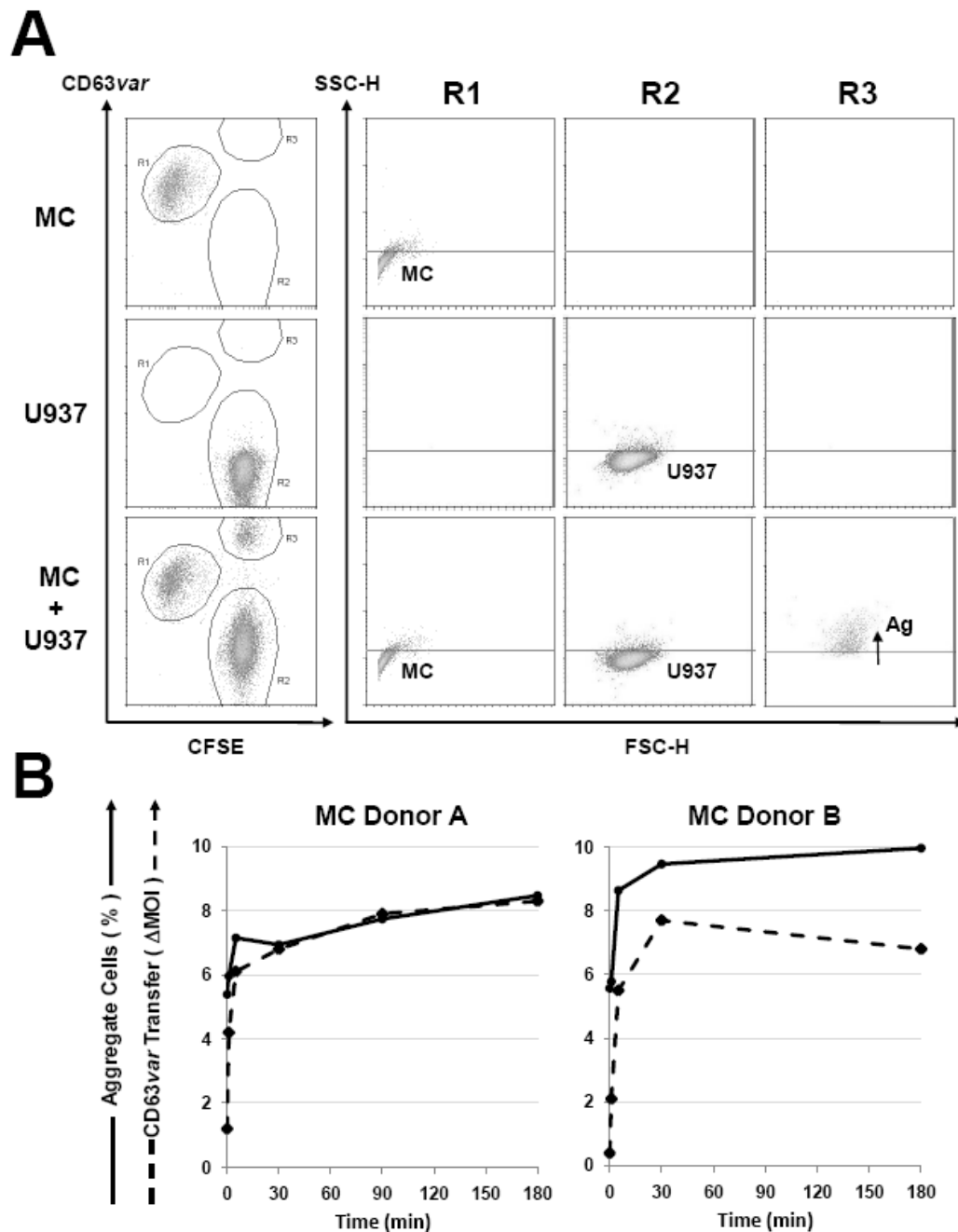
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Supplemental Figure 1 | Cell-to-cell contact is a functional prerequisite of epitope transfer.

(A) Human mast cells, CFSE-labeled promonocytic U937 cells, or co-cultures of both were stimulated with 25ng/ml PMA and 500ng/ml ionomycin for 1.5h and stained with antibody NIBR63/1 to detect CD63 var epitopes ($n \geq 20$). Dot plot analyses based on staining (left) consistently identify three independent populations in the assay: CD63 var positive mast cells (R1), CFSE positive U937 cells (R2), and a double positive fraction (R3). Re-examination of these populations according to their size/granularity distribution using an FSC vs. SSC plot verified that the R3 fraction contained aggregate particles whose size clearly exceeded that of single mast cells and single U937 cells (arrow). In contrast, appearance of the CD63 var epitope on U937 cells (R2 population) is clearly not due to cell aggregation. **(B)** Time course of epitope transfer ($n=2$): cell aggregation (% total cells, solid line) and epitope transfer (mean fluorescence intensity shift of acceptor cells in the CD63 var channel, dashed line) of stimulated (PMA/ionomycin treatment) over non-stimulated (mock medium) co-cultures closely correlate in two independent transfer experiments.

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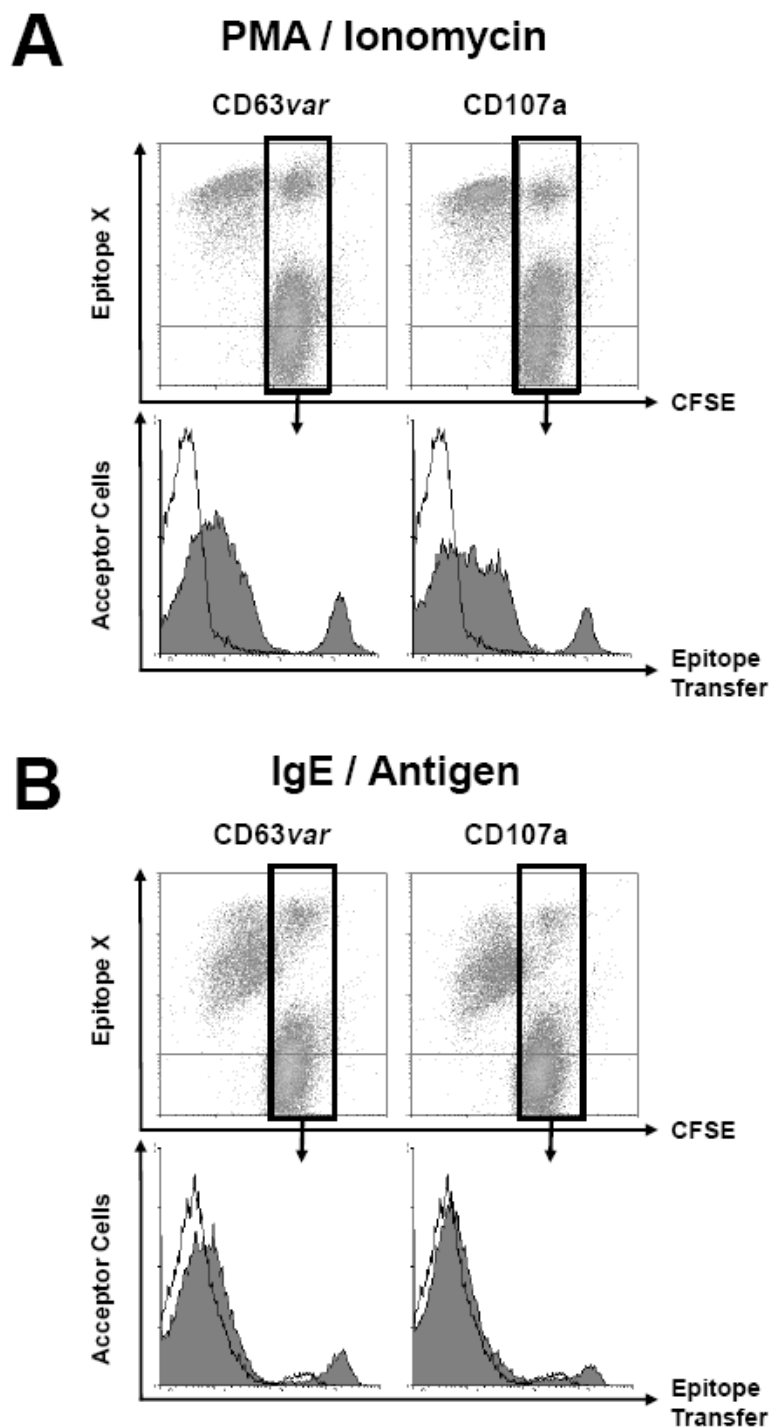
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Supplemental Figure 2 | Epitope transfer in response to different activation stimuli.



Donor mast cells and CFSE-labeled acceptor cells were incubated in presence of different activating stimuli to induce mast cell degranulation (n=3). Aggregate formation and molecule transfer were followed with antibodies directed against the granular mast cell components CD63 var and CD107a, respectively. Transfer was induced **(A)** with PMA (25ng/ml) and ionomycin (500ng/ml); or **(B)** via Fc ϵ RI by IgE crosslinking (B11 and Le27, 5ug/ml each) for 1.5h at 37°C. Depicted are dot plot analysis (upper blocks) and histogram images of the gated (black square)

CFSE $^{+}$ acceptor cells (lower blocks). Stimulated co-cultures (gray histograms) are compared to quiescent control cells (1.5h in mock medium, open graph).

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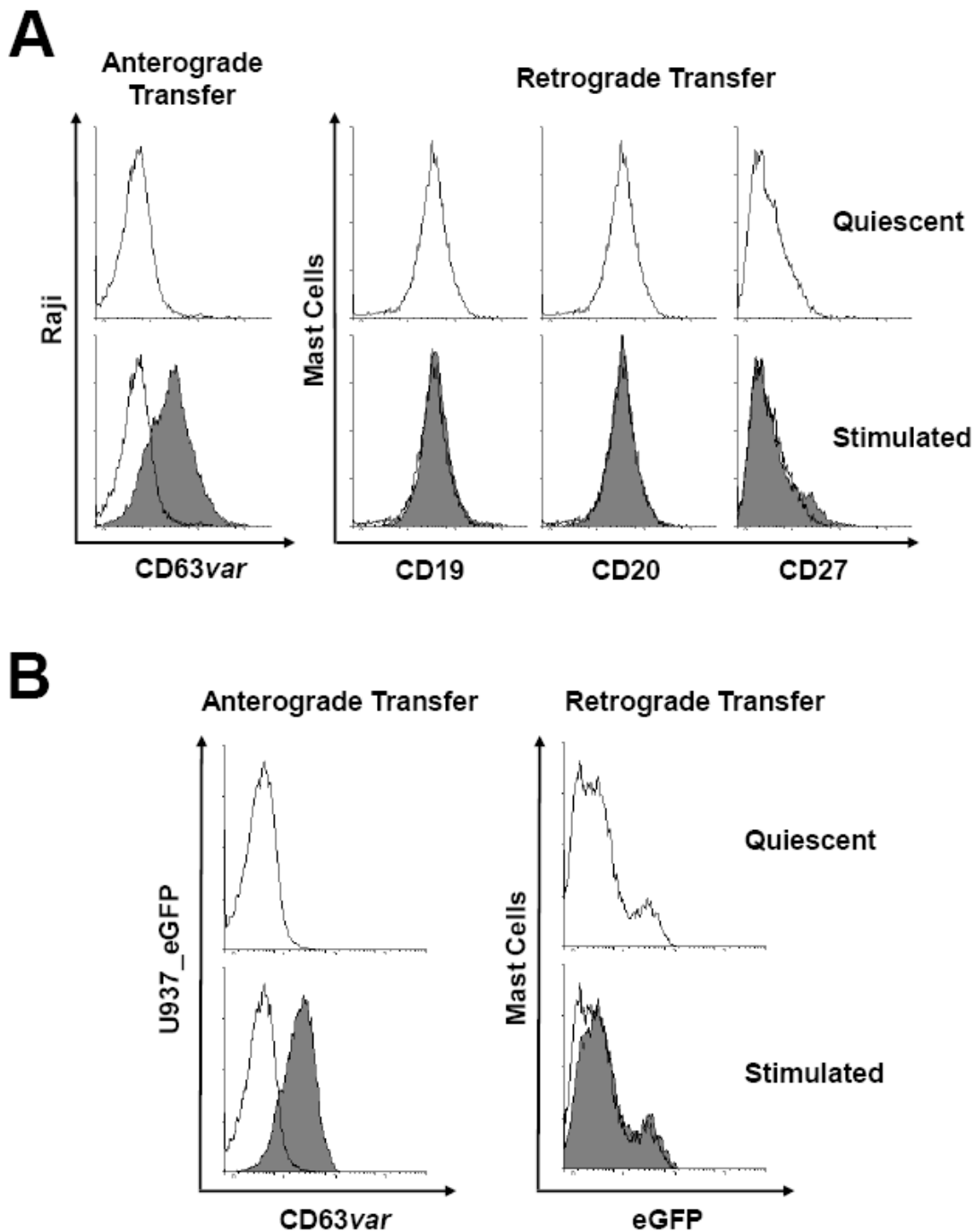
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Supplemental Figure 3 | Epitope transfer is essentially uni-directional.



Mast cells and acceptor cells were co-cultivated and either incubated in mock medium (quiescent; top panels, open graphs) or stimulated with 25ng/ml PMA and 500ng/ml ionomycin for 1.5h at 37°C to induce degranulation (stimulated; bottom panels, grey overlays). **(A)** Anterograde transfer of CD63var epitopes onto Raji B-cells was verified by staining with antibody NIBR63/1 (left). In contrast, no retrograde exchange of the membrane-bound B-cell molecules CD19, CD20, or CD27 onto mast cells is seen (right). Data are representative of two independent experiments using either Raji or Ramos B-cells (n=2). **(B)** Anterograde transfer of CD63var epitopes onto U937 transfectant cells expressing soluble cytoplasmic eGFP at high concentration (left). Retrograde influx/uptake of cytosolic eGFP into mast cells remains insignificant (right).