

A granular variant of CD63 is a regulator of repeated human mast cell degranulation

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Abstract

Background: Mast cells are secretory immune cells whose degranulation can provoke acute allergic reactions. It is presently unclear, however, whether an individual mast cell can repeatedly degranulate or turns dysfunctional after a single antigen stimulus. This work thus aims to better define the mast cell life cycle, with particular focus on new target structures for therapeutic or diagnostic approaches in allergy.

Methods: Monoclonal antibodies were raised against degranulated cord blood-derived human mast cells. A subset of these antibodies that exclusively recognized degranulated mast cells, but did not cross-react with quiescent mast cells or other hematopoietic cell types, became key reagents in subsequent experiments.

Results: We identified a granular variant of tetraspanin CD63 as an exclusive molecular marker of degranulated human mast cells. Mutant analyses indicate that a cysteine cluster around residue C170 and protein glycosylation at residue N172 account for the antibody specificity. Here, we show that mast cells, which underwent an initial FcεRI-mediated degranulation, can be degranulated for at least another cycle *in vitro*. Repeated degranulation, however, requires an IgE/antigen stimulus that differs from the preceding one. Furthermore, the new variant-specific anti-CD63 antibodies effectively impair repeated cycles of mast cell degranulation.

Conclusion: Our findings indicate that mast cells are stable, multiple-use cells, which are capable of surviving and delivering several consecutive hits. Surface expression of the novel CD63 variant is a distinguishing feature of such primed cells. Reagents directed against this molecular hallmark may thus become valuable diagnostic and therapeutic agents.

Mast cells are secretory immune cells with pivotal importance in inflammation, allergy, and autoimmune diseases, like asthma bronchiale, neurodermitis, or rheumatoid arthritis (1). They are a major source of immunoreactive substances, including histamine, chemokines, and cytokines, that promote vasodilatation in inflamed tissue, attract and costimulate lymphocytes at infectious sites, and provide a primary defense line against pathogens and parasites in mucosa and epithelia (2, 3). These and further bioactive mediators are spatially compacted in mast cell granuli, whose activation-dependent release has been elucidated in considerable detail. In nature,

degranulation generally commences with an antigen-induced cross-linking of IgE molecules that are bound on the surface of an individual mast cell by FcεRI, the high-affinity anti-IgE receptor (4). This receptor launches an intracellular signaling cascade, which in a Ca²⁺-dependent manner results in a massive cellular remodeling and fusion of plasma membrane and granuli (5–7). In addition, also secretion of entire intact granuli has been reported (8, 9).

Significantly less is known about the subsequent fate of mast cells, about how, if at all, they recover from degranulation. Although a few historic reports on possible survival and regeneration of mast cells post degranulation exist (10–12), generally mast cell degranulation is viewed as a terminal process. The notion that mast cells would die after degranulation is based on the low proliferation and difficult survival of explanted mast cells *in vitro* and is corroborated with what we know about the fate of other granular cell types, like

Abbreviations

CBHMC, cord blood-derived human mast cell; DSαHMC-mAb, degranulation-specific anti-human mast cell monoclonal antibody; MC, mast cell; RBL, rat basophil leukemia cell; TEM, tetraspanin-enriched microdomain; TM4SF, tetraspanin superfamily.

neutrophils or platelets. Nevertheless, recovery of degranulated human mast cells is still an option, although repeated activation of human mast cells has not been formally shown.

To better define the mast cell life cycle, we raised monoclonal antibodies against degranulated cord blood-derived human mast cells (CBHMCs). Surprisingly, one predominant epitope identified from this approach was CD63, a broadly expressed transmembrane protein previously already detected on mast cells. CD63 belongs to the tetraspanin superfamily (TM4SF) that comprises various members in different species (32 in mammals, 35 or more in drosophila, and 21 in worms) (13). 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) (14). Tetraspanins are required for successful mammalian fertilization, coregulate signaling through growth factor receptors, regulate neuronal-astrocyte interactions in brain, facilitate neuromuscular synapse formation in *Drosophila* embryos, and coregulate integrin-dependent cell migration by strengthening adhesion (15). CD63 is expressed on a variety of hematopoietic cell types like monocytes, macrophages, and T cells and was shown to directly interact with integrins, syntenin-1, and membrane metalloproteases when localized to TEMs (16, 17). Cell adhesion, cell motility, and phagocytosis/endocytosis are thus thought to be influenced by CD63 (18). A substantial fraction of CD63 localizes to secretory vesicles, lysosomes, and late endosomes in platelets, dendritic cells, and melanocytes and reaches the plasma membrane in response to cell activation and secretion (19, 20). The same applies to basophils, whose surface exposure of CD63 serves as a diagnostic marker in allergic diseases (21–23). Interestingly, also retrograde trafficking of CD63 has been demonstrated, which makes this protein a prime candidate to study the replenishment of granuli and the reactivation of granular immune cells (24).

Here, we use our novel anti-CD63 monoclonal antibodies to demonstrate that human mast cells can survive an Fc ϵ RI-induced degranulation and are capable of further sequential degranulation cycles. By characterizing the particular CD63 epitope, we provide experimental evidence that two structurally distinct isoforms of the human CD63 protein exist: one characteristic of vesicles and another expressed on the cell surface. Antibodies that differentiate these two isoforms may thus become powerful diagnostic tools. In addition, the novel anti-CD63 antibodies selectively impaired the repeated degranulation of pre-activated (i.e., partially degranulated) human mast cells *in vitro*. These data underscore the significance of CD63 as a diagnostic marker of mast cell activation, and as a potential target for anti-allergic therapy.

Materials and methods

Cell culture and transfectants

Human mast cells were differentiated from cord blood-derived progenitor cells according to established procedures (25–27). In brief, stem cells were isolated from heparinized human cord

blood by Ficoll gradient centrifugation and magnetic enrichment for CD133-positive cells (CD133 Micro Bead kit; Miltenyi Biotec, Bergisch Gladbach, Germany). The resultant mononuclear cell fraction was propagated for 3 weeks in serum-free expansion medium (Stem Cell Technologies, Vancouver, Canada) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamate, and a cytokine cocktail consisting of 100 ng/ml rhSCF, 100 ng/ml rhIL-6, and 30 ng/ml rhIL-3 (Peprotech, Rocky Hill, NJ, USA). Cultivation was then continued in medium without IL-3 for two additional weeks. Finally, cultures were shifted to RPMI 1640 medium supplemented with 10% decompartmented FBS (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamate, 100 ng/ml SCF, and 100 ng/ml IL-6 for at least another 6 weeks before degranulation experiments were performed. Rat basophil leukemia (RBL) cells were cultivated in complete DMEM containing 10% FBS and antibiotics. cDNAs of the human CD63 and CD82 genes were obtained from the IMAGE collection (clones IRAL10m10 and IRAL2m8, respectively) and subcloned into a pEK series expression vector. CD63 mutants were generated by PCR site-directed mutagenesis. For primer sequences see Supplementary Table S2. All constructs were verified by sequencing and transfected into RBL cells using Eugene6 (Roche, Basel, Switzerland) reagent according to the manufacturer's instructions. Stable transfectants were selected using mCD8a as a co-expressed marker by multiple rounds of magnetic sorting (BD IMag) and final flow sorting on a FACS Aria cell sorter (Becton Dickinson (BD), Franklin Lakes, NJ, USA).

Generation of monoclonal antibodies

Pathogen-free Balb/c mice were immunized multiple times with phorbol 12-myristate 13-acetate (PMA)/ionomycin-treated CBHMCs homogenized in Titer-Max adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Spleen cells were fused with SP2/0 mouse myeloma cells using standard techniques, and antibodies were purified from the culture supernatant on rProteinG fast protein liquid chromatography (FPLC) columns (GE Biosciences; GE Health Care, Piscataway, NJ, USA).

Degranulation experiments

CBHMCs were incubated overnight with 5 μ g/ml coating antibody (B11, Jw8, or anti-phosphocholine mAb) and 25 ng/ml IL-4 in conditioned medium. Cells were washed the next day to remove excess IgE and resuspended in assay medium (RPMI 1640 w/o phenol red, 25 mM HEPES pH 7.2, 0.5% BSA, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamate) to an approximate cell density of 1×10^6 /ml. Degranulation was induced by the addition of respective antigens (5 μ g/ml cross-linking antibody Le27, or 100 ng/ml NIP-BSA, or 100 ng/ml phosphocholine-BSA), and the incubation continued for at least 2.5 h at 37°C and 7.5% CO₂. For sequential degranulation experiments, cells were washed after the first round to remove excess IgE/antigen and resuspended in fresh medium supplemented with

25 ng/ml IL-4 and 5 µg/ml of the second coating antibody. The second degranulation cycle was induced essentially the same way as the first one described previously. Total cell lysates were obtained by the addition of 1% Triton X-100 (Sigma, T8787). Alternatively, degranulation was also induced with 25 ng/ml PMA (Sigma, P1585) and 500 ng/ml ionomycin (Sigma, I3909). The release of β -hexosaminidase was determined from degranulation supernatants using 1 mM 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide (Sigma) as substrate in 96-well flat bottom, white microlite TCT plates. Fluorescence emission was measured on a Tecan Ultra Spectrophotometer. Cytokines were measured using a multiplex cytokine bead kit (Milliplex MAP; Millipore, Billerica, MA, USA).

Cytospin staining

Approximately $1 \times E5$ CBHMCs were immobilized on polylysine-coated glass slides and fixed with MeOH/acetone (1 : 1) for 5 min at RT. Slides were washed with TBS buffer, pH 7.6 and incubated overnight at 4°C with different anti-CD63 mAbs. For these and other antibodies see Supplementary Table S1. The APAAP detection system (Dako Cytomation, Glostrup, Denmark) was then used for color development.

Homology Model Building

The structure of CD81 (28), PDB entry 1IV5, was used as a template to build a homology model for CD63. Only residues from F113 to H202 (native residue L202) were conserved. The SCWRL routine v2.9 was then used to mutate all residues into the CD63 sequence (29). Eventually, insertions and deletions were rectified manually. The two disulfide bonds (C145-C191 and C146-C169) were closed; the N-terminus was capped by acetyl (-CO-CH₃) (ACE) and the C-terminus by *N*-methyl (-NH-CH₃) (NME) to avoid artifacts from charged terminal groups. The resulting coordinates were further refined by conjugate gradient minimization, using the *parm03* data set of the AMBER force field and a Generalized-Born implicit-solvent model (corresponding to *igb* = 5 in AMBER modules). The energy-refined structure was passed through *procheck* (30) and did not reveal severe deviations from standard values. The finalized structure was visualized and illustrated using the PYMOL software package (DeLano Scientific, Palo Alto, CA, USA).

Results

Degranulation-specific anti-human mast cell antibodies

To follow the fate of postdegranulation-state mast cells and to question whether mast cells are capable of multiple rounds of degranulation, we needed reagents that reliably distinguish native (i.e., *in vitro* differentiated, uninduced cells) from degranulated CBHMCs. Although several such markers have been described, like CD107a or CD203c (31), their sensitivity and signal-to-noise ratio was suboptimal for observations with single-cell resolution.

We have therefore generated mAbs that selectively recognized degranulated, but not native CBHMCs (Fig. 1A). To identify the molecular target structures for these new cell state-specific antibodies, publicly available gene expression data (GEO entry numbers GSE1933 and GDS1520) were screened for candidate genes, which were either upregulated in response to mast cell degranulation or were generally overexpressed in CBHMCs (data not shown). Based on these criteria, RBL cells were stably transfected with selected candidate genes and screened for potential surface recognition by the novel antibodies. One surface protein successfully identified through this gene expression strategy was tetraspanin CD63. Rat basophil leukemia transfectants stably expressing CD63, but not the structurally related tetraspanin CD82, were selectively recognized by state-specific anti-human mast cell Abs NIBR63/1, /2, and /3 (Fig. 1B). Antibody binding increased markedly, when the cells had been pre-incubated with PMA/ionomycin to induce degranulation (Fig. 1C).

As CD63 is known to be widely expressed on a number of cell types and was implicated in the progression of melanoma (32, 33), a thorough mapping of target and species selectivity was particularly important. From a broad collection of cell lines we have tested, the sole candidate that displayed considerable cross-reactivity with the novel anti-CD63 mAbs was the myeloid precursor cell line THP-1 (Fig. S1A). However, as with mast cells, surface staining was only seen after PMA/ionomycin treatment, while native THP-1 cells were not recognized. Only insignificant binding was observed with whole blood-derived human platelets and the melanoma cell line MalMe3, whereas no staining was seen for human promonocytic U937 cells, Jurkat T cells, lymphoblastoid cell line (LCL) B cells, or on fresh peripheral blood mononuclear cells (PBMCs) (Fig. S1, and data not shown).

Degranulation-specific anti-human mast cell mAbs interact with a granular isoform of CD63

Mast cells have been reported before to express CD63 (26), but the remarkable selectivity of the new anti-CD63 mAbs toward degranulated CBHMCs suggested that differently modified isoforms of this protein may be present within human mast cells. The subcellular localization of DSHMC-mAbs-related epitopes was therefore examined by cytospin staining (Fig. 2). In line with the preceding data, degranulation-specific anti-human mast cell (DS α HMC)-linked epitopes are highly enriched in human mast cells but absent from Jurkat control cells (Fig. 2A). More importantly, however, the DS α HMC-mAb signal locates to dotted inclusions that spread throughout the mast cell cytosol. This observation strongly suggests that the corresponding epitope is carried specifically by a granular isoform of CD63. Cell activation finally leads to diminished cytoplasmic CD63 signal and to a redistribution of reactivity. Interestingly, punctate staining was also found in the extracellular space upon degranulation, suggesting that exosomes or secreted entire granuli can carry the functional epitope with them (Fig. 2B). Importantly, the co-existence of differently modified CD63 variants within CBHMCs was also verified by FACS. Although only a minor

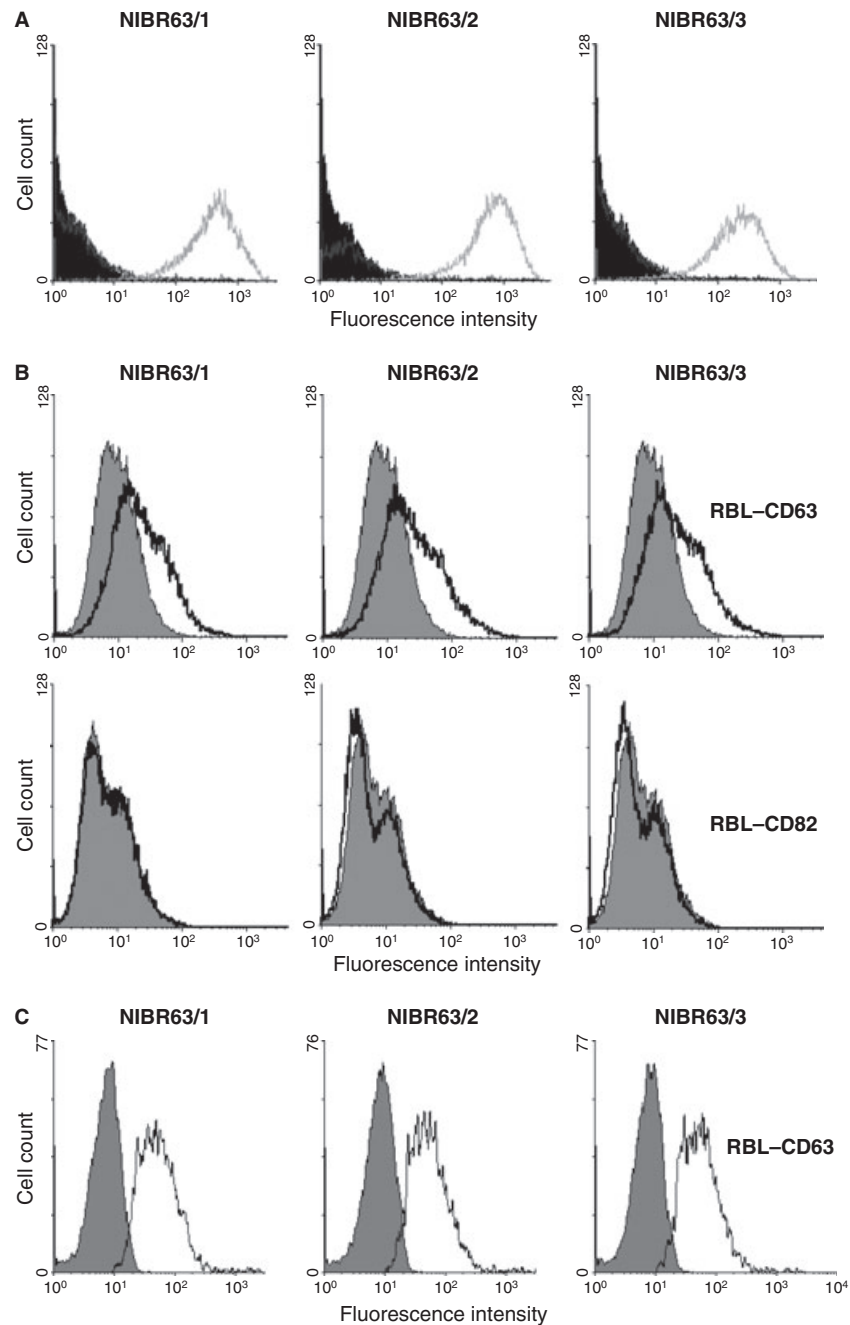


Figure 1 Novel anti-CD63 mAbs specific for degranulated human mast cells. (A) Generation of degranulation-specific anti-human mast cell Abs. Human cord blood-derived mast cells were either stimulated with PMA/ionomycin to induce degranulation (open graphs) or were left untreated (black), and subsequently stained with selected degranulation-specific antibodies. (B) Verification of antibody targets through expression cloning. Rat basophil leukemia (RBL) cells were stably transfected with cDNAs of the human CD63 (upper row) and CD82 genes (lower panels), and without further treatment stained with anti-human mast cell mAbs NIBR63/1, NIBR63/2, and NIBR63/3. Note selective surface staining only for

CD63-transfected cells, but not for cells expressing the related tetraspanin CD82. Nontransfected RBL cells are shown for reference (gray areas). (C) Activation-dependent CD63 surface exposure on RBL transfectant cells. RBL cells expressing the human CD63 gene were either stimulated with PMA/ionomycin to induce degranulation (open graphs) or were left untreated (gray), and subsequently stained with indicated degranulation-specific anti-CD63 mAbs. Note a significant fluorescent intensity shift of stimulated over unstimulated cells, suggesting activation-dependent surface exposure of the respective epitope also in this heterologous expression background.

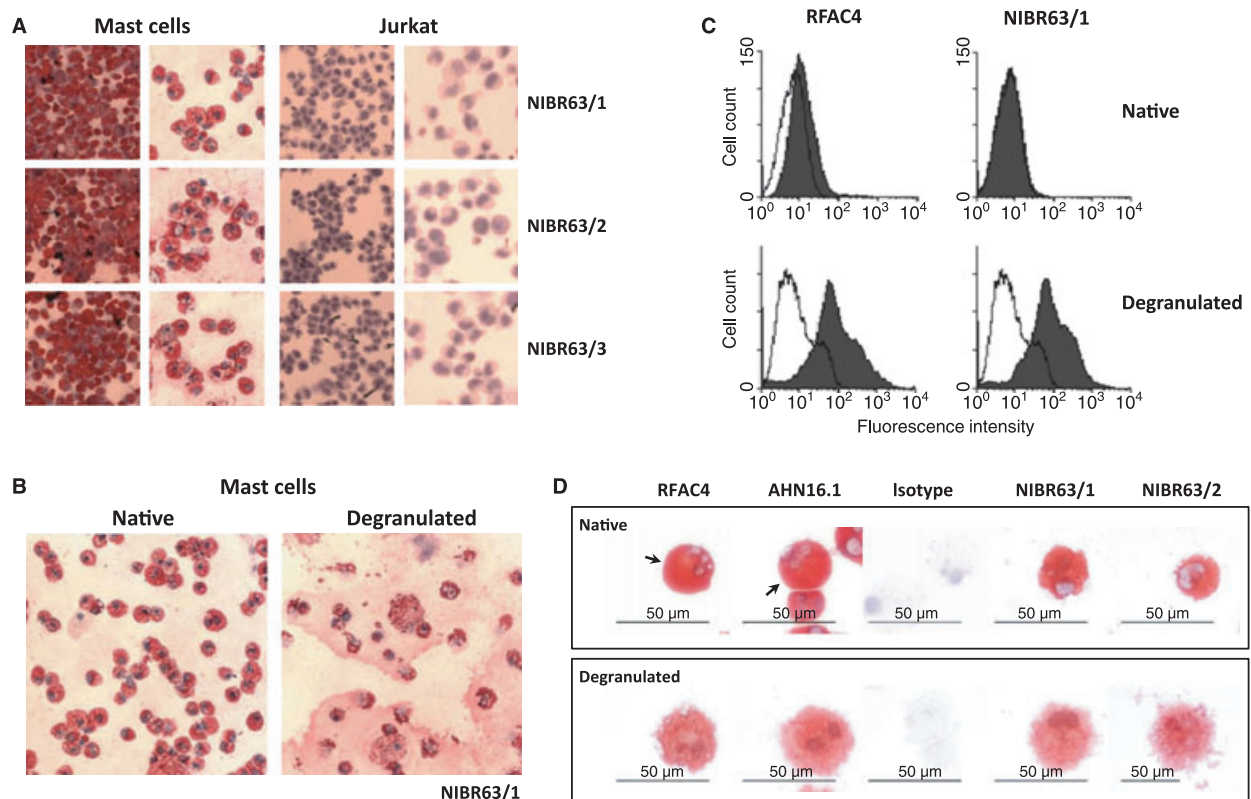


Figure 2 Subcellular localization of CD63 within native and degranulated human mast cells. (A) Mast cells are highly enriched with tetraspanin CD63. Human cord blood-derived mast cells (left panels) and Jurkat control cells (right) were analyzed for expression and subcellular localization of CD63 on cytospin preparations using degranulation-specific mAbs. (B) Degranulation-dependent release of CD63 upon cell activation. Human cord blood-derived mast cells were either incubated in mock buffer or stimulated with PMA/ionomycin to induce degranulation. The activation-dependent subcellular localization of CD63 was subsequently analyzed by APAAP cytospin staining. Whereas degranulation-specific anti-human CD63 mAb NIBR63/1 stains native mast cells predominantly intracellularly (left), staining spreads out to the extracellular space following degranulation (right). (C) Activation-dependent mAbs stain a subpopulation of mast cell CD63. Human cord blood-derived mast cells were either left untreated (native, top), or stimulated with PMA/ionomycin to

induce degranulation (bottom), and subsequently stained with a conventional CD63 mAb (RFAC4, left), or with a degranulation-specific anti-human CD63 mAb (NIBR63/1, right). Whereas the commercial Ab interacts with native and degranulated mast cells, mAb NIBR63/1 reacts only with induced mast cells. (D) Activation-dependent anti-CD63 mAbs do not bind the surface of native mast cells. Mast cells were either left untreated (native) or stimulated with PMA/ionomycin to induce degranulation. Single-cell resolution images of APAAP-stained cytospin preparations are shown using the following staining antibodies (from left to right): conventional anti-CD63 mAbs RFAC4 or AHN16.1; isotype control (murine IgG1); degranulation-specific anti-human CD63 mAb NIBR63/1 or NIBR63/2. Whereas the conventional antibodies showed a distinct rim staining on native mast cells (arrows), degranulation-specific anti-CD63 mAbs apparently discriminate against the cell surface and only associate with intracellular structures.

fraction of CD63 locates to the surface of quiescent mast cells, both native and degranulated MCs are recognized by commercial anti-human CD63 Abs (e.g., RFAC4). DS α HMC-mAbs on the other hand discriminate against the quiescent cell state, and only stain degranulated CBHMCs (Fig. 2C). Additional proof for the co-existence of at least two CD63 variants came from single-cell resolution images of cytospin-fixed native mast cells (Fig. 2D). While the intracellular compartment is intensely stained by all tested CD63 antibodies, a distinct rim-like membrane association was found only with the conventional anti-CD63 mAbs RFAC4 and AHN16.1. The border of DS α HMC-mAbs-labeled cells on the other hand appeared rough and edgy, suggesting that

these antibodies do not bind to CD63 incorporated into the cell membrane. Altogether, these data strongly suggest the co-existence of at least two CD63 variants within human mast cells and an activation-dependent redistribution of a granular isoform of the protein.

A topological hot spot within a surface lobe of CD63

CD63 molecules sorted to granuli can easily be subjects of secondary modifications. For example, *N*-glycosylation had been reported to affect at least three residues within the CD63 protein chain (34). Thus, an extensive mutant analysis was initiated to locate the epitope of specific DS α HMC anti-

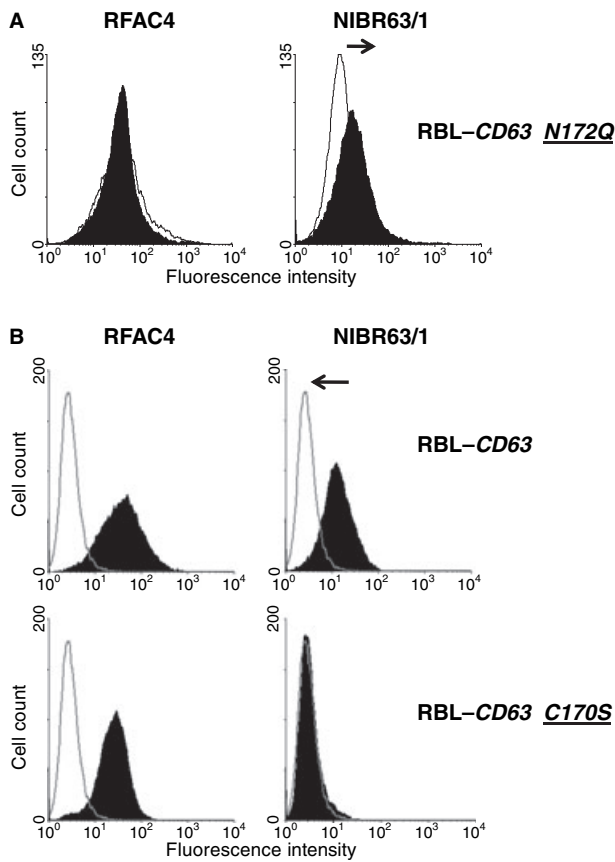


Figure 3 Mutant-based epitope mapping for degranulation-specific anti-human mast cell CD63 mAbs. (A) A CD63 N172Q single site mutation alters the binding characteristics of a degranulation-specific anti-CD63 Ab. Rat basophil leukemia cells stably expressing either a wild-type copy of human CD63 (white area), or a N172Q mutant allele (black area), were incubated in the presence of PMA/ionomycin and subsequently stained with the indicated mAbs. An arrow indicates a fluorescent intensity shift of mutant over wild-type cells, when stained with the degranulation-specific anti-human mast cell Ab NIBR63/1. In contrast, the staining efficiency of the commercial anti-CD63 Ab RFAC4 is not affected by the N172Q mutation (left panel). (B) A C170S single site mutation selectively abrogates CD63 surface recognition by degranulation-specific Abs. Rat basophil leukemia cells stably expressing either a wild-type copy of human CD63 (upper panels), or a C170S mutant allele of the same protein (lower panels), were incubated in the presence of PMA/ionomycin and subsequently stained with the commercial anti-CD63 mAb RFAC4 (left), or with the degranulation-specific mAb NIBR63/1 (right). A mouse IgG1 isotype control is shown for reference (open line).

body association. First, the predicted CD63 glycosylation sites N130, N150, and N172 were successively replaced by glutamine, and the mutant alleles were stably transfected into RBL cells and subjected to FACS analysis (Figs 3A and S2). Point mutations of the first two residues had no significant effect on antibody recognition (Fig. S2). In contrast, a N172Q mutation clearly and selectively improved the reactiv-

ity of degranulation-specific antibody NIBR63/1 while the interaction with conventional anti-CD63 Abs was not changed (Fig. 3A). Improved NIBR63/1 surface staining was also seen with independently cloned double and triple glycosylation mutants, whenever position N172 was included (Fig. S2).

Structure and sequence comparisons of CD63 with related tetraspanins indicated a cysteine cluster motif in the external loop II region of the protein (Fig. S3). C170 of this cluster is localized in utmost sequence neighborhood of the critical glycosylation site N172. We therefore decided to also create CD63 C170S mutant cells. While this mutation was fully tolerated by the conventional anti-CD63 mAbs, binding of DS α HMC-mAbs was completely abolished (Fig. 3B). Thus, cysteine C170 and a glycosylation of asparagine N172 are critical determinants of the degranulation-specific CD63 surface epitope.

C170 is surface accessible and not buried in an obligatory disulfide bridge

Most human tetraspanins contain four universally conserved cysteine residues in their external loop II domain [(13) and Fig. S3]. As has been demonstrated for the previously crystallized CD81 protein, these residues are likely to engage in two structurally determining disulfide bridges (see PDB entry 1IV5 for comparison). Other tetraspanins, including CD63, contain six or even eight cysteines in their external loop II domain [(13) and Fig. 3]. The even total number of cysteines strongly implies the possibility of additional disulfide bridges. In the case of CD63, for example, a covalent association of residue C170 with C177 appeared possible. A series of mutants were thus cloned in which each of the remaining cysteines was individually replaced by serine, expressed in RBL cells, and analyzed by FACS (Fig. 4B). In good agreement with the predicted disulfide interactions between C145–C191 and C146–C169, point mutations of each respective residue pair had comparable effects on CD63 protein expression and antibody recognition. In particular, C145S and C191S point mutations both reduced commercial anti-CD63 binding and cell staining with NIBR63/1 by a factor of 10 when compared to the wild-type allele (MOI shifts from 145 to 14/12, and from 31 to 3.5/3.6, respectively). Similarly, the association of the commercial antibody was reduced by half (MOI shifted from 145 to 65/63), and that of NIBR63/1 by a factor of 4 (MOI shifted from 31 to 7.9/7.1) both in C146S and C169S mutant strains. Clearly disfavoring an obligatory third cysteine bond, however, point mutations C170S and C177S had remarkably dissimilar effects on CD63 antibody binding. Whereas the C170S allele completely abolished CD63 surface staining by DS α HMC-mAbs (see Fig. 3B for comparison), the association of commercial and degranulation-specific antibodies equaled wild-type levels in C177S mutant cells. In conclusion, residue C170 does not seem to participate in an obligatory cysteine bond but rather contributes to a surface accessible epitope *per se*.

With experimental evidence for conserved cysteine bridges C145–C191 and C146–C169 and free residues C170 and C177,

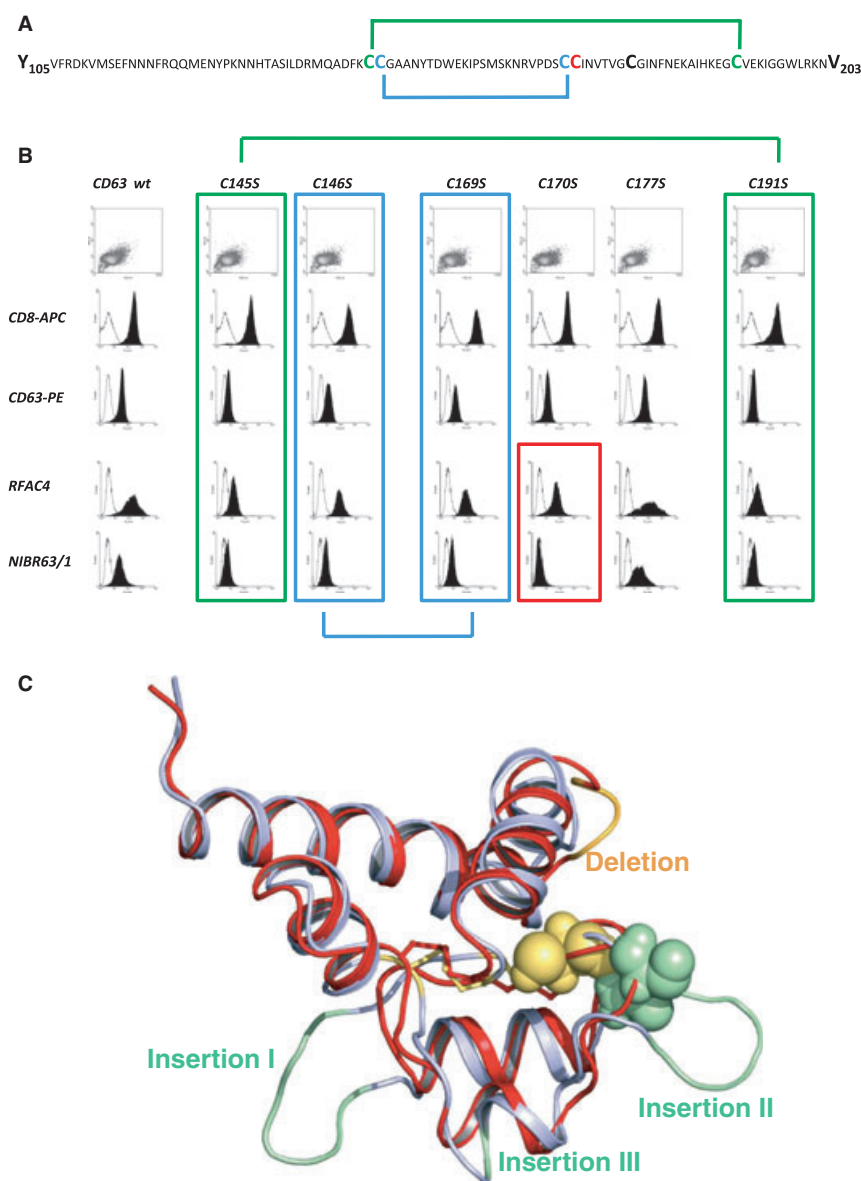


Figure 4 C170 and N172 are critical determinants of degranulation-specific anti-human mast cell CD63 antibody binding. (A) Sequence detail of the human CD63 protein. Depicted is the external loop II region of CD63 (residues Y₁₀₅ throughout V₂₀₃). Cysteine residues in bold. Suggested disulfide bridges C145/C191 (green) and C146/C169 (blue) as indicated. (B) Cysteine mutations suggest distinct disulfide pairing within CD63. FACS analysis of PMA/ionomycin-treated rat basophil leukemia cells stably expressing either a wild-type copy or various cysteine mutants of the human CD63 gene. Cells had been sorted and normalized on basis of mCD8a (Ly-2) cotransfection (anti-CD8-APC, first row). Total surface expression of CD63 was deduced from direct labeling with the commercial anti-CD63-PE antibody Immunotech No.1914 (second row). CD63 staining was performed either with the commercial Ab RFAC4 (third row) or with the state-specific anti-CD63 Ab NIBR63/1 (bottom row). Histograms depict cell counts (events) as a function of fluores-

cence intensity in the respective detection channels. Similar staining characteristics for mutant pairs C145/C191 (green) and C146/C169 (blue) suggest cysteine bond formation between the indicated residues. A CD63 C170S mutation selectively abrogates cell surface staining by state-specific mAb NIBR63/1, but not by the commercial reference antibody (enclosed in red). (C) Homology model of the human CD63 protein external loop II domain. Structural details of the human CD63 protein external loop II region. Residues Y105 throughout V203 are shown (cyan). This is a homology model based on the crystal structure of the related tetraspanin CD81 (PDB entry 1IV5 in red). Note one deletion (orange) and three sequence insertions (turquoise) with respect to CD81. Conserved disulfide bridges C145-C191 and C146-C169 form a central axis and are highlighted in yellow. Critical residues C170 (yellow) and N172 (turquoise) colocalize to the base of a surface lobe at insertion point II, which may be inherently flexible.

we built a homology model of the CD63 external loop II domain on the basis of the published crystal structure of tetraspanin CD81 (Fig. 4C). Our calculation places both residues critical for DS α HMC antibody binding, C170 and N172, in surface-exposed juxtaposition. Interestingly, both residues locate to a characteristic sequence insertion within the CD63 protein that clearly distinguishes this tetraspanin from CD81 (Fig. 4C). Apparently surface exposed, this lobe forms a structural hallmark on the CD63 protein and a signature structure for antibody recognition. Thus, antibodies NIBR63/1, /2, and /3 recognize a characteristic surface feature of the human CD63 protein, of which residues C170 and N172 are essential constituents.

Repeated cycles of mast cell activation

Published data consent that in general only a minor fraction of the total granular enzymatic content of a mast cell can be mobilized *in vitro* upon Fc ϵ RI α cross-linking (5, 6). We have quantified degranulation in cells from three independent donors using various stimuli (Fig. 5A). Cells were coated with different IgE molecules (B11, Jw8, or antiphosphocholine antibodies) overnight, and subsequently a corresponding cross-linker agent was added (Le27, NIP-BSA, and PC₁₇-BSA, respectively). The activation-dependent release of β -hexosaminidase from mast cell granuli was then quantified. We find that the IgE-driven response is in the same range in all three donors and is comparable to published values. More importantly, also the three different triggering systems were comparable in efficiency. Particularly, we find that a single degranulation stimulus induces a net release of no more than 15% total enzymatic activity, independent of mast cell donor and IgE/antigen pair (Fig. 5A). Noteworthy, these values reflect experimental conditions, wherein cell density, molar ratio of both IgE antibody and antigen, and incubation times had been optimized in titration experiments beforehand (data not shown).

Altogether, these data strongly suggested that mast cells retained a major part of their granular content, which could be released upon subsequent stimulation. To test whether repeated mast cell degranulation *in vitro* was possible, cells that underwent a primary degranulation (Jw-8/NIP-BSA system) were collected and allowed to recover over night. Medium and supplements were exchanged during the recovery phase and the cell surfaces recoated with a different IgE species (antiphosphocholine). A second degranulation was induced by the addition of the corresponding secondary antigen (PC₁₇-BSA; Fig. 5B,C). In all cases tested, mast cells also responded to the second degranulation stimulus with substantial levels of β -hexosaminidase release. Although the absolute enzymatic turnover during degranulation cycle 2 varied from donor to donor, the relative mobilization of β -hexosaminidase activity always equaled 50–70% of the first stimulation. A second stimulation using the same IgE/antigen system (i.e., a repeated coating with Jw8 antibody, and triggering with NIP-BSA) however did not elicit a further response (Fig. 5B). Thus, human cord blood-derived mast cells can undergo iterative degranulation reactions *in vitro*.

CD63 can modulate repeated mast cell degranulation

Anti-rat CD63 antibodies have previously been shown to inhibit the degranulation of RBL cells (35, 36). Although from a different species background, these data prompted us to test whether anti-human CD63 Abs would influence the activation of human mast cells as well. Degranulation of CBHMCs was induced *in vitro*, either in the presence or absence of anti-human CD63 mAbs (Fig. 6). Not unexpectedly, several commercially available anti-CD63 mAbs significantly impaired the degranulation efficiency of CBHMCs (Fig. 6A). The degranulation-specific anti-human mast cell CD63 mAbs NIBR63/1, /2, and /3 on the other hand had no effect on primary degranulation, likely so, because these antibodies do not bind to native mast cells *per se*. Instead, when added during a secondary degranulation cycle to CBHMCs which were previously degranulated, also the new antibodies had a significant and reproducible inhibitory effect (Fig. 6B). In particular, the net release of β -hexosaminidase activity was reduced to 65–75% of uninhibited control cells, when a second degranulation was performed in the presence of DS α HMC-mAbs NIBR63/1 or NIBR63/2. These values are directly comparable to what can be achieved with conventional anti-CD63 Abs (e.g., AHN16.1), thus there is essentially no functional difference between anti-CD63 antibodies in the impairment of repeated mast cell degranulation.

To provide additional evidence for iterative cycles of mast cell activation and for a functional significance of CD63 in this context, we followed mast cell degranulation also by activation markers different from β -hexosaminidase. In particular, we have simultaneously assessed serum concentrations of various cytokines 6 h after addition of the first or second triggering antigen (Fig. S4B). Similar to the preceding enzyme release data, repeated secretion pulses were also found for several of the investigated cytokines, e.g., GM-CSF, IL-6, and IL-13. While the first secretion cycle was preferentially impaired by the commercial anti-CD63 mAbs MEM-259 and AHN16.1 only, conventional and state-specific anti-CD63 mAbs strongly inhibited iterative rounds of cytokine release. Anti-CD63 mAbs had only a marginal effect on TNF α release during the first secretion cycle. However, mast cells hold preformed stocks of this particular cytokine (37), and the secretion of TNF α is not strictly dependent on antigen (38). Thus, both enzymatic data and the secretion profiles of various cytokines strongly support the idea that iterative rounds of mast cell activations are possible, and also a functional role of tetraspanin CD63 in this context.

From these experiments, it was initially unclear, however, whether indeed individual cells are capable of serial degranulation, or what we have seen reflects the degranulation of separate cell subsets. We therefore included FACS sorting after an initial round of degranulation to subdivide the cell population on basis of CD63 surface exposure (Fig. 7A). Following an initial degranulation cycle, cells were thus briefly stained with an anti-CD63 mAb. Although essentially all cells had responded to the first activation cycle, the absolute amount of CD63 surface expression varied significantly within the population. Cells were therefore separated into low, intermediate,

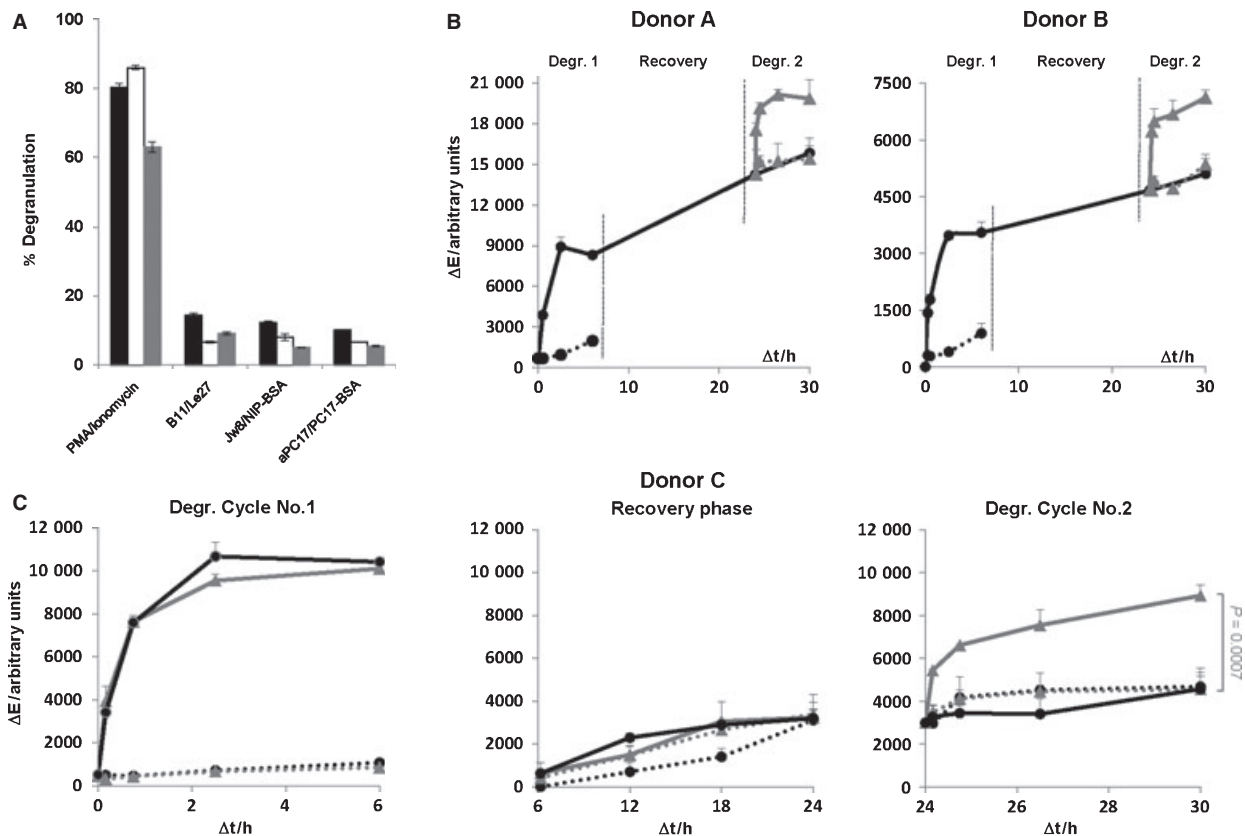


Figure 5 Iterative IgE/antigen-induced human mast cell degranulation *in vitro*. (A) Comparative analysis of mast cell degranulation *in vitro*. Depicted is the degranulation efficacy of three independent CBHMC cultures (black, white, and gray bars) in response to different activation stimuli. Degranulation was either induced with PMA/ionomycin (chemical induction), with a cross-linking antibody system B11/Le27, or with one of the multivalent antigen systems Jw8/NIP-BSA or α PC₁₇/PC₁₇-BSA, respectively. Activity of β -hexosaminidase released into the medium is shown in percent of total, with the enzymatic activity of the whole cell lysate set to 100%. Note that IgE-based degranulation systems allow for mobilization of only 5–15% of the total enzymatic content. (B) Cord blood-derived human mast cells undergo iterative degranulation cycles. Native mast cells were surface coated with humanized IgE antibody Jw8, and then a first degranulation was induced by the addition of cognate NIP-BSA antigen. After a recovery phase in presence of new coating Ab (either Jw-8 or α PC₁₇-IgE), a second degranulation stimulus was set by addition of the corresponding antigen (NIP-BSA or PC₁₇-BSA, respectively). Activity of β -hexosaminidase released into the medium is shown (y -axis: fluorescence counts)

for (i) spontaneous degranulation of Jw-8-coated cells (dotted black line); (ii) NIP-BSA induced first degranulation and subsequent spontaneous degranulation (solid black line); (iii) NIP-BSA induced first degranulation followed by a secondary stimulation again with Jw8/NIP-BSA (dotted gray line); (iv) NIP-BSA induced first degranulation followed by an α PC₁₇/PC₁₇-BSA induced second degranulation (solid gray line). Two independent mast cell batches (Donor A and B) are shown for comparison. (C) Comprehensive temporal resolution of repeated mast cell activation *in vitro*. The same experimental procedure was performed with cells from a third donor, and activity was recorded with a higher time resolution. The following experimental conditions are depicted: (i) Spontaneous degranulation of uncoated cells (dotted black line); (ii) spontaneous degranulation of Jw-8-coated cells, recovery, and spontaneous degranulation after α PC₁₇ coating (dotted gray line); (iii) NIP-BSA induced degranulation first, recovery, and spontaneous degranulation after α PC₁₇ coating (solid black line); (iv) NIP-BSA induced degranulation first, recovery, and PC₁₇-BSA induced second degranulation (solid gray line). Statistical significance of spontaneous over antigen-induced degranulation in the second cycle as indicated ($P = 0.0007$).

and high CD63 expressing fractions. Each population was independently recovered, coated with IgE molecules required for the second cycle, and subjected to another round of degranulation (Fig. 7B). Although the efficiency of the second degranulation reaction varied, degranulation could be re-induced in all three fractions. Most remarkably, high responders of the first cycle degranulated also very efficiently in the second cycle, while low responders of the first round

responded also lower thereafter. Thus, in spite of their intrinsic heterogeneity, persisting responsiveness to IgE/antigen stimuli is an inherent characteristic of CBHMC *in vitro* cultures.

Nonetheless, we found no evidence for specialized mast cell subclasses, which preferably respond to one degranulation stimulus or the other. Instead, the addition of anti-CD63 Abs to low, intermediate, and high CD63 expressing fractions reproducibly diminished the efficiency of repeated mast cell

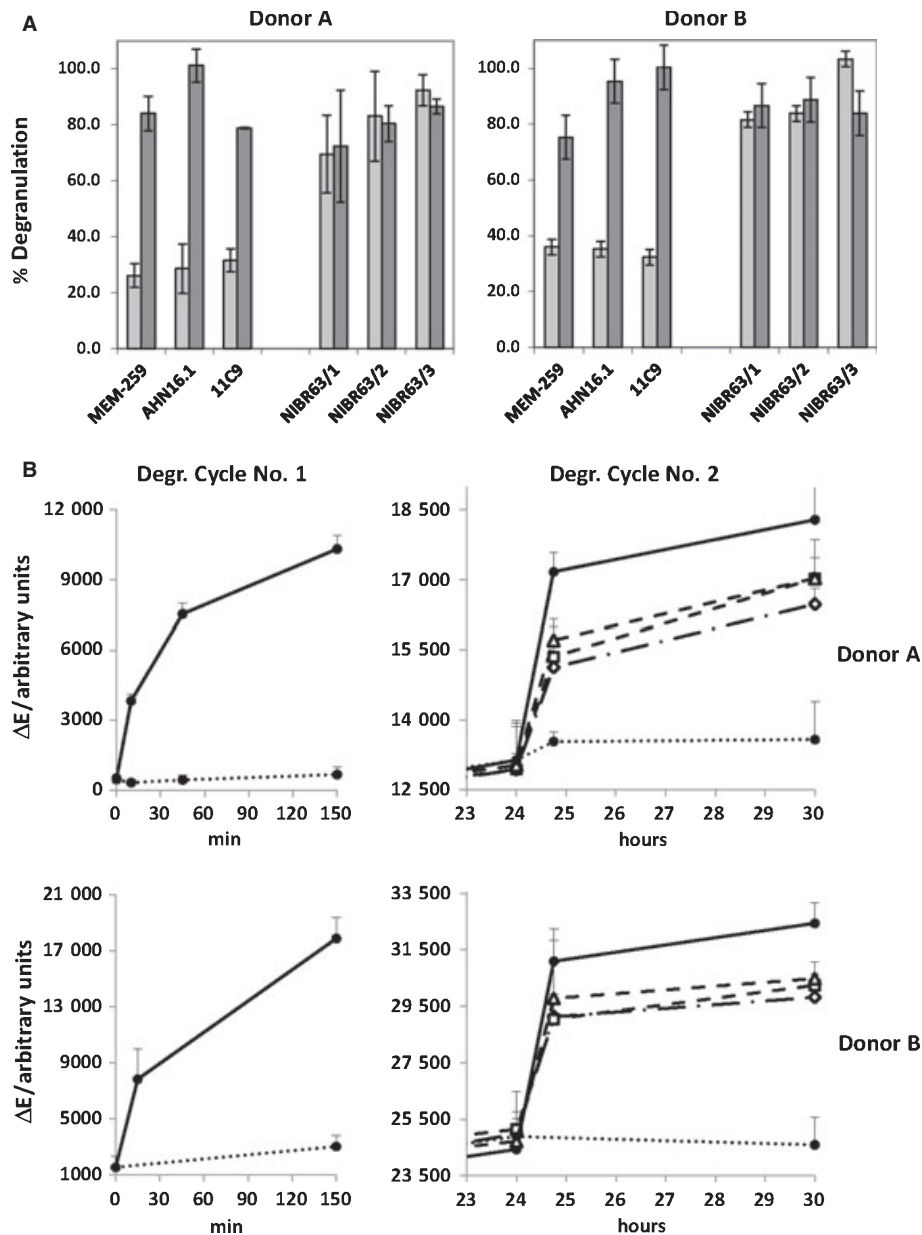


Figure 6 Novel anti-human CD63 mAbs selectively inhibit the degranulation of pre-activated mast cells. (A) Various commercial anti-CD63 Abs impair human mast cell degranulation. Human cord blood-derived mast cells were preincubated in the presence (light bars) or absence (dark bars) of the indicated anti-CD63 mAbs for 1 h before triggering IgE/antigen-induced degranulation. Note significantly impaired degranulation efficiency in the presence of commercial mAbs MEM-259, AHN16.1, or 11C9, but not for the state-specific anti-human CD63 mAbs NIBR63/1-3. The amount of reporter enzyme β -hexosaminidase that was released from uninhibited

control cells was set to 100% degranulation efficiency. (B) State-specific anti-human CD63 mAbs selectively impair repeated mast cell degranulation. A first degranulation cycle (left panels) was induced with Jw-8/NIP-BSA (solid black line). Spontaneous degranulation of IgE-coated cells is shown as dotted line. After recovery, a second degranulation cycle was induced with α PC₁₇/PC₁₇-BSA (right panels) in the presence of no blocking antibody (solid line), NIBR63/1 or NIBR63/2 (dashed lines), or AHN16.1 (dashed/dotted line). Spontaneous degranulation in the second activation cycle is shown as dotted line again.

degranulation by 20–35% (Fig. 7B). We also kept aliquots of low, intermediate, and highly degranulated cells in culture and observed them in regular intervals. While mature mast cells hardly proliferate, the majority of sorted cells survived

for several weeks (data not shown). This is a further indication that degranulation is not inevitably coupled with cell death and in general, native mast cells may undergo several activation cycles.

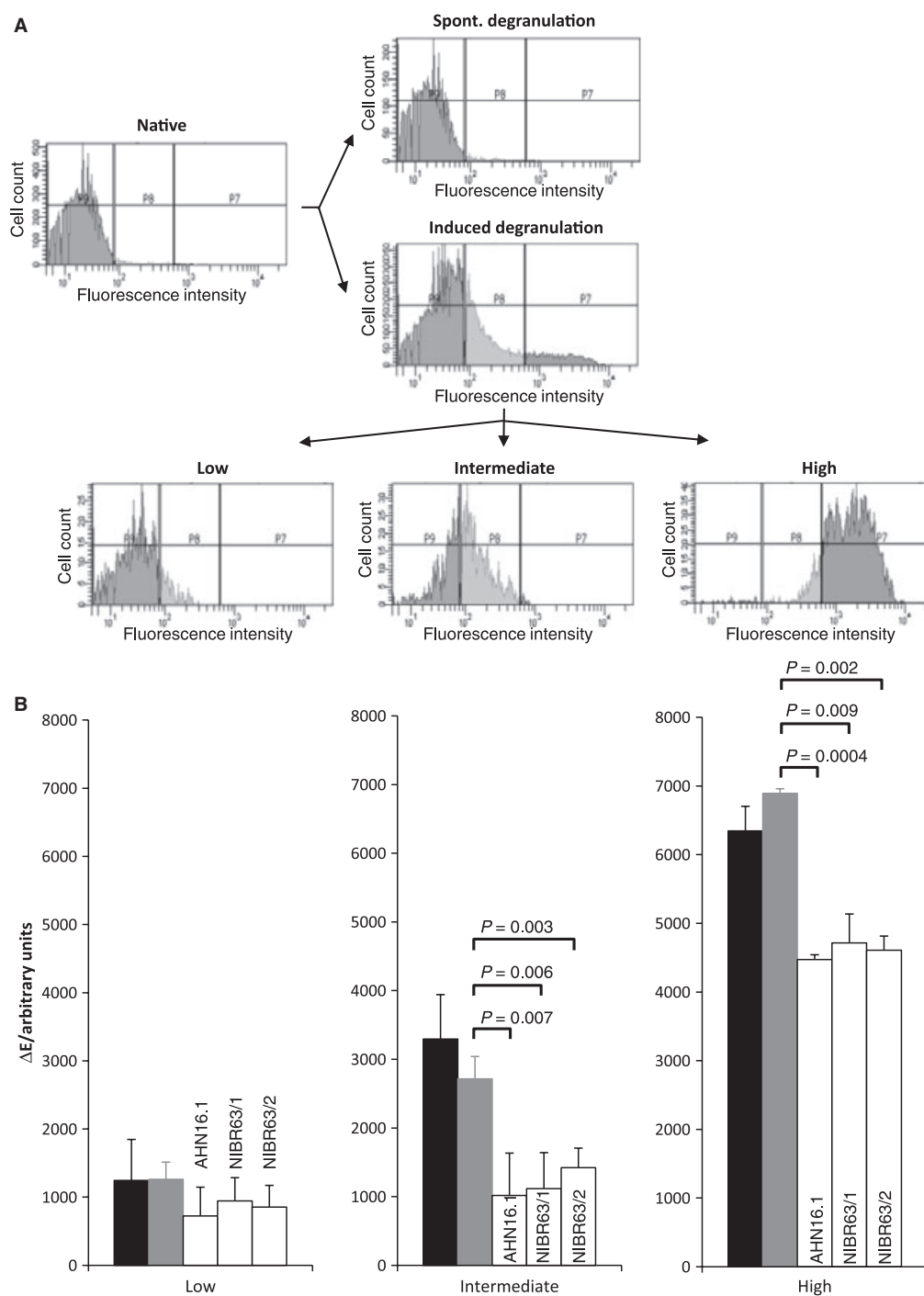


Figure 7 State-specific anti-human CD63 mAbs inhibit repeated mast cell degranulation. (A) CD63 expression-based fractionation of activated human mast cells. IgE-coated human mast cells were antigen treated and subjected to FACS sorting after a degranulation period of 1 h. Based on CD63 surface expression, low, intermediate, and high content fractions were separated. Their homogeneity was verified by analytic separation (bottom three panels), and then each sample was subjected to another round of cell activation. (B) State-specific and commercial anti-CD63 Abs impair iterative

mast cell degranulation. Pretreated (Jw8/NIP-BSA), FACS-separated human mast cell fractions were restimulated with α PC/PC₁₇-BSA. This repeated mast cell degranulation was performed either in the absence of antibodies (black), or in the presence of an isotype control mAb (gray), or with anti-CD63 mAbs (white; from left to right AHN16.1, NIBR63/1, NIBR63/2, as indicated). To indicate significance of inhibition in intermediate and high responder fractions, *P*-values are given.

Discussion

Only accidental observations have suggested that mast cells may undergo more than one cycle of activation and degranulation, but no convincing experimental evidence has been brought forward thus far. One critical aspect of established model systems is that they mobilize only a subfraction of the total granular content when mast cell degranulation is induced *in vitro*. Even under optimized conditions, the documented net release from human mast cells in general stays below 20% of the total enzymatic activity (5). This value is somewhat higher with cultured murine mast cells and cell lines (39), and releases of up to 60% have been described after antigen-specific triggering in exceptional cases (40).

Our data for three independent IgE/antigen systems used on mast cells of several independent human donors are closer to the lower end, and thus confirm this perception. Although potentially reflecting the limitations of experimental research, this finding implies that mast cells may keep reserves of granuli. Thus, they could have the potential to undergo several degranulation cycles, which ultimately may be an indication for persisting responsiveness. Mast cells may actively retain the majority of granules for subsequent cell activation or may even contain specialized receptor-granuli subsets that respond to selected degranulation signals. We now provide first experimental evidence of iterative degranulation activity in cultured human mast cells.

Interestingly, repeated mast cell stimulation seems to necessitate alternating antigen stimuli. A repeated response to the same antigen trigger was not observed. Importantly, even when cells had been recoated with the respective IgE species again, the same antigen did not allow for a second degranulation reaction. These observations clearly suggest that mast cell activation also triggers an attenuation mechanism, which may serve to prevent a full-blown degranulation under common circumstances.

Contradicting a paradigm according to which degranulation and cell death may be inevitably coupled, survival of postdegranulation-state mast cells has occasionally been observed (10, 11). Based on the analysis of microscopic images, which suggested extensive intracellular re-organization and the appearance of multivesicular bodies, even a recovery of postdegranulation-state mast cells was proposed. Somewhat more recently, the survival of antigen-treated murine mast cells was followed on the single-cell level by time-lapse microscopy (12). Also we find that activated, partially degranulated human mast cells survive in this resting state *in vitro* for at least several days and may undergo a next round of activation if they encounter an alternative antigen. *In vivo*, one could therefore assume that partly degranulated mast cells can persist at the site of activation and can amplify a subsequent response if another antigen is sensed. Taken together, one can speculate that a full-blown mast cell response may be very rare in real life and could only be induced if multiple polyvalent antigens occur at the same time and place. In addition, mast cells may have the capacity to replenish and reload their granuli. In possible support of this, CD63 was found to be involved in vesicular targeting of

various proteins, e.g., neutrophil elastase (41). Thus, a bidirectional exchange of CD63, from granuli to plasma membrane and back, can be envisioned. Such retrograde transport may support the re-uptake of granular cargo; either to replenish the cells enzymatic content or for autocrine regulation. Ultimately, the re-uptake of CD63 in postdegranulation mast cells may also have a functional counterpart in cell differentiation and ontological granule formation.

To better characterize the mast cell life cycle, we developed novel tools to unequivocally distinguish between resting and degranulated mast cells. Among the more than 100 mAbs that we raised against degranulated CBHMCs, a group of antibodies with high specificity turned out to recognize CD63. This was initially surprising, because CD63 is known to be present also on the surface of native mast cells, which our antibodies clearly do not detect. However, in granular cells like platelets, dendritic cells, and mast cells, a major fraction of this protein is also retained within secretory vesicles (20). Moreover, cross-reactivity experiments indicated that of all cell lines tested, only myeloid precursor THP-1 cells (42) significantly reacted with the novel DS α HMC antibodies, but as with mast cells, this surface staining was strictly activation dependent. Thus, our antibodies are apparently specific for a granular variant of the protein and discriminate against the plasma membrane fraction. At least two requirements are thus needed for the successful detection of the epitope, (i) a particular biosynthetic mechanism, found only in mast cells, THP-1 cells, and (although significantly less effective) in RBL cells transfected with the human CD63 gene; and (ii) a controlled subcellular localization. Accordingly, the particular isoform of CD63 that our antibodies bind to may be selectively sorted into granuli, or formed directly in the granuli, and/or also actively excluded from the cell surface. Interestingly, activated mast cells were still tested positive when the staining antibody was added hours after the degranulation reaction. Taken together, our observations identify a conformationally distinct, inherently stable isotype of CD63 that does not spontaneously convert into the plasma membrane form of the protein. A mere redox-switch for example would not serve to explain this longevity. Rather, the new isoform of CD63 may either resemble a specialized folding variant, whose biosynthesis pathway targets late endosomes, and does not reach the cell surface, or must be the product of particular intragranular modification. Mast cell granuli are highly enriched with various enzymatic activities, among which sugar-modifying enzymes like glycosidases, epimerases, and sulfotransferases are particularly abundant (43, 44). With mutant constructs and homology modeling, we were able to locate the DS α HMC antibody-binding epitope to a surface lobe of CD63 that extends from the external loop II domain. Two residues of particular importance for antibody binding locate to the base of this protrusion and have been identified as C170 and N172. Further mutant analyses suggest that cysteine 170 may be surface accessible and not buried in an obligatory disulfide bond. Even a putative secondary modification of this amino acid (e.g., nitrosylation or oxidation) is thinkable and may prevent the formation of a disulfide bridge with neighboring C177. Asparagine N172 on the other

hand is a target of protein N-glycosylation. Our data seem to suggest that an elimination of this sugar modification supports the association of antibodies NIBR63/1-3. However, the data were obtained with a protein expressed heterologously in RBL cells, where its sugar modifications may not be identical to the human form. In conclusion, antibody recognition is determined by surface accessibility of the region around C170 and N172.

Several commercial anti-CD63 mAbs inhibit the degranulation of basophil and mast cells, whereas our novel mAbs do not interfere with mast cell degranulation *per se*. Primed (i.e., previously activated and partially degranulated) mast cells on the other hand are effectively targeted, and their repeated degranulation significantly impaired by the new antibody generation. Importantly, this inhibition is not restricted to the release of granular enzymatic cargo exemplified by β -hexosaminidase, but also effects the formation and repeated secretion of various cytokines, like TNF α , GM-CSF, IL6, and IL-13. The novel anti-CD63 antibodies could therefore have an impact not only on mast cell survival, differentiation, and regranulation, but may also affect the attraction and paracrine regulation of other immune cells. In addition to this, CD63 has an established history as diagnostic marker of allergy. The basophil activation test (BAT) for example exploits CD63 surface expression to assess the severity of insect venom or rubber latex allergy in man (22, 23). Based on a pronounced functional homology of basophils and mast cells, we expect that our antibodies may also be used in such diagnostic procedures. Moreover, their characteristic activation-dependency may provide a distinguishing advantage over existing BAT systems, as far as stringency and signal-to-noise ratio are concerned.

In conclusion, this work provides experimental evidence that human mast cells can undergo repeated activation and degranulation reactions. A hitherto unknown, granular

variant of CD63 is a reliable surface marker of such pre-activated cells. Antibodies targeting this structure impair repeated mast cell degranulation *in vitro* and may thus have therapeutic or diagnostic applications in allergy.

Acknowledgments

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Supporting Information

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

Figure S1. A degranulation-specific anti human mast cell mAb does not cross-react with platelets, MalMe3, or U937 cells.

Figure S2. Protein N-glycosylation affects CD63 protein binding by DS α HMC-mAbs.

Figure S3. Cysteine cluster alignment of human tetraspanin proteins.

Figure S4. Effects of anti CD63 mAbs on the release of β -hexosaminidase and selected cytokines from activated human mast cells.

Table S1. Inventory of antibodies used in this Report.

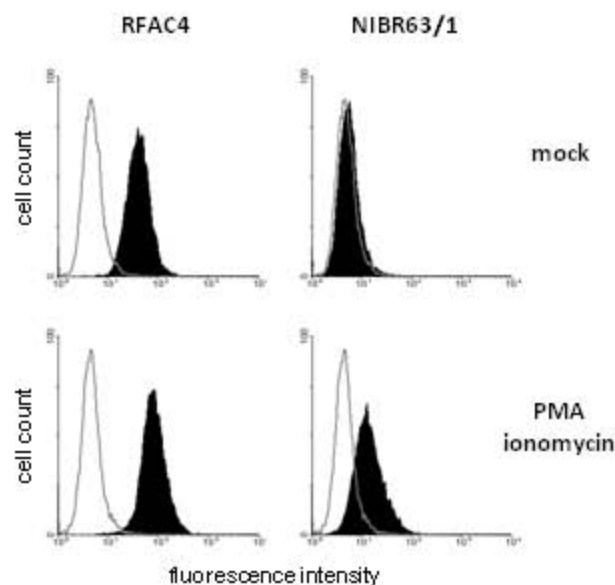
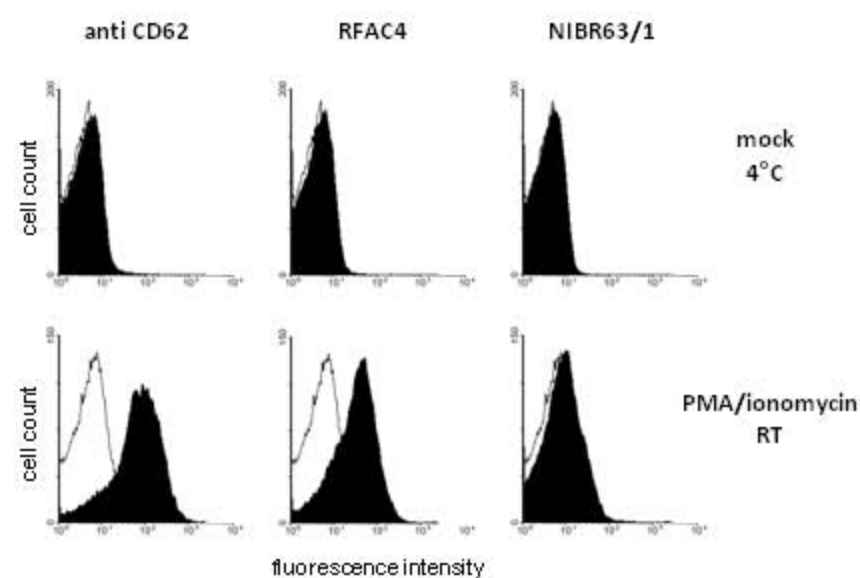
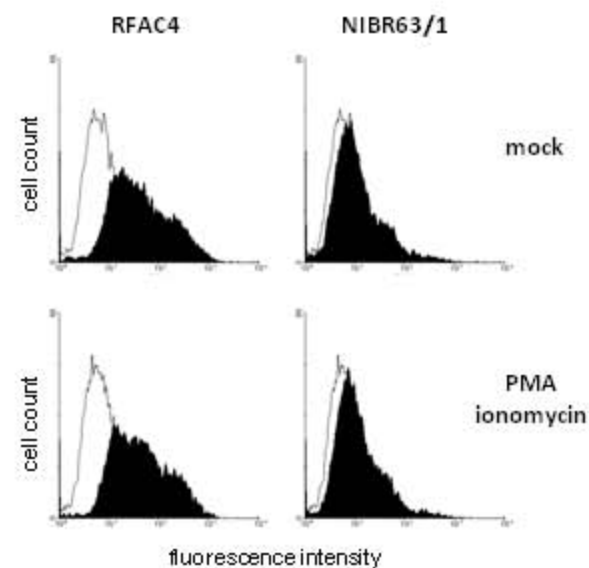
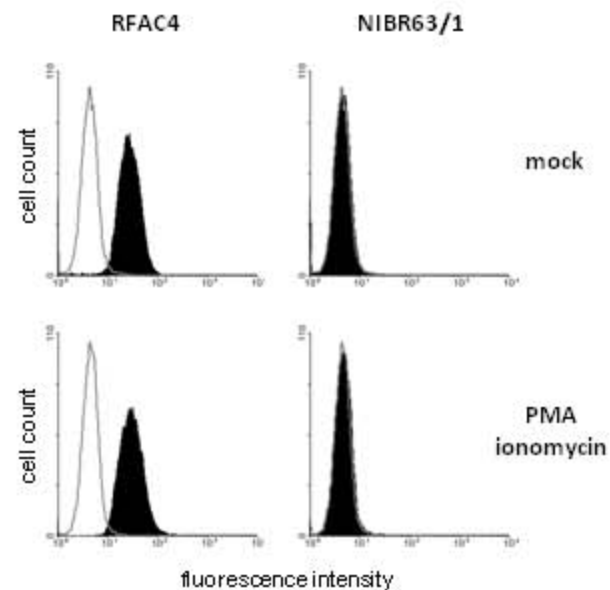
Table S2. List of oligonucleotides (primers) used in this Report.

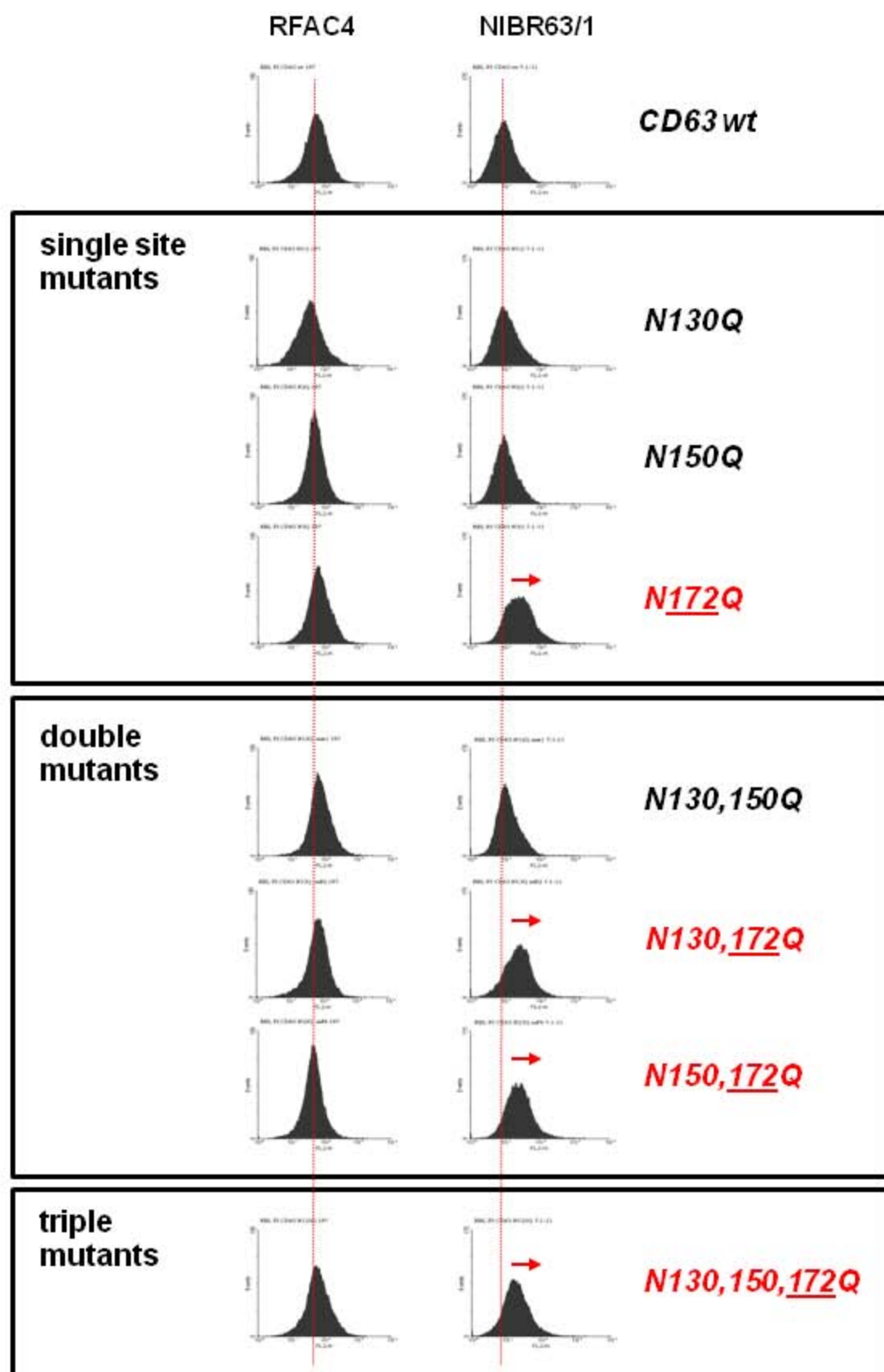
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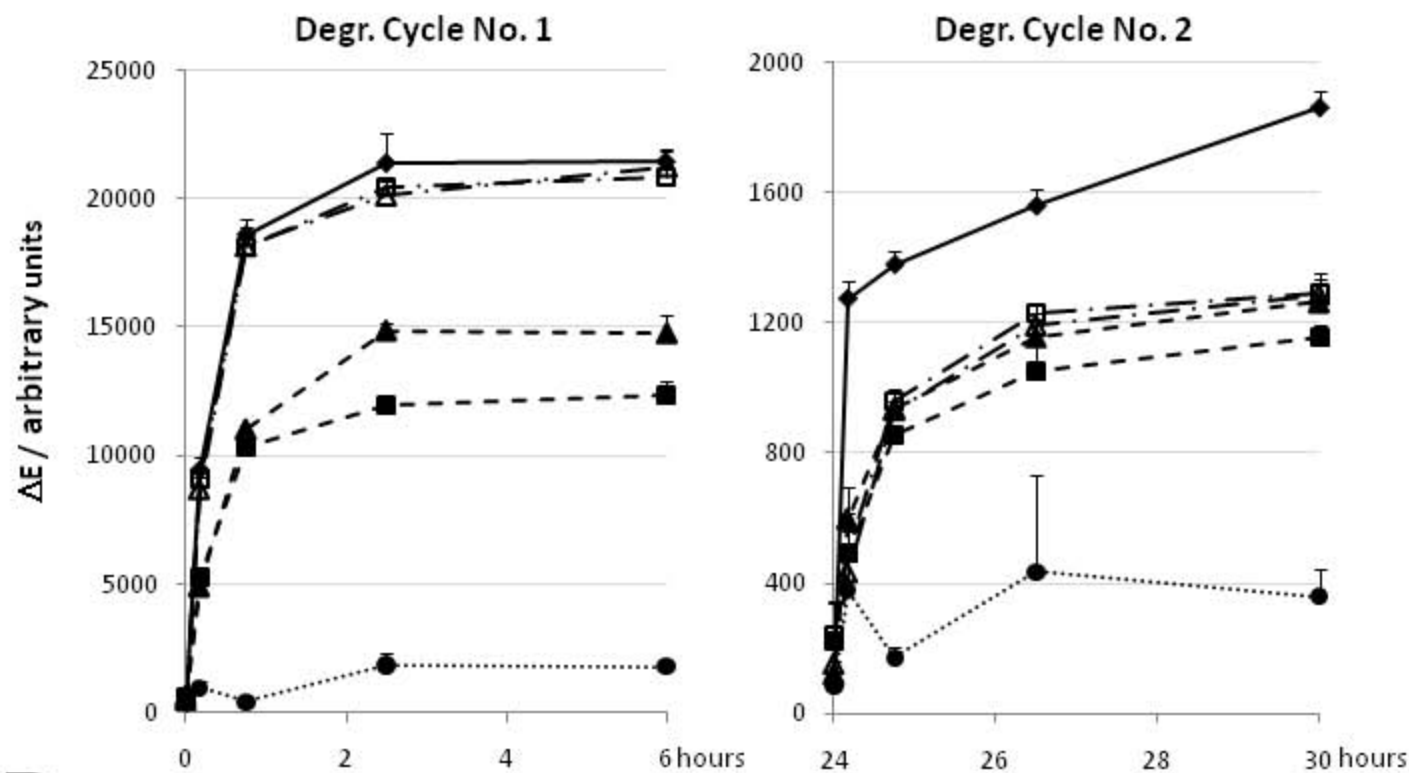
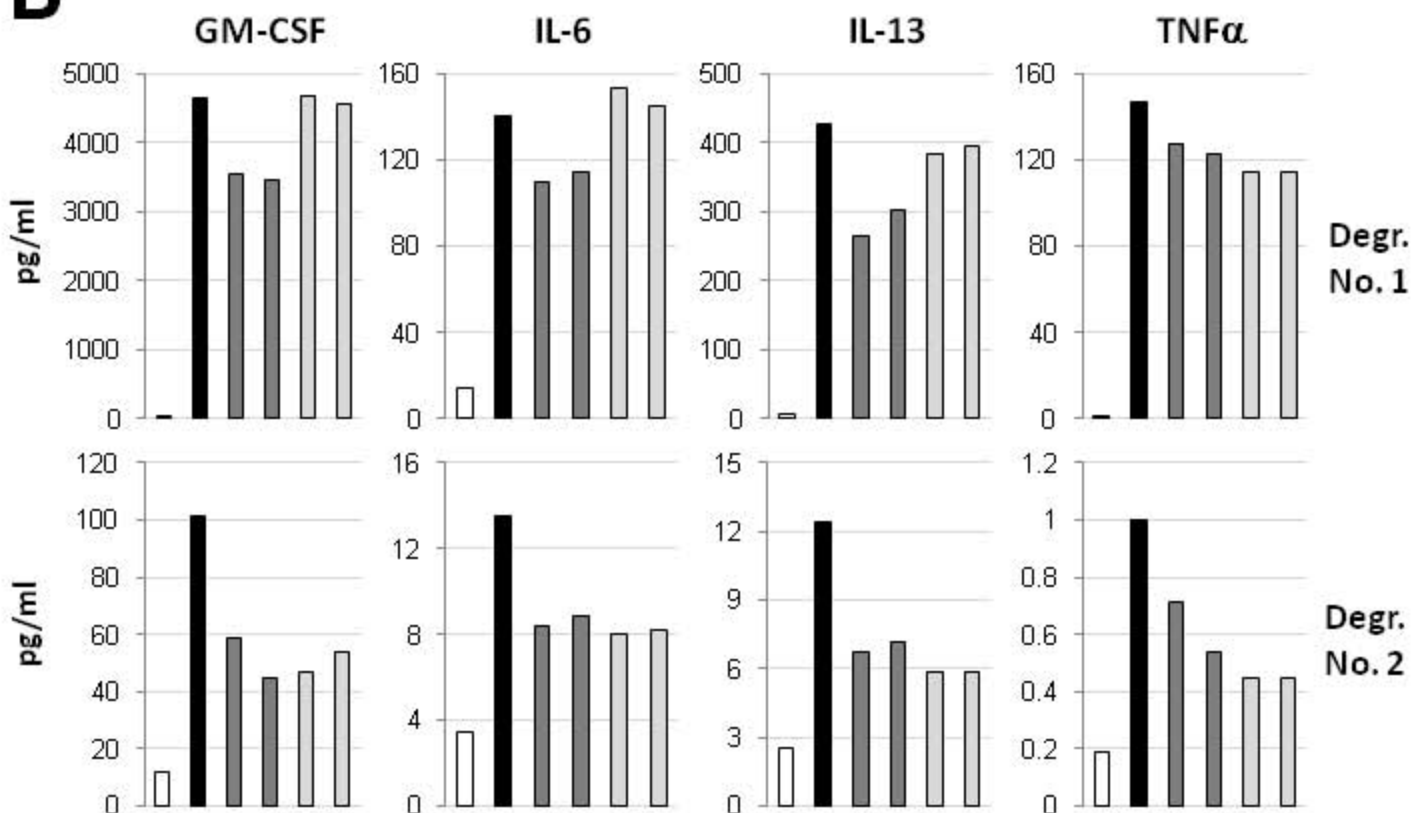
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A**THP-1****B****platelets****C****MalMe3****D****U937**



A **β -Hexosaminidase****B**

Schäfer et al., 2010
 Supplementary Figure 4

Effects of anti CD63 mAbs on the release of β -hexosaminidase and selected cytokines from activated human mast cells

Supplementary Information

A granular variant of CD63 is a regulator of repeated human mast cell degranulation

Running title

CD63 and Mast Cell Life-Cycle

Authors

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Supplementary Figure Legends

Supplementary Figure 1

A degranulation-specific anti human mast cell mAb does not cross-react with platelets, MalMe3, or U937 cells

- A) **NIBR63/1 interacts with THP-1 cells in an activation-dependent manner.** Myeloid precursor-like THP-1 cells were either incubated in mock buffer, or degranulated in a buffer supplemented with PMA/ionomycin. Cell surface staining with a commercial anti-CD63 mAb RFAC4 indicated significant CD63 protein expression. Although activated THP-1 cells clearly cross-react with the degranulation-specific anti human mast cell CD63 Ab NIBR63/1, virtually no surface staining was found for native THP-1 cells.
- B) **Degranulation-specific anti human mast cell Ab NIBR63/1 does not bind to platelets.** Human whole blood derived platelets were either incubated at 4°C to prevent cell activation, or in a buffer supplemented with PMA/ionomycin to induce degranulation at RT. Cell surface staining was performed with an anti human CD62P mAb (BD Pharmingen, Cat. No. 556087, activation control), the commercial anti human CD63 mAb RFAC4, or with the degranulation-specific mAb NIBR63/1. Incubation of the activated platelet fraction with NIBR63/1 induced a fluorescent intensity shift of $\Delta\text{MFI} \leq 3$ and was therefore considered as insignificant. An isotype control is given for reference (white).
- C) **Degranulation-specific anti human mast cell Ab NIBR63/1 does not bind to MalMe3 cells.** Human MalMe3 melanoma cells were incubated in mock buffer or in a buffer system supplemented with PMA/ionomycin to induce degranulation. Cell surface staining was performed either with the commercial anti CD63 mAb RFAC4 or

a degranulation specific anti human CD63 Ab NIBR63/1. Independently of the activation state, only insignificant surface staining was observed with NIBR63/1 ($\Delta\text{MFI} \leq 3$). Isotype control for reference (white).

D) Degranulation-specific anti human mast cell Ab NIBR63/1 does not bind to U937 cells. Promonocytic U937 cells were either incubated in mock buffer, or degranulated in a buffer supplemented with PMA/ionomycin. Cell surface staining with the commercial anti CD63 mAb RFAC4 indicated significant CD63 protein expression. In contrast, no reactivity with the degranulation-specific anti human mast cell CD63 Ab NIBR63/1 was found.

Supplementary Figure 2

Protein N-glycosylation affects CD63 protein binding by DS α HMC mAbs

Cell surface staining of PMA/ionomycin-treated rat basophile leukemia (RBL) cells stably transfected with a wild type copy or indicated glycosylation-site mutants of the human CD63 gene. Cells were either stained with the commercial anti CD63 antibody RFAC4, or with the degranulation-specific mAb NIBR63/1. The latter shows a fluorescent intensity shift of mutant over wild-type cells, whenever a N172Q mutation is included. Depicted are cell counts (events) as a function of fluorescence intensity.

Supplementary Figure 3

Cysteine cluster alignment of human tetraspanin proteins

Sequence detail alignment of indicated human tetraspanin proteins. Depicted is the central part of the external loop II region, which contains a cysteine cluster motif consisting of 4, 6, or 8 individual residues. Universally conserved cysteines (1-4, red), semi-conserved cysteine residues (A-D, magenta) are indicated. Whereas the

Hepatitis C virus receptor CD81 contains 4 cysteine residues, the human CD63 protein has two additional cysteines in the respective protein area.

Supplementary Figure 4

Effects of anti CD63 mAbs on the release of β -hexosaminidase and selected cytokines from activated human mast cells

A) Anti CD63 Abs affect the release of β -hexosaminidase from iteratively stimulated

mast cells. Cord blood derived mast cells were subjected to two iterative activation cycles, and the release of reporter enzyme β -hexosaminidase recorded over time. Whereas the first degranulation reaction was induced with Jw-8/NIP-BSA, the second stimulus was set with α PC17/PC17-BSA. Uninhibited degranulation (solid lines), spontaneous degranulation (dotted lines), mast cell degranulation in presence of the commercial α CD63 mAbs MEM-259 (solid triangles), or AHN16.1 (solid squares), or in presence of cell state-specific anti CD63 mAbs NIBR63/1 (open triangles), or NIBR63/2 (open squares) is shown.

B) Anti CD63 Abs affect the release of various cytokines from iteratively stimulated

mast cells. Experimental samples from [A] were analyzed for cytokine secretion using a multiplex immunoassay (Milliplex MAP, Millipore; single analysis was done on samples pooled from three independent experimental setups). Depicted is the medium concentration of indicated cytokines 6h after degranulation cycles 1 (top) or 2 (bottom panels). Values are shown for spontaneous release (white), uninhibited degranulation (black), cell activation in presence of commercial α CD63 mAbs MEM-259 or AHN16.1 (dark grey), or in presence of state-specific anti CD63 mAbs NIBR63/1 or NIBR63/2 (light grey).

Supplementary Table I

Inventory of Antibodies used in this Report

A) Degranulation-specific anti Human Mast Cell mAbs (DSaHMC-mAbs):

NIBR63/1 (int. clone ref. No. 7-1-11, this study)
 NIBR63/2 (int. clone ref. No. 7-2-21, this study)
 NIBR63/3 (int. clone ref. No. 7-4-28, this study)

B) Commercial / conventional anti CD63 mAbs:

Serotec, MCA2142, MEM-259 mouse anti human CD63
 Ancell, 215-020, AHN16.1 mouse anti human CD63
 Med.Univ.Vienna, 11C9 mouse anti human CD63
 Chemicon Int., CBL553, RFAC4 mouse anti human CD63
 Immunotech, #1914 anti human CD63, PE-conjugate
 Beckman Coulter, PNIM1165U anti human CD63, FITC-conjugate

C) Further Primary Antibodies:

BD Pharmingen, #553033 anti murine CD8a (Ly-2), PE-conjugate
 BD Pharmingen, #553035 anti murine CD8a (Ly-2), APC-conjugate
 BD Pharmingen, #556087 anti human CD62 (P-selectin), PE-conjugate

D) Isotype Control:

BD Pharmingen, #557273 mouse IgG1

E) Secondary Antibodies:

Biosource International, AMI4407 goat F(ab')₂ anti mouse IgG, PE-conjugate
 Jackson ImmunoRes., 715-136-150 goat F(ab')₂ anti mouse IgG, APC-conjugate

F) IgE/Antigen Systems of Mast Cell Degranulation:

Novartis, NIBR B11/Le27
 Novartis, NIBR Jw8/NIP₈-BSA
 Novartis, NIBR anti Phosphocholine/PC₁₇-BSA

G) Cytospin immunostaining:

Dako cytomation, M7052 mouse anti human mast cell tryptase
 Dako cytomation, K0670 APAAP kit
 Dako cytomation, Z0259 rabbit anti mouse IgG
 Dako cytomation, D0651 APAAP, mouse monoclonal
 Dako cytomation, K0597 chromogenic substrate, Fast Red

H) MILLIPLEX MAP anti human Cytokine Assay:

Millipore, MXHPMX14 Human Cytokine/Chemokine Premixed Beads
 Millipore, MXH1060-2 Human Cytokine/Chemokine Detection Antibodies
 Millipore, L-SAPE3 Streptavidin-Phycoerythrin Dye

Supplementary Table II**List of Oligonucleotides (Primers) used in this Report****A) N-glycosylation site mutagenesis:**

CD63 <i>Asn130Gln</i> FW	CCGAAAAACCAGCACACTGCTTCGATCCTG
CD63 <i>Asn130Gln</i> BW	AGCAGTGTGCTGGTTTTTCGGGTAAATTCTC
CD63 <i>Asn150Gln</i> FW	GGGGCTGCTCAGTACACAGATTGGGAGAAA
CD63 <i>Asn150Gln</i> BW	AATCTGTGTACTGAGCAGCCCCACAGCAC
CD63 <i>Asn172Gln</i> FW	TGCTGCATTCAGGTTACTGTGGGCTGTGGG
CD63 <i>Asn172Gln</i> BW	CACAGTAACCTGAATGCAGCAGGAGTCGGG

B) Cysteine cluster mutagenesis:

CD63 <i>Cys145Ser</i> FW	GCAGGCAGATTTTAAGTCCTGTGGGGCTGCTAACT
CD63 <i>Cys145Ser</i> BW	AGTTAGCAGCCCCACAGGACTTAAAATCTGCCTGC
CD63 <i>Cys146Ser</i> FW	GGCAGATTTTAAGTGCTCTGGGGCTGCTAACTACA
CD63 <i>Cys146Ser</i> BW	TGTAGTTAGCAGCCCCAGAGCACTTAAAATCTGCC
CD63 <i>Cys169Ser</i> FW	CGAGTCCCCGACTCCTCCTGCATTAATGTTACT
CD63 <i>Cys169Ser</i> BW	AGTAACATTAATGCAGGAGGAGTCGGGGACTCG
CD63 <i>Cys170Ser</i> FW	GTCCCCGACTCCTGCTCCATTAATGTTACTGTG
CD63 <i>Cys170Ser</i> BW	CACAGTAACATTAATGGAGCAGGAGTCGGGGAC
CD63 <i>Cys177Ser</i> FW	AATGTTACTGTGGGCTCTGGGATTAATTTCAAC
CD63 <i>Cys177Ser</i> BW	GTTGAAATTAATCCCAGAGCCCACAGTAACATT
CD63 <i>Cys191Ser</i> FW	GATCCATAAGGAGGGCAGTGTGGAGAAGATTGGGG
CD63 <i>Cys191Ser</i> BW	CCCCAATCTTCTCCACACTGCCCTCCTTATGGATC

C) Amplification of CD63 / Flanking primers for cloning

CD63 up (EFa)	AATTCTCCTTGGAATTTGCC
CD63 down (MuLV)	AAGCTTCCAGAGGAACTGCT