Flow-based allergen testing : can mast cells beat basophils?

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Running title: Can MAT beat BAT

Abstract

The basophil activation test (BAT)has emerged as a reliable complementary diagnostic to document IgE-dependent allergies and to study cross-reactivity between structural homologues. However, the BAT has some weaknesses that hinder a wider application. The BAT requires fresh blood samples and is lost as a diagnostic in patients showing a non-responder status of their cells. The BAT is difficult to standardize mainly because of the difficulty to perform batch analyses. In contrast, mast cell activation tests (MATs), using passively sensitized mast cells (MCs) with patients' sera (henceforth indicated as passive MAT; pMAT), use serum samples that can be frozen, stored, and shipped to a reference center experienced in MC lines and/or cultures and capable of offering batch testing. With the recent recognition of the mas-related G protein-coupled receptor X2 (MRGPRX2) occupation as a putative mechanism of immediate drug hypersensitivity reactions, the MAT has another advantage compared to the BAT. MCs, in contrast to resting basophils, express the MRGPRX2 and can therefore be used to study this IgE-independent mechanism. This review provides a status update of pMAT in the diagnosis of allergic IgE-mediated hypersensitivity and speculates how direct activation of MCs via the MRGPRX2 receptor could advance paradigms for this non-allergic hypersensitivity.

Keywords: mast cells, basophils, hypersensitivity, flow cytometry, MRGPRX2, IgE

Introduction

Since the seminal description of CD63 as a surface degranulation marker [1], we have witnessed a progressive increase of flow-assisted basophil activation tests to document allergic reactions originating from cross-linking of allergen-specific IgE (sIgE) antibodies bound to their high-affinity receptor (FcεRI) of basophils [2-6]. From these reviews it emerges that the BAT has evolved to a safe and reliable diagnostic for some drugs classes, but certainly not all the classes. Moreover, flow-based *ex vivo* basophil activation tests using patients' cells (henceforth called direct BAT, dBAT) have been shown to be more than a diagnostic aid . Actually, dBAT can also advance insights in sometimes complex IgE-(cross)-reactivity patterns between structural homologues and monitor immunotherapy and desensitization protocols, especially in immediate drug hypersensitivity reactions (IDHRs) [4-6].

However, the dBAT technique leaves us with some significant shortcomings and weaknesses such as the preference for analyses within 4 hours after sampling [7], the basophilic non-responder status observed in 10-15% of our patients [8, 9] and quick negativization post event, as observed for β-lactam antibiotics [10]. In an attempt to circumvent these issues, different groups have focused on the development of passive BAT (pBAT) [11, 12]. In the pBAT, donor basophils are stripped and subsequently passively sensitized with patients' sera prior to stimulation with the relevant allergen(s). Although the pBAT is a step forward, some limitations remain. First, pBAT seems less sensitive than the traditional dBAT, with cells only responding if incubated with titres of sIgE exceeding 1 kUA/L [13, 14]. The need of such high amounts of sIgE is an important limitation as titres of sIgE for drugs are mostly low. Second, the outcome of pBAT is highly dependent on the donor whose cellular responder status is unpredictable and can only be determined *ad hoc*. Third, dBAT and pBAT do not allow to study direct activation by occupation of the mas-related G protein coupled receptor X2 (MRGPRX2)

by drugs [15]. Actually, unlike mast cells (MCs), resting basophils – that are the starting point in traditional dBAT – barely express this receptor [16, 17]. Therefore, in the mechanistic predictive algorithm of IDHRs, contribution of dBAT is currently restricted to documentation of sIgE-mediated reactions [5].

In pursuit of resolving these shortcomings, different authors have started exploration of the potential of flow-based *in vitro* mast cell activation tests (MATs). In the MAT, cultured donor MCs can be activated either directly (dMAT) or indirectly after passive sensitization with patients' sera (pMAT).

The main application of dMAT is exploration of MC activation and degranulation independent from sIgE/FcεRI signalling, e.g. by occupation of MRGPRX2 or anaphylatoxin receptors C3a and C5a. The pMAT allows to demonstrate the presence of serum sIgE antibodies and to evaluate their MC activating capacity [18-21]. In the auto-immune MAT (aMAT), unlike in pMAT, donor MCs are incubated directly with patients' sera to depict anti-FcεRI autoantibodies or after incubation with donor IgE-containing sera to depict anti-IgE autoantibodies as frequently observed in different kinds of auto-immune urticaria [22, 23].

Although the use of MATs as a novel *in vitro* diagnostic is in its infancy, we felt appropriate to outline its current status, as the technique looks promising. For an introductory review on MC sources and MC cultures – essential for MAT – the reader is referred elsewhere [24].

Mast cells

Mast cells (MCs) are derived from multipotent $CD34^+$ hematopoietic stem cells that enter the circulation. After transmigrating to peripheral tissues, the progenitor cells differentiate and mature into tissue-specific MC subpopulations expressing distinct phenotypes and functional properties [25-30]. Traditionally, human MCs are classified into two different subtypes:

tryptase-containing mucosal MCs (MC_T) and tryptase- and chymase-containing connective tissue MCs (MC_{TC}). MC_T mainly reside in mucosal tissue, such as the lungs, while the MC_{TC} are located in the connective tissues, such as the skin and lymph nodes [26, 31]. These subtypes also differ significantly in their response to endogenous and exogenous secretagogues [25, 32-36].

Mast cell proliferation, maturation and differentiation depends on various cytokines and tissue factors; amongst them, stem cell factor (SCF), interleukin (IL)-6 and IL-3 are the three most important. IL-6 has been reported to enhance MC proliferation, maturation and reactivity after cross-linking of the FcεRI [37]. IL-3 is responsible for the differentiation and proliferation [38, 39]. SCF is a ligand for the CD117 (c-kit) receptor, a tyrosine-kinase transmembrane receptor located on the membrane of the MC. Binding of SCF to the c-kit receptor causes improved survival, growth and cell migration and improves the effector functions of the MCs [38-41]. During SCF-induced differentiation, MC progenitor cells produce cell-specific enzymes such as tryptase and chymase that are stored in their granules and start to express FcεRI on their surface membrane. The production of these enzymes and the expression of FcεRI is dependent on the maturation stage of the cell. For example, the synthesis of tryptase starts at an early stage of MC development, while the membrane expression of FcεRI and synthesis of chymase occurs at a later stage [28, 41].

In addition to their expression of FcεRI, tryptase and chymase, mature MCs also invariably express the previously mentioned SCF receptor (CD117) and CD203c [41-45]. CD203c (or ENPP-3) is an ectonucleotide pyrophosphatase/phosphodiesterase family member. Recently, it has been shown that MRGPRX2 is highly expressed on the cell surface of MC_{TC}, while only poor expression is reported on MC_T cells [30, 40]. Besides the activation receptors FcεRI and MRGPRX2, MCs also express inhibitory receptors like CD300a and the low-affinity IgG receptor

(CD32) [46, 47]. Resting MCs barely express the lysosomal-associated membrane protein CD107a (LAMP1) and CD63 (LAMP3). As a matter of fact, increased surface CD63 and CD107a expression mirrors MC activation/degranulation [1, 18, 48, 49]. Moreover, in line with the observations in basophils, up-regulation of these lysosomal-associated membrane proteins is accompanied with compound degranulation and quantifiable exteriorization of granular content [50, 51]. Up-regulation of CD203c without augmented expression of LAMPs might merely reflects piecemeal degranulation [52, 53].

Flow cytometric immunophenotyping

For decades, exploration of MC biology has principally relied on measurement of granular mediators (e.g. β-hexosaminidase, histamine, tryptase) exteriorized in the supernatant, collected after centrifugation of the activated cells. Although this approach has largely contributed to our insights in the processes that govern MC activation and degranulation, it does not allow to analyse subtle responses of individual cells nor does it enable to study small subpopulations, as the results represent an average of all degranulating cells. In this regard, multicolour flow cytometry allowing simultaneous analyses on a single cell level of surface alterations (e.g. up-regulation or appearance of activation/degranulation markers, exteriorization of granular content) and intracellular changes (phosphorylation of signalling molecules, trapping of cytokines and mRNA thereof, calcium staining, mediator content) is a significant asset. In most studies, MCs have been identified by double positive staining for the stem cell factor receptor CD117 (c-KIT) and the ectoenzyme NPP3 (CD203c) or as double positive for CD117 and the high affinity receptor for IgE (FcεRI) [16, 18, 20, 49, 54-56]. As shown in figure 1, activation/degranulation of MCs can subsequently be explored by the appearance/up-regulation of specific markers such as the LAMPs CD107a and CD63 and/or

CD203c [16, 57-61], changes in intracellular calcium [56, 57, 61, 62] or histamine content [51], the visualization of anionic proteoglycans form exteriorized MC granule matrix [56, 59, 61, 63], and intracellular measurement of newly synthesized chemokines [59].

Allergic sIgE-mediated hypersensitivity

Mast cell lines

In an attempt to circumvent the limitations of the dBAT/pBAT and to streamline functional evaluation of sIgE antibodies to trigger effector cell activation/degranulation, many have focused on the development and optimization of different MC and basophil lines. These are comprehensively described elsewhere [64]. From this review it is clear that there is only a limited number of fully human MC lineages available and only few have been used in sIgE-dependent allergy research. To the best of our knowledge, the first exploration of the pMAT as a potential additional *in vitro* diagnostic dates back from 2018 [19]. In this study, it was shown that pMAT using the LAD2 cell line, generated by Kirschenbaum et al. [65], is comparable to traditional BAT in terms of specificity (98%) but displays lower sensitivity (73%) to document peanut allergy. Alternatively, pMAT could document peanut allergy in patients with unresponsive basophils.

Furthermore, pMAT, along with stripped basophil experiments enabled to demonstrate the inhibitory capacity of IgG4 antibodies in peanut tolerant children sensitized to major peanut allergens [60] and also identified patients susceptible for more severe reactions during food challenges [19]. Data about the effect of blocking antibodies were confirmed in a peanut oral immunotherapy study. By using LAD2 cells and basophils, it was shown that this treatment does not alter the functional characteristics of sIgE [66]. Igarashi et al., described the usefulness of human induced pluripotent stem cells (iPSC) (201B7), and tested the ability of

these cells to be triggered by cedar pollen, mite and house dust mite following passive sensitization [67].

Cultured primary human mast cells

The utility of the pMAT, using human cultured MCs that are passively sensitized with patients' sera, to explore and document sIgE-mediated allergies seems promising. Bahri et al., showed that MCs derived from $CD34^{+}CD117^{+}$ human progenitor cells that were sensitized with sera from patients allergic to peanut, grass pollen and wasp venom demonstrated allergen-specific degranulation, as revealed by the upregulation of the lysosomal associated membrane proteins CD107a and CD63, and release of β-hexosaminidase and prostaglandin D2. For peanut, the pMAT was found to have a superior diagnostic accuracy to determine clinical reactivity compared to traditional sIgE, skin tests and BAT [18].

Regarding to IDHRs, the previous mentioned limitations of the dBAT, together with our search for better understandings of the underlying pathomechanisms, urged us to explore the potential of the pMAT in this complex domain. In this respect, we recently provided the proof of concept that the technique can also be applied to study MC responsiveness to the biguanide antiseptic chlorhexidine (figure 2) [20]. As indicated by the accompanying Editorial [68], our proof of concept highlights that the application of the pMAT extends beyond the study of sensitization and allergy to peptidergic allergens that are generally considered more potent effector cell secretagogues than small molecules (i.e. haptens). Another peculiarity of this study relates to the observation that pMAT shows potential to discriminate between clinically relevant and irrelevant drug-sIgE antibodies in their ability to elicit MC degranulation, and therefore discriminate between allergy and sensitization reflected by a positive sIgE result in isolation. Recently, these proof of concept data were confirmed in a larger series that included

30 chlorhexidine-allergic patients and 20 uneventfully exposed control individuals and revealed that the pMAT had a high analytical sensitivity [21]. In addition, we recently evidenced that pMAT can also benefit the exploration of the chlorhexidine sIgE sensitization profile [69] that clearly extends beyond recognition of its chloroguanide endings [14]. Actually, it was shown that sera from patients with true chlorhexidine allergy but not from individuals with a clinically irrelevant sensitization to chlorhexidine activates MCs in response to the biguanide antiseptic alexidine and the bispyridine cationic antiseptic octenidine. Alexidine consists out of two (2-ethylhexyl) guanide units linked by a hexamethylene bridge, whereas octenidine has only the hexamethylene part in common with chlorhexidine. One could argue it is far to go from this encouraging data in chlorhexidine allergy and sensitization to mainstream application. In this regard, we recently started exploration of pMAT as a supplementary diagnostic in IDHRs to the neuromuscular blocking agent (NMBA) rocuronium. Figure 3, shows a representative dBAT and pMAT for a patient who experienced anaphylaxis to rocuronium. Moreover, we showed that the pMAT can benefit diagnosis in patients with unresponsive basophils (figure 4). Important to mention is that MC responsiveness is not exclusively dependent on the sIgE titer, but is also regulated by additional parameters such as avidity or sIgE reactivity and specificity profiles [18]. Likely, the sensitivity of the pMAT also depends on the surface density of FcεRI, the cells' intrinsic sensitivity to aggregation of these receptors and the sIgE-to-total IgE ratio.

Other relevant articles on human MC activation and degranulation in response to cross-linking of sIgE/FcεRI-complexes and other secretagogues (e.g. anaphylatoxins C3a, C5a) relate to the exploration of IL-33, produced on epithelial contact with an allergen, as a primer of individual cell responsiveness [49, 59, 70]. These studies show that IL-33 amplifies MC responses by both enhancing the responsiveness of individual cells and increasing the number of responding

cells. Thereby providing additional data for targeting IL-33 in Th2-mediated diseases [71]. Similar but less pronounced effects were seen with IL-6 [49] and SCF [72]. Note that IL-33 and IL-6 do not potentiate MRGPRX2-dependent MC activation/degranulation [49], whilst MRGPRX2 is negatively targeted by SCF and IL-4 [73].

Non-allergic MRGPRX2-mediated hypersensitivity

Besides the use of MC lines and primary human MCs to study IgE/FcεRI-signalling, MCs are also increasingly employed to explore alternative innate mechanisms of MC activation and degranulation. Since the first description by McNeil et al., increasing lines of evidence indicate that MC degranulation by occupation of MRGPRX2 likely constitutes a novel endotype of IDHRs [43]. Drugs that might act as MRGPRX2 agonists are various NMBAs, fluoroquinolones, icatibant and opiates such as morphine [15, 74]. Many drugs, but certainly not all, harbor a tetrahydroisoquinoline (THIQ) motif which could dramatically increase potency [75].

However, current evidence for activation of the MRGPRX2 receptor mainly comes from animal studies, and translation of these findings into clinical relevance in humans is difficult and should be critically interpreted. Based on current models, the MRGPRX2-agonistic potency of NMBAs is different and does not correspond to their potency to activate the mouse orthologue. For example, rocuronium is approximately 12 times less potent at the MRGPRX2 receptor in humans than in mice.

Mast cell lines

In an attempt to circumvent the intrinsic limitations of animal studies, MC lines appear to be an attractive alternative. For a recent review on the MC lines that have currently been used to explore MC responses to MRGPRX2 occupation by drugs see Elst et al., [24]. Briefly, most

research groups use the LAD2 cell line or a transfected HEK cell line. While there is some controversy, most studies showed MRGPRX2-mediated activation for different NMBAs and fluoroquinolones. Although these studies added significant insights to the mice data, translation to the clinics might still not be straightforward. First, because of the heterogeneity of human MC subpopulations with respect to MRGPRX2 receptor expression and function [36]. Second, MC lines do not behave exactly as primary human MCs. For example, the MRGPRX2 agonist compound 48/80 effectively induced degranulation of HMC1 as measured by CD63 membrane expression and β-hexosaminidase release, albeit in lower levels than for LAD2 or human MCs. HMC1, LAD2 and human MCs each had different degranulation kinetics upon stimulation with compound 48/80 [76].

Cultured primary human mast cells

Given the earlier indicated limitations of animal and MC line models, we took advantage of our expertise with flow-based analyses of peripheral blood cultured MCs [49, 51, 55, 58] to further explore the MRGPRX2 agonistic activity of different drug classes. In summary, based upon a combined analyses of $MRGPRX2^{+}$ and $MRGPRX2^{-}$ subpopulations together with selective receptor silencing, MRGPRX2 agonistic activity was demonstrable for the neuromuscular blocking agents atracurium, the fluoroquinolones ciprofloxacin, levofloxacin and moxifloxacin and the natural opiate morphine. In contrast, no MRGPRX2-depdendent degranulation was demonstrable for the neuromuscular blocking agents succinylcholine and rocuronium nor for the β-lactam antibiotic amoxicillin (figure 5) [57, 62]. Recently, whilst exploring the sIgE reactivity profile of chlorhexidine we found sIgE reactive to polyhexamethylene biguanide (PHMB) to be clinically irrelevant, but PHMB, unlike chlorhexidine, alexidine and octenidine, triggered direct degranulation of MCs. This

degranulation was inhibited selective silencing of ther MRGPRX2 receptor using dicer small interference RNA [69].

Summary and perspectives

Over the last two decades flow-based dBAT has evolved to a pervasive diagnostic and undoubtedly deepened our knowledge in the processes that govern activation of basophils. However, there remains room for improvement and the introduction for adjunct diagnostics, mainly because of unresolvable limitations inherent to *ex vivo* experiments necessitating viable patients' cells. Although still nascent, it seems that with the development of the MAT – including direct and passive MC activation experiments – progression is made to cover some of these limitations. Admittedly, it is far to go from these pilot studies to a performant diagnostic and MATs might remain out of reach for many years. Nevertheless, it seems that laboratories interested in MC biology should be capable of offering this adjunct diagnostic. Actually, when equipment and experience are available, development of a MAT platform as a complementary safe *in vitro* diagnostic should not pose a major hurdle. One might notice that, as with any development of this nature, a major difficulty could be to accurately identify a group of patients with clinical significant allergy and to compose an appropriate control group. However, unlike the dBAT, the MAT does not necessitate viable cells but only correct collection, storing and shipping of serum samples allowing deferred testing. It is re-emphasized that validation of a novel diagnostic cannot be considered appropriate when it failed to identify other conditions that could confound the outcome. In this respect, it is reassuring that pMAT already proved to discriminate between clinically relevant and irrelevant sIgE results and that expression of MRGPRX2 can be effectively and selectively silenced without affecting sIgE/FcεRI signalling. In addition, in parallel with the recommendations for other cellular tests, one should establish optimal allergen-specific

thresholds and abandon predefined arbitrarily decision thresholds. Second, the MAT might further benefit from optimization, standardization and harmonization of the assay conditions. Another problem of the validation procedure is the situation where full-dose challenges are obsolete and comparative analyses are to be made with imperfect diagnostics. In this respect, patients and control individuals should be carefully selected on the basis of positive results for a combination of tests and where the clinical features meet consensus criteria for an "almost certain" diagnosis [77].

In conclusion, the MAT appears a promising adjunct technique to safely assess the clinical relevance of positive sIgE results. However, unlike the dBAT, the technique could have broader applications, such as identifying the drugs responsible for direct activation of MCs, e.g. by direct activation of the MRGPRX2 receptor. However, it is unlikely that the dMAT could advance diagnosis of MRGPRX2-dependent IDHRs, as MRGPRX2 agonists are already active via wild type MRGPRX2 and preliminary results indicate that cultured MCs of patients with IgE-independent anaphylaxis to rocuronium were similar in their MRGPRX2 expression and function, to those of patients with IgE-mediated anaphylaxis [62]. Unless, it would turn out that the results with rocuronium are not generalizable and that MCs of patients with IgE-independent anaphylaxis are more susceptible to MRGPRX2 agonists and would differ in their MRGPRX2 expression and function, to those of patients with IgE-mediated anaphylaxis.

Author contribution

Didier G. Ebo: conceptualization, writing – original Draft; **Kevin Heremans**: writing – review and editing; **Michiel Beyens**: writing – review and editing; **Marie-Line M. van der Poorten**: writing – review and editing; **Athina L. Van Gasse**: writing – review and editing; **Christel**

Mertens: Investigation, writing – review and editing; **Michel Van Houdt**: Investigation, writing – review and editing; **Vito Sabato**: conceptualization, writing – original Draft; **Jessy Elst**: conceptualization, investigation; writing – original Draft, visualization

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Figures

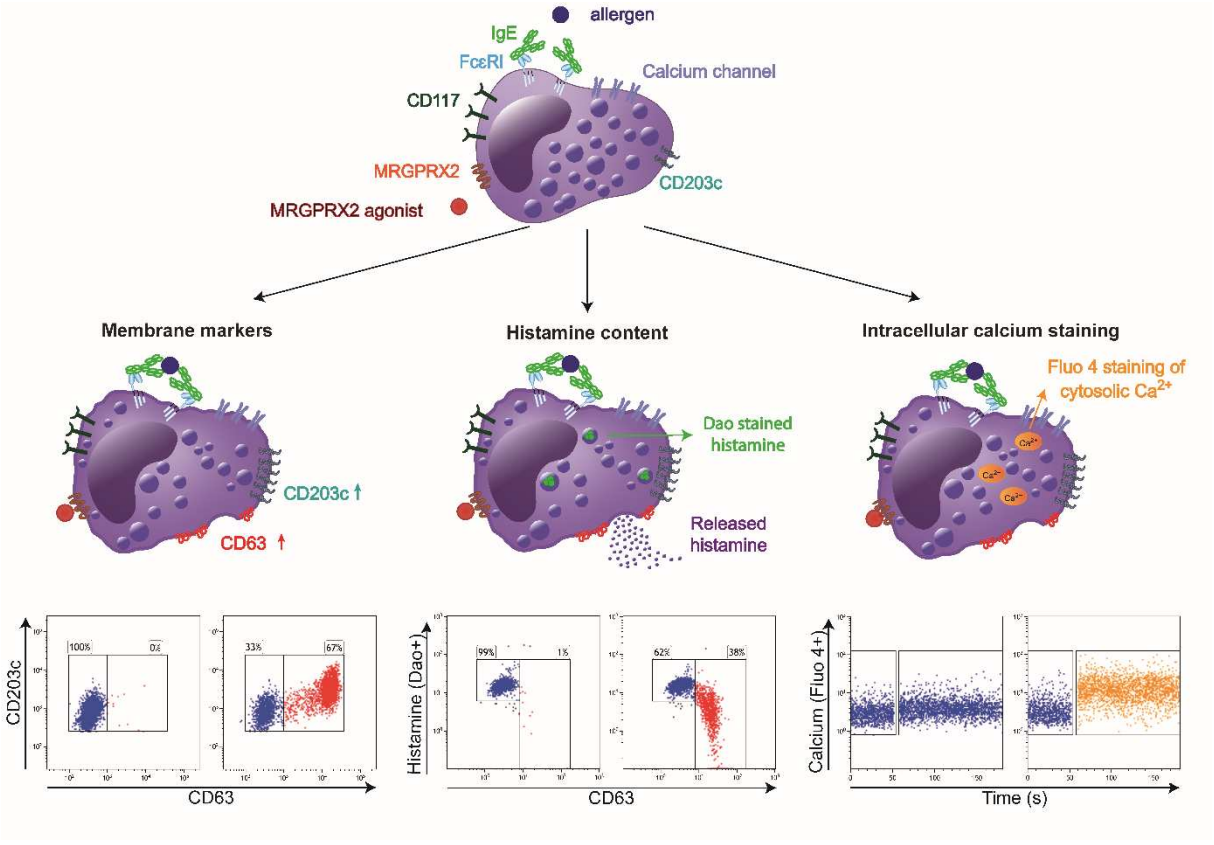
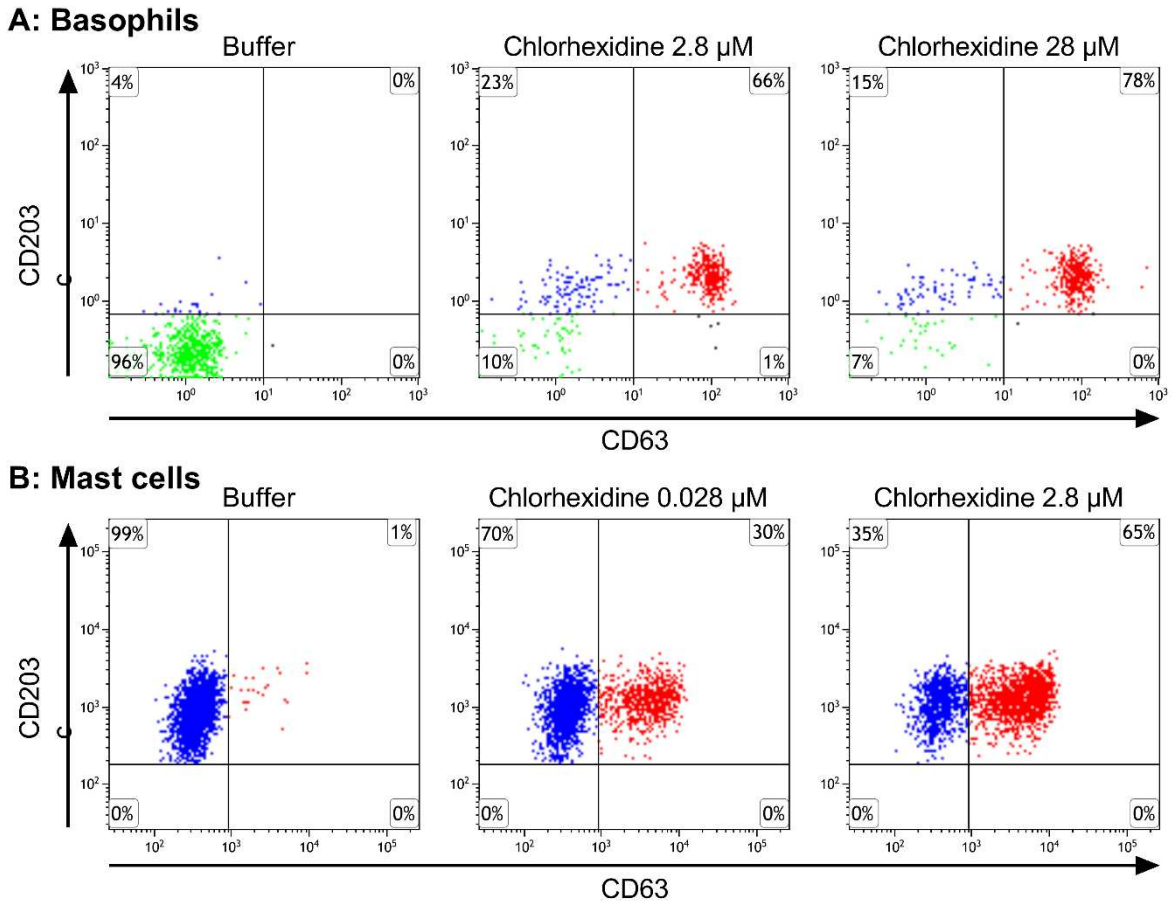


Figure 1: Flow cytometric readout markers for mast cell activation/degranulation

Individual mast cell activation/degranulation can flowcytometrically be measured via (1) appearance or upregulation of specific surface markers such as CD63 and CD203c, (2) changes in mediator content such as decrease in histamine, and (3) increased intracellular calcium staining.



544

545 **Figure 2 : Activation of basophils and mast cells with chlorhexidine**

546 **(A)** Resting basophils (incubated with stimulation buffer) do not degranulate (do not express
547 the lysosomal protein CD63). Basophils of a chlorhexidine-allergic patient upregulate, in a
548 dose-dependent manner, CD63 after incubation with chlorhexidine. **(B)** MCs, which are
549 passively sensitized with serum of the same patient barely express CD63 after incubation with
550 buffer. In contrast, after stimulation with chlorhexidine, passively sensitized MCs upregulate
551 in a dose-dependent manner CD63.

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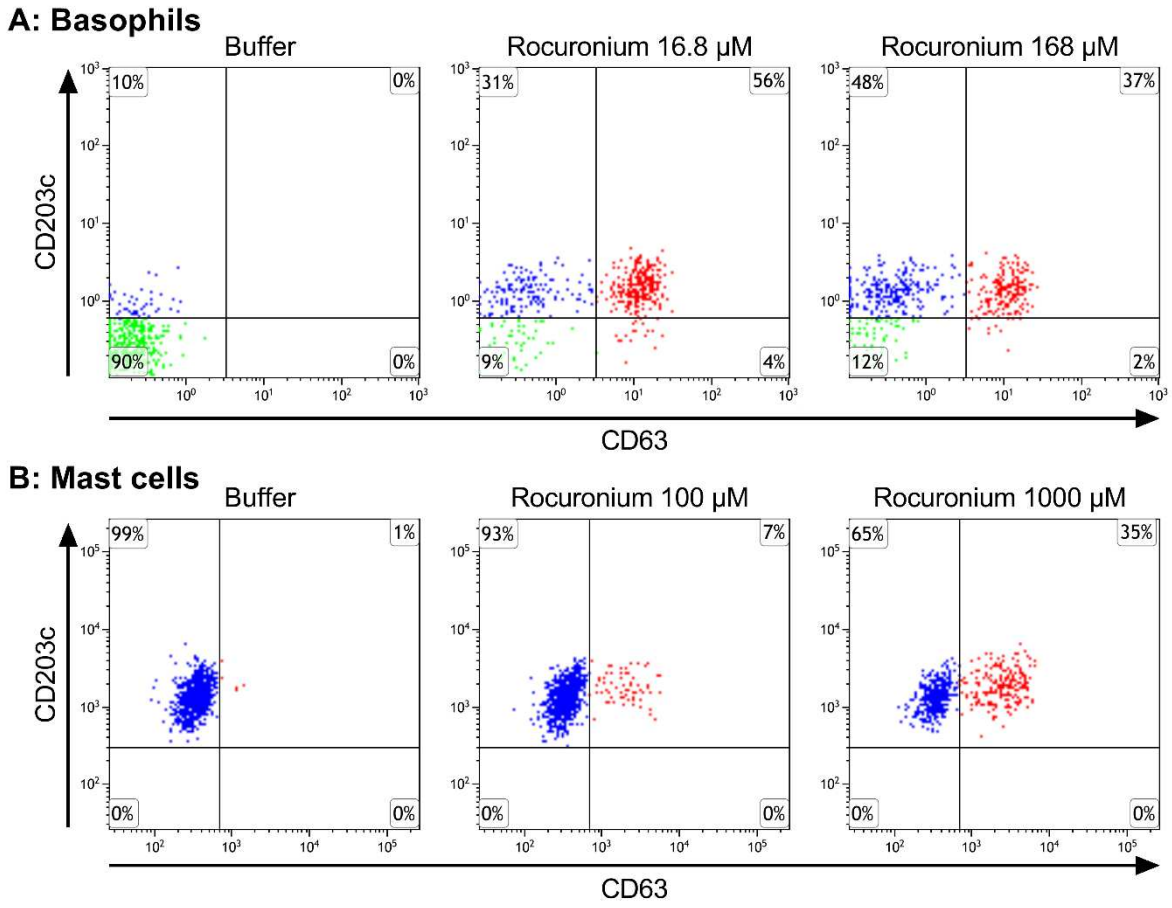

553

554 **Figure 3: Activation of basophils and mast cells with the neuromuscular blocking agent**

555 **rocuronium**

556 **(A)** Basophils of a rocuronium allergic patient stimulated with buffer do not express the

557 degranulation marker CD63. After incubation with rocuronium, the basophils upregulate in a

558 dose-dependent manner CD63. **(B)** MCs, which are passively sensitized with serum of the

559 same rocuronium-allergic patient, barely express CD63 after incubation with buffer. After

560 stimulation with rocuronium, passively sensitized MCs upregulate in a dose-dependent

561 manner CD63.

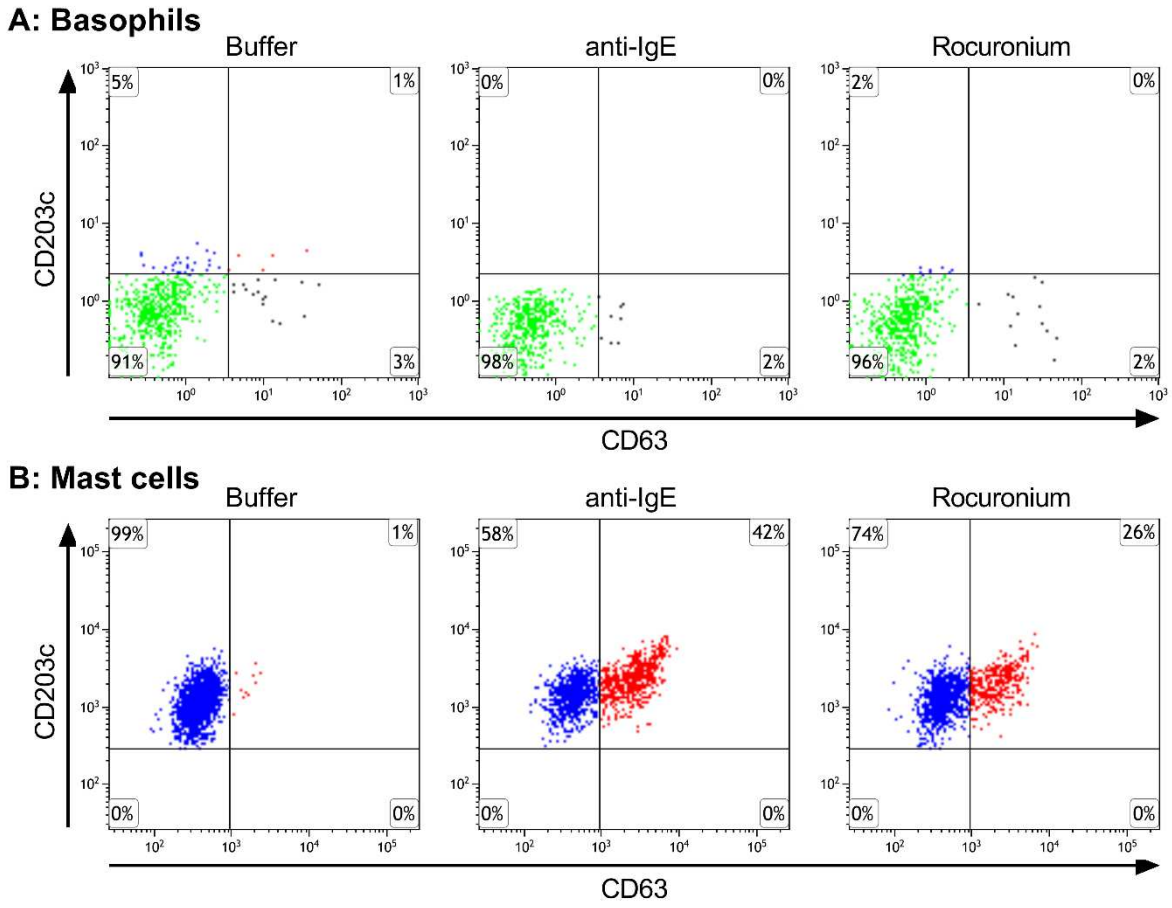

Figure 4: Representative plots of the activation of basophils and mast cells of a non-responder patient in the basophil activation test with the neuromuscular blocking agent rocuronium

(A) Resting basophils do not express the lysosomal degranulation marker CD63. Basophils of a rocuronium-allergic patient with non-responsive basophils do not upregulate CD63 after stimulation with the positive control anti-IgE, which cross-links surface-bound IgE antibodies. Such basophils, generally, do also not upregulate CD63 after stimulation with allergen such as rocuronium. **(B)** Passively sensitized MCs barely express CD63 after incubation with buffer. In contrast, MC which are passively sensitized with serum form the same patients, upregulate CD63 after stimulation with the positive control anti-IgE and rocuronium. The end concentration of rocuronium is 164 µM for basophil stimulation and 1000 µM for mast cell stimulation.

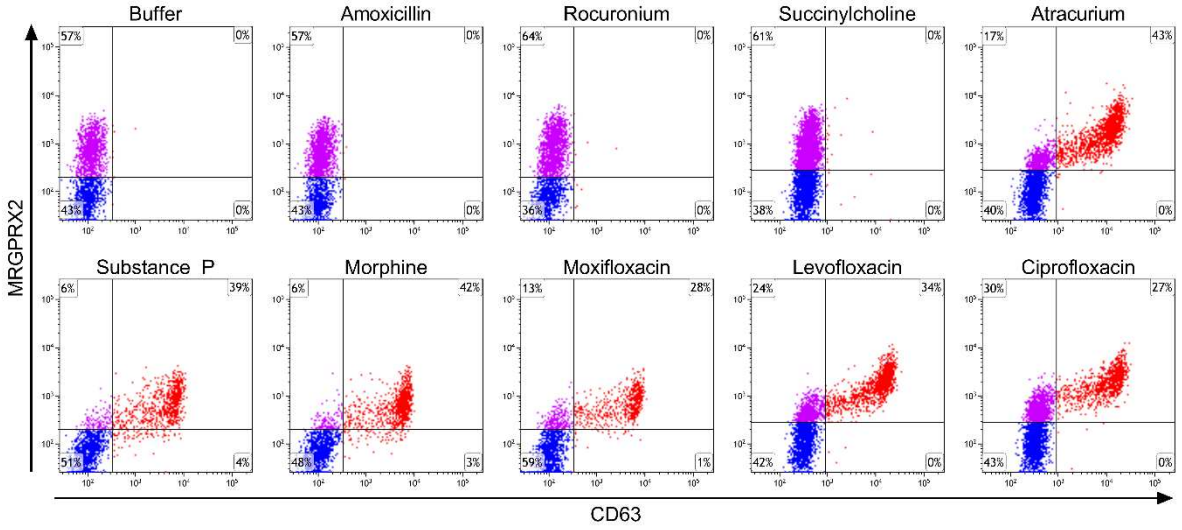

Figure 5: Representative plots of direct mast cell activation

Direct mast cell activation by MRGPRX2 agonists substance P, the opiate morphine and the fluoroquinolones moxifloxacin, ciprofloxacin and levofloxacin and the neuromuscular blocking agent atracurium. Note that amoxicillin and the neuromuscular blocking agents rocuronium and succinylcholine do not induce degranulation of cultured human MCs.