Mast cell activation tests by flow cytometry : a new diagnostic asset?

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Short title: Mast cell activation test: potentials and limitations

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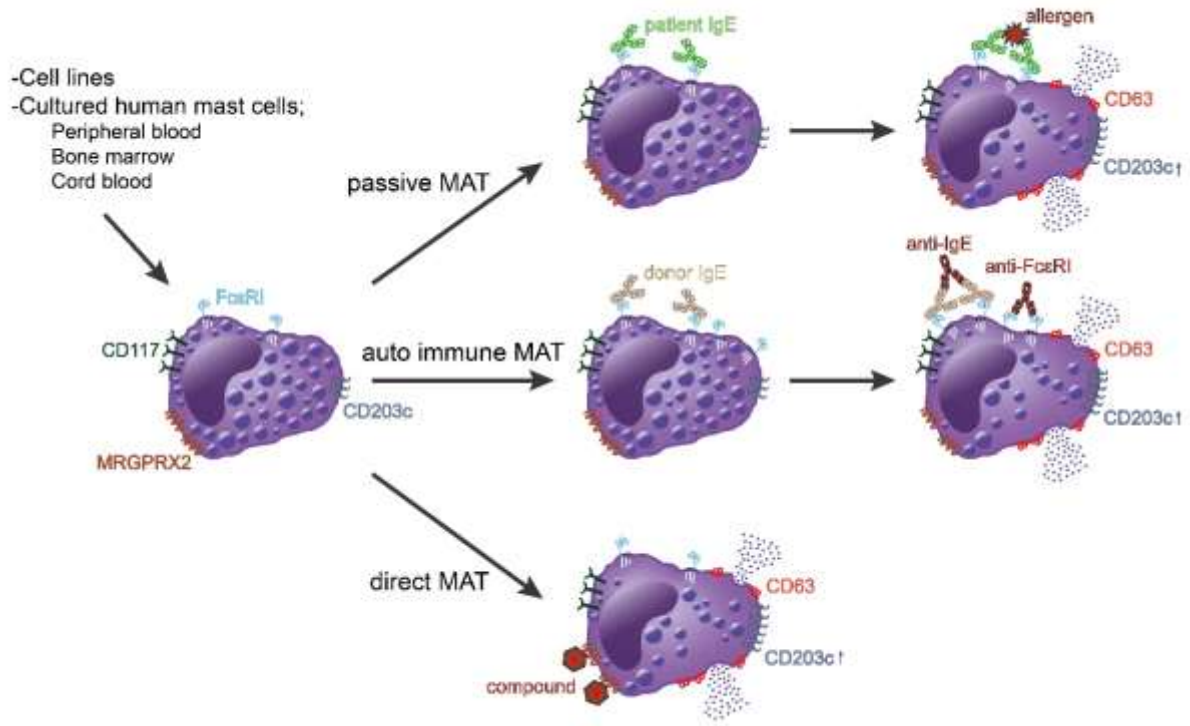
Key messages

- Mast cell activation test (MAT) is a promising diagnostic for IgE-mediated allergies and auto-immune urticaria.
- MAT allows simultaneous analysis of MRGPRX2+ and MRGPRX2- subpopulations and silencing of the receptor.
- In combination with BATs, MATs might advance our understanding of effector cell activation and degranulation.

Graphical abstract



The passive mast cell activation test (MAT), which uses mast cells (MCs) that were passively sensitized with patients' sera, is a promising diagnostic for IgE-mediated allergies. For auto-immune urticaria, in the auto immune MAT, MCs can directly be activated with patients' sera or after passively sensitization with donor IgE. Besides, the MCs can directly be activated through MRGPRX2 with simultaneous analysis of the MRGPRX2+ and MRGPRX2- subpopulations. MC degranulation can be studied via upregulation of specific degranulation markers or mediator release.

Abstract

Since the late nineties, evidence has accumulated that flow-assisted basophil activation test (BAT) might be an accessible and reliable method to explore the mechanisms governing basophil degranulation and diagnostic allowing correct prediction of the clinical outcome following exposure to the offending allergen(s) and cross-reactive structures for different IgE-dependent allergies and particular forms of autoimmune urticaria. Although the BAT offers many advantages over mediator release tests, it is left with some weaknesses that hinder a wider application. It is preferable to perform the BAT analysis within four hours of collection and the technique does not advance diagnosis in patients with non-responsive cells. Besides, the BAT is difficult to standardize mainly because of the difficulty to perform large batch analyses that might span over several days. This article reviews the status on flow cytometric mast cell activation test (MAT) using passively sensitized MCs with patients' sera or plasma (henceforth indicated as passive MAT; pMAT) using both MC lines and cultured MCs in the diagnosis of IgE-dependent allergies. In addition, this paper provides guidance for generating human MCs from peripheral blood $CD34^+$ progenitor cells (PBCMCs) and correct interpretation of flow cytometric analyses of activated and/or degranulating cells. With the recent recognition of the mas-related G-protein coupled receptor X2 (MRGPRX2) occupation as a putative mechanism of immediate drug hypersensitivity reactions (IDHRs), we also speculate how direct activation of MCs (dMAT) - that is direct activation by MRGPRX2 agonists without prior passive sensitization - could advance paradigms for this novel endotype of IDHRs.

Keywords: allergy, anaphylaxis, CD63, flow cytometry, human cultured mast cells, IgE, mast cell activation, MRGPRX2

List of abbreviations

aMAT: autoimmune mast cell activation test

ASST: autologous serum skin test

Atra: atracurium

BAT(s): basophil activation test(s)

BM: bone marrow

BMCMC(s): bone marrow cultured mast cell(s)

CB: cord blood

Cipro: ciprofloxacin

Cisatra: cisatracurium

dMAT: direct mast cell activation test (without prior passive sensitization with patients' sera or plasma)

DsRed: *Discosoma sp.* red fluorescent protein

ENPP: ectonucleotide pyrophosphatase/phosphodiesterase

FBS: fetal bovine serum

FcεRI: high affinity receptor for IgE

FCM: flow cytometry

IDHR(s): immediate drug hypersensitivity reaction(s)

IDT(s): intradermal test(s)

IL: interleukin

pMAT: passive mast cell activation tests (after passive sensitization of MCs with patients' sera or plasma)

IMDM: Iscove's Modified Dulbecco's Medium

IPSC: induced pluripotent stem cells

LAMP: lysosome associated molecular protein

Levo: levofloxacin

MAT(s): mast cell activation test(s)

MC(s): mast cell(s)

MC_{TC}: connective tissue mast cells

Moxi: moxifloxacin

MRGPRX2: Mas-related G-protein coupled receptor X2

Mths: months

ND: not determined

NMBA(s): neuromuscular blocking agent(s)

PB: peripheral blood

PBCMC(s): peripheral blood cultured mast cell(s)

RBL: Rat Basophilic Leukaemia

Rocu: rocuronium

SCF: stem cell factor

sIgE: specific IgE antibody

SPT(s): skin prick test(s)

ST(s): skin test(s)

THIQ: tetrahydroisoquinoline

Introduction

Although tissue resident mast cells (MCs) have been recognized as primary effector cells in IgE-dependent allergies, evidence about the utility of mast cell activation test (MATs) using passively sensitized MCs (passive MAT; pMAT) is relatively new. Actually, most studies on a functional assay for allergy diagnosis have focused on circulating basophils, as these cells are more accessible for *ex vivo* experiments. Together with the advent of flow cytometers and the discovery of easy to measure basophil identification and activation/degranulation markers that paved the way for the basophil activation test (BAT). The clinical utility of the BAT in the diagnosis and monitoring of allergic diseases has widely been studied and reviewed (1, 2). These studies and reviews show that the BAT, although offering many advantages over mediator release tests, is left with some weaknesses that hinder a wider application. Although the BAT can be executed up to 24 hours of collection (3), it is preferable to perform the analyses within 4 hours (4) and the technique does not advance diagnosis in patients with non-responsive cells. Besides, the BAT is difficult to standardize mainly because of the difficulty to perform large batch analyses that might require repetitive analyses spanning over several days.

As already indicated, MC studies should ideally analyses of tissue-resident cells of interest (5, 6). However, such studies are hindered by technical issues such as isolating sufficient numbers of viable, mature and functionally MCs and poor clonal expansion of such cells *ex vivo*. Furthermore, functional behavior of tissue MCs can be influenced by the applied isolation techniques (7-9). To circumvent the limitations encountered with BAT and tissue MCs, different groups have adopted passive sensitization techniques using specific basophil and MC cell lines (10-12), stripped basophils (13, 14) or primary human MCs cultured from donor progenitor cells (10, 15) (16-18).

This article reviews the status on flow cytometric pMAT using both MC lines and cultured cells in the diagnosis of IgE-dependent allergies. Table 1 summarizes the main differences between traditional flow cytometry (FCM)-assisted BAT and pMAT in their application to explore the effector cell activating potential of IgE antibodies and diagnosis of IgE-dependent allergies. In addition, this paper provides guidance for generating MCs from peripheral blood $CD34^+$ progenitor cells (PBCMCs) and correct interpretation of flow cytometric analyses of activated and/or degranulating cells. With

the recent recognition of the mas-related G-protein coupled receptor X2 (MRGPRX2) occupation as a putative mechanism of immediate drug hypersensitivity reactions (IDHRs), we also speculate how direct activation of MCs (dMAT) - that is direct activation by MRGPRX2 agonists without prior passive sensitization - could advance paradigms for this novel endotype of IDHRs. Finally, we provide a proof of concept for application of the MAT to depict the presence of autoantibodies autoimmune urticaria in a technique called autoimmune MAT (aMAT).

Flow cytometric analysis of activated and/or degranulating humanized cells and primary human MCs

For decades, exploration of MC functionality has predominantly relied on quantification of released mediators or proteases (e.g. β-hexosaminidase, histamine, etc.) in the supernatant, collected after centrifugation of stimulated cells (10, 17-53). However, these techniques do not allow to analyze (subtle) responses of individual cells nor do they enable to study (small) subpopulations, as their results represent an average of all stimulated cells. In this respect, multicolor FCM enabling to couple surface alterations (e.g. up-regulation of activation and degranulation markers, exteriorization of granular content) and intracellular changes (e.g. phosphorylation of signal molecules, trapping of chemokines, calcium staining) is a significant asset. As shown in Figure 1, traditional FCM-based MAT relies upon cellular identification and quantification of activation and/or degranulation markers on the surface membrane. These changes are detectable and quantifiable using specific fluorescent-labelled monoclonal antibodies. In most studies, MCs have been identified by positive staining for surface markers such as CD117/CD203c (15, 16, 54-56) or as CD117/FcεRI (10, 19, 20, 22, 40, 44). Subsequently, after activation, the up-regulation of specific markers, such as CD63 and CD107a or CD203c is measured (10, 11, 15, 16, 21, 22, 44, 54-58). CD63 and CD107a are membrane proteins of the lysosome associated molecular protein (LAMP) tetraspanin family that are barely expressed by resting MCs. Up-regulation of CD63 is closely associated with mediator release during degranulation (6, 10, 59-61). CD203c (or ENPP-3), an ectonucleotide pyrophosphatase/phosphodiesterase family member, is already expressed on the cell surface of quiescent MCs and is up-regulated after stimulation (15, 59, 61, 62). However, the upregulation of CD203c and CD63 is clearly different. Actually,

upregulation of CD63, unlike upregulation of CD203c, is generally bimodal with a subpopulation of degranulating cells that express CD63 with a high intensity versus a resting subpopulation with a significant lower expression. Degranulation of MCs can also be explored by measuring the exteriorization of granule matrix. Briefly, anionic proteoglycans from exteriorized MC granule matrix are stained by cationic fluorescent avidin probes (19-22). MC degranulation can also be measured by flow cytometric techniques to quantify intracellular histamine and its release. In this technique, the intracellular content of histamine is quantified using the affinity of diamine oxidase (DAO) for its substrate histamine (63, 64). By coupling the histaminase DAO to fluorochromes, intracellular histamine content and consequently release of histamine can be measured by FCM at a single cell level (54). Newly synthesized chemokines such as CCL4 or CXCL8 can be trapped and measured intracellularly (22). Next, phosphotyrosine staining, a parameter associated to cell protein kinase function, can be used to study MC activation (22). Finally, MC activation can also be measured by imaging changes of intracellular calcium over time (20, 22, 30, 57, 65).

Basophil and mast cell lines

For a recent comprehensive compendium on humanized basophil and fully human MC lines the reader is referred elsewhere (12). Tables 2 and 3 summarize the different humanized rat leukemia and the fully human MC lines and indicate those applied in pMAT studies. Table 4 shows the cell lines expressing MRGPRX2 that have been used to study MRGPRX2-dependent MC activation and degranulation. As most of these studies have used alternative non-flow cytometric read-outs, they are only touched briefly.

Mast cell lines: utility in IgE-dependent allergy

In an attempt to streamline the functional assessment of sIgE antibodies to trigger effector cells, much effort has been put in the development and optimization of cultured cell lines. Nevertheless, these cell lines still present some important shortcomings that generate a lack of acceptance and skepticism by clinicians toward this experimental tool. The first lineage used in this domain have been humanized or transfected cell lines derived from the Rat Basophilic Leukaemia (RBL) cell line and expressing human FcεRI chains (48-50) such as, RBL SX-38 and RBL-48 (47-52, 65-71) (Table 2).

However, as earlier explained, most of these studies used mediator release as a read out. Only Taudou et al. used FCM to demonstrate that a RBL line transfected with the α-chain of the human FcεRI produce a calcium response when sensitized with patients' sIgE and subsequently incubated with house dust mite (65).

Although for years some of these humanized RBL cell lines have significantly contributed to our understandings of the mechanisms that govern IgE-dependent effector cell degranulation, their clinical application is limited. First, like the native RBL cells, all the chimeric clones show reduced ability to bind purified IgE after approximately 2 weeks in culture (12). Second, these cell lines often need high serum titers of sIgEs in the donor's serum to reach satisfactory results (e.g. >10 kUA/mL for peanut sIgE in RBL SX-38) and require a high percentage of receptor occupancy (>10% for RBL-2H3 E5.D12.8) (49, 72). Third, human xenoreactive IgGs present in patients' sera can cause cell death and induce background degranulation in up to 25% and laboratory procedures used to limit this shortcoming (i.e. serum dilution) could lead to suboptimal MC sensitization and lead to false negative results (71, 73).

As shown in Table 3, there is only a limited number of fully human MC lineages and only few have been used in IgE-mediated allergy research. Of relevance for this review, is the LAD-2 cell line, generated by Kirshenbaum and co-workers in the early 2000s, and that has been validated to assess peanut allergy and to monitor the effect of peanut oral immunotherapy of in children (11). In a first study, Santos et al (11), have shown that pMAT using passively sensitized LAD-2 cells is comparable to BAT in terms of specificity (98%) but has lower sensitivity (73%) to diagnose peanut allergy (11). However, pMAT provided results for those patients with unresponsive basophils. Indirect MAT also identified patients at risk of severe reactions during food challenges (11) and, along with stripped basophil experiments, enabled to demonstrate the inhibitory capacity of IgG4 antibodies in peanut-tolerant children sensitized to peanut major allergens (58). In a second study, the same group confirmed their previously reported findings on the induction of blocking antibodies with peanut oral immunotherapy and disclose that this treatment does not change the functional characteristics of sIgE, as determined by the ability of LAD-2 cells and basophils to respond to peanut allergen (74).

Igarashi et al. (39) described the use of human induced pluripotent stem cells (iPSC) cell line (201B7) to generate short-lived human iPSC-MC line to use in the pMAT. The authors tested the ability of this cell line to be triggered in a dose-dependent manner by cedar pollen, mite and house dust following appropriate sensitization. iPSC-MC could not be sensitized by ragweed. Due to the human origin of these cells, serum could be used undiluted without cellular damage. However, iPSC-derived cells could be maintained in culture only for maximum eight months, this might limit their routine use as a diagnostic tool.

To the best of our knowledge, humanized or human MC lines have only been used to study the pMAT with proteinaceous allergens and not in the field of IDHRs yet. However, in the clinics seldomly additional diagnostic tests are needed for this proteinaceous allergens, but are more than welcome in IDHRs.

Mast cell lines: utility in MRGPRX2-mediated hypersensitivity

Besides the use of MCs lines to study the IgE-dependent activation, cell lines are also increasingly used to study the IgE-independent activation mechanisms of MCs. Since the seminal description by Mc Neil et al. [40], increasing lines of evidence indicate that MC degranulation via occupation of MRGPRX2 might constitute a new endotype of IDHRs independent from cross-linking of IgE/FcεRI complexes [20, 42-44, 75-78]. Examples of drugs that might act via MRGPRX2 engagement are neuromuscular blocking agents (NMBAs), fluoroquinolones, icatibant and opiates such as morphine. Many of these drugs harbor a tetrahydroisoquinoline (THIQ) motif (40). Table 4 summarizes the cell lines, including human MC lines that have been used to study the MRGPRX2-dependent activation in IDHRs per drug class. Note that FCM was only used by Navines-Ferrer et al. (44). Although these *in vitro* studies have nourished our insights in MRGPRX2-dependent IDHRs and anaphylaxis, interpretation of the sometimes-incongruent findings and translation into the clinical setting is difficult. Based on the current models, the MRGPRX2-activating capacity and potency of different drugs is left with some uncertainties. First, it appears that its MRGPRX2-activating capacity is different and difficult, if at all, predictable by its effects on its mouse orthologue. For example, rocuronium is ~ 12 times less potent at the MRGPRX2 receptor in humans than in mice [40]. The difficulties of translating findings with Mrgprb2 – the murine orthologue of MRGPRX2 – into humans and a clinical setting has been addressed

elsewhere [75] and probably reflect adaptive evolution of the human MRGPRX2 gene [79]. Second, results in LAD-2 cells are sometimes inconsistent and therefore difficult to interpret or translate (41, 43, 77, 80-82). The reason for these observations could relate to the observation that LAD-2 cells express variable levels of MRGPRX2 as compared to human mature skin MCs (81, 83, 84) and that LAD-2 cells show different responsiveness to MRGPRX2 agonists as compared to primary human MCs (81, 84-86). In addition, the research groups often use different concentrations which make interpretation of the results difficult (43, 81). Besides, the LAD-2 cells demonstrate a relatively slow growth rate and express very low levels of tryptase and chymase (83). Furthermore, the cytokine generation is deficient in the cells, making the LAD-2 cells only intermediately differentiated as compared to human mature skin MC (83, 87). In addition to the disadvantage regarding the MRGPRX2-pathway, the LAD-2 cells are also not always suitable for studying the IgE-dependent activation as LAD-2 cells grown for prolonged periods display excessive clumping or slower growth, may lose responsiveness to biotinylated IgE/FcεRI crosslinking, and have reduced activation and degranulation (87). As recently described, the HMC-1 cell line also expresses the MRGPRX2 although at lower levels compared to LAD-2 and human cultured MCs and with lower reactivity (84). Because of these drawbacks there is a need for a representative human MC model to study simultaneously the sIgE/FcεRI- and MRGPRX2-dependent activation. Finally, another important aspect to mention is the surface expression of particular GTP-binding proteins that might alter ligand affinity of the MRGPRX2 receptor. Not well indicated in the McNeil paper (40) was that their results required a specific co-transfection of HEK cells with a specific GTP-binding protein. This suggest that not only the MRGPRX2 expression levels are important, but also will be the profile of GTP-binding proteins being expressed.

Primary human mast cells

Culture techniques

At the beginning, different research groups developed strategies to culture primary human MCs starting from mononuclear cells isolated out of different source materials such as cord blood (CB), bone marrow (BM), fetal liver cell or rarely out of peripheral blood (PB) (34-38, 88-94). However, starting from mononuclear cells can be challenging and pose some difficulties. First, the most used sources are CB and fetal

liver cells, which are not easily and commonly available. Second, the recovery of MCs out of mononuclear cells is low and cultures are often contaminated with other cells (37, 89). Next, most of the obtained MCs do not express chymase (92-94). Circumventing these disadvantages, several research groups have developed protocols for culturing sizeable numbers of primary MCs from different progenitor cells.

Table 5 summarizes the main culture protocols starting from progenitor cells mainly obtained from BM, CB and PB. Bone marrow and CB contain high amounts of progenitors (95). However, as already indicated, BM and CB are not easily accessible. Furthermore, CB derived MCs (CBDMCs) are often immature with a low expression of FcεRI and CD203c, contain little histamine and are poorly dedicated for IgE-mediated activation (33, 38, 87, 96). On the other hand, PB derived MCs (PBCMCs) are more easily and repeatedly accessible making the (PBCMC) cultures probably the preferred method to study MCs function. As shown in Figure 2, PBCMCs, cultured for 4 weeks in the presence of SCF and IL-3, are characterized by CD117 and CD203c expression (62). Besides, these PBCMCs uniformly express other membrane markers as the FcεRI, CD300a and CD32. MRGPRX2 is partially expressed, creating two distinctive subpopulations, $MRGPRX2^+$ and $MRGPRX2^-$cells. PBCMCs also contain intracellular markers like histamine, tryptase and chymase and thereby displaying a MC_{TC}-like phenotype. However, as already indicated in table 5, there exist different culture protocols for PBCMCs and it cannot be excluded that MC obtained from different cultures display phenotypic and functional dissimilarities, mainly because of considerable differences in culture time (3-12 weeks) and in maturation protocols (different growth medium, growth factors and cytokine mixtures). The cultures that are already used to study IgE-mediated allergies or MRGPRX2 mediated IDHRs are indicated with an asterisk in table 5. Note that the outcomes of pMAT and dMAT might differ markedly depending on whether MC_T or MC_{TC}-like cells are used. Our experience is mainly based on MC_{TC}-like cells that includes both a $MRGPRX2^+$ and a $MRGPRX2^-$ subpopulation enabling comparative analyses. For a comparison between the phenotype of MCs and basophils the reader is referred elsewhere (97).

Passive mast cell activation tests (pMATs): clinical utility in IgE-mediated allergy

The utility of the pMAT that uses passively sensitized human cultured MCs to study allergen-specific IgE-mediated MC activation, although nascent, is promising. In the

study by Bahri et al (10), MCs derived from $CD117^{+}CD34^{+}$ human progenitor cells and sensitized with sera from patients allergic to peanut, grass pollen, and wasp venom allergy demonstrated allergen-specific degranulation by quantification of CD63 and CD107a, and release of prostaglandin D2 and β-hexosaminidase. Moreover, for peanut, pMAT conferred superior diagnostic accuracy compared to sIgE, SPTs and BAT in distinguishing between patients with and without clinical reactivity. Findings also implied that MC responsiveness is not exclusively dependent on the sIgE titer, but also regulated by additional elements such as avidity or IgE reactivity and specificity patterns. Likely, the sensitivity of the pMAT also depends on the FcεRI receptor expression levels, the cell's intrinsic sensitivity to aggregation of FcεRI and the sIgE to total IgE ratio. Two other relevant articles about in vitro MC activation relate to the exploration of interleukin (IL)-33 as a primer of individual cell responsiveness (15, 22). Synthetic analyses reveal that this cytokine – which is produced on epithelial contact by an allergen (review in (98)) – potentiate IgE-mediated MC responses by both increasing the number of responding cells and enhancing the responsiveness of individual cells. A similar, but less pronounced priming effect was seen with IL-6 (15) and SCF (99). Note that IL-33 and IL-6 did not potentiate MRGPRX2-dependent MC activation (15), while SCF can dampening MRGPRX2-dependent activation.

The reference standard for diagnosis of drug allergy is a controlled drug challenge with administration of incremental doses of drug or placebo. However, such challenges are not devoid of risk and sometimes almost impossible to be performed because of the resources required to manage the pharmacologic effects of some drugs (e.g. curarizing neuromuscular blocking agents) (100, 101). Furthermore, their predictive value is not absolute (102). Therefore, drug hypersensitivity reactions are usually diagnosed using detection of drug-reactive sIgE, skin tests (STs), and by BAT (103-105). However, as reviewed elsewhere (106), the sIgE assay is only available for a limited number of drugs and show poor performance for some drug classes (e.g. β-lactams). Besides, skin testing is still associated with some uncertainties, especially for nonspecific histamine releasers that might act via off-target MRGPRX2 occupation (e.g. opiates and quinolones) [104, 107-109]. The BAT has emerged as a performant diagnostic tool for some, but certainly not all, drug(s) (classes) and sensitivity and specificity is highly variable between the different drug(s) (classes) (103, 105, 110-112). As already mentioned, the BAT is left with some limitations, these limitations,

together with the search for a better understanding of the mechanisms of IgE-independent IDHRs, has urged us to explore the potential of pMAT in this domain. Although, currently limited to a single proof of concept with the biguanide antiseptic chlorhexidine (16), it appears that the pMAT cannot only be successfully applied to proteinaceous allergens such as pollen (10, 15), food (10, 11) and Hymenoptera venom (10), but also to drugs. Actually, from our proof of concept it appears that the pMAT has a high analytical sensitivity enabling to depict low titers of functionally active drug-reactive sIgE antibodies and that the pMAT discriminates between allergy, and merely sensitization as reflected by an isolated positive sIgE result. Unlike PBCMCs passively sensitized with sera from CHX-allergic patients with positive STs and BATs, PBCMCs passively sensitized with sera from CHX-sensitized individuals with negative STs and BATs, did not respond to subsequent incubation with the antiseptic. Finally, the pMAT can also be used to study the cross-reactivity profiles of CHX-reactive sera. As shown in Figure 3, and already demonstrated by Mueller-Wirth et al (113), this cross-reactivity profile can involve the structural homologues alexidine and octenidine.

Passive mast cell activation tests (pMATs) in IgE-mediated allergy: more than a diagnostic

As indicated in the title, we expect that the pMAT might also become a valuable asset to study many domains of IgE-mediated allergies that remain poorly understood. An example of such an application of the pMAT lies in its capability to detect and identify major allergens and antibody recognition structures, as well as the affinity between epitope and paratope. For example, Hemmings et al (114), showed that Ara h 2, a major allergen from peanut (*Arachis hypogeae*), induce greater maximal MC reactivity and has a lower half maximal effective concentration than Ara h 6 when testing co-sensitized patients. Recently, and as shown in Figure 4, we applied pMAT to identify Can s 2 (profilin) and Can s 5 (a Bet v 1 homologue) as new allergens of *Cannabis sativa* (115). As indicated in the introductory paragraph, basophil and MC activation/degranulation can result from distinct IgE-dependent and IgE-independent mechanisms. Therefore, it is likely that comparative analyses of dMAT and pMAT experiments, along with BATs (110), might advance our understandings in the mechanisms that govern effector cell activation and degranulation. For example, evidence has emerged that IgE-mediated activation is not only achievable by traditional allergens, but can also occur by lectins with a binding specificity that

matches the glycosylation of IgE and/or FcεRI, or by other molecules such as super allergens (protein Fv, HIV gp120) or S. mansoni IPSE/alpha-1 (116, 117). How dMAT and pMAT can contribute to the exploration of mechanistic endotypes of IDHRs and discriminate between clinically indistinguishable IgE-dependent and MRGPRX2-dependent reactions is discussed in the section below.

The autoimmune MAT (aMATs) in autoimmune urticaria

Acute and chronic histaminergic urticaria and angioedema result from MC and basophil degranulation via diverse innate and adaptive immune responses, including auto-immune processes (118, 119). At present, in chronic spontaneous urticaria, two groups of MC degranulating signals have been identified, that is IgE autoantibodies to auto-allergens and IgG autoantibodies that target FcεRI or IgE/FcεRI complexes present on the MC surface. The presence of such anti-IgE or anti-FcεRI anti-bodies can be assessed functionally using patients' sera in an autologous serum skin test (ASST), via autoimmune BAT and by MAT (18, 120-122). Figure 5 shows our proof of concept of the aMAT in which the presence of anti-FcεRI and/or anti-IgE autoantibodies or IgE autoantibodies towards auto-allergens is depicted via FCM.

Mast cell activation tests: clinical utility in MRGPRX2-mediated allergy

As earlier mentioned, the recognition of off-target occupation of the MRGPRX2 MC receptor heralds a new and attractive domain in our understandings of IgE-independent IDHRs. However, as evidence for this pathway has mainly been gathered in mutated animals and via *in vitro* studies using different cell lines (40, 76), it remains uncertain whether findings also apply in humans (55). For the time being, there is little direct clinical evidence for a MRGPRX2-dependent mechanism of IDHRs in humans. To the best of our knowledge, description of patients who might possibly have suffered from a MRGPRX2-dependent reaction is restricted to a single series of patients who experienced anaphylaxis from rocuronium (123). In these patients, in contrast to those who had suffered from a documented IgE-dependent rocuronium anaphylaxis, diagnosis could only be established by a positive ST (quantification of sIgE and BAT rocuronium were negative). In other words, MRGPRX2-dependent reactions are

undetectable by traditional BAT. The reason for this has to be sought in the fact that traditional BAT uses resting peripheral blood basophils that barely express MRGPRX2 (about 5%) (56, 124). In contrast to isolated basophils that express MRGPRX2 in about 13-23% of the cells (125). Whether the MAT that uses non-passively sensitized cells (LAD-2 or MC_{TC}-like donor MCs) could advance diagnosis of MRGPRX2-dependent reactions seems unlikely, because drugs acting as MRGPRX2 agonists are already active via wild-type MRGPRX2 (Figure 6). In other words, for the time being there is no method that enables to unambiguously and directly document a MRGPRX2-dependent IDHR. However, the combination of positive STs with negative quantification of sIgE, BAT and eventually MAT, albeit indirectly, could point to an MRGPRX2 reaction for drugs known to be potent MRGPRX2 agonists (2, 110, 123). Figure 7 shows an integrated approach using STs, sIgE immunoassays, BAT, MAT/pMAT and MRGPRX2 silencing experiments to deepen our insights in the pathomechanisms of IgE- and MRGPRX2-dependent IDHRs. As cutaneous MCs express significant numbers of MRGPRX2, there is a general belief that STs to drugs that might act via this receptor do not discriminate between both these endotypes of IDHRs. However, we observed that sIgE- and MRGPRX2-dependent reactions might show different ST patterns (123). In patients with a combined positive IgE to morphine and a positive rocuronium BAT suggestive of an IgE-dependent mechanism, skin MCs appeared to be more sensitive. Actually, in 30/36 (83%) such patients STs were generally positive in SPTs or intradermal test (IDT) dilutions beyond the concentrations required to trigger non-specific ST responses. In contrast, in patients with negative sIgE to morphine and negative rocuronium BAT, STs were positive in only 12/25 (48%) of patients in SPT settings, and for IDTs, higher non-irritative concentrations were required to reach positivity. Whether this observation could reflect topographic differences in MRGPRX2 expression by cutaneous MCs remains elusive.

Conclusions and perspectives

FCM-assisted BATs have emerged as a robust tool to diagnose and monitor allergic diseases. However, the BAT has some limitations; it requires fresh samples and the technique does not advance diagnosis in patients showing a non-responder status of their cells. Besides, resting basophils barely express MRGPRX2, making it difficult to study this mechanism. In contrast, MATs use serum or plasma samples that can be frozen, stored, and shipped to a reference center experienced in MC lines and/or

cultures, and allow (deferred) batch testing with necessary quality controls. In addition, the MCs express MRGPRX2, allowing to study the potential of drugs to stimulate MCs through this receptor. Therefore, the MAT can be a promising diagnostic approach, especially in difficult cases where traditional tests are unavailable or yield uncertain results. However, there is far to go from the current experience to the creation of a new robust diagnostic and its entrance in mainstream use. For example, for pMAT, further validation will need exploration of the effects of FcεRI receptor expression levels, the cell's intrinsic sensitivity to aggregation of FcεRI and the sIgE-to-total IgE ratio. All parameters that are likely to depend on MC source and culture conditions. With respect to MRGPRX2 signaling, it will be critical to know more than its expression levels, but also deepen our insights in the effects of GTP-binding protein surface expression on its ligand specificity and affinity. Nevertheless, although currently restricted to a proof of concept and some preliminary results, the pMAT appears to be highly sensitive and has a good discrimination performance between true drug allergy and cases with indeterminate diagnostics (e.g. sensitization as reflected by an isolated positive sIgE result). Admittedly, additional larger studies are required to confirm and critically verify these encouraging results and to assess whether the technique could become a cost-effective new diagnostic. Further automation of data analyses and bioinformatic tools should advance standardization and quality assurance and thus accelerate transition to the clinics. Although the non-diagnostic applications of MAT are still in their infancy, with increasing employment, it is expected that the technique might become an attractive and valuable asset to study various domains of MC activation/degranulation biology, especially when used in combination with other tools such as IgE binding studies, skin testing and BATs. Actually, the MAT, could become an attractive complementary method to further accelerate our insight into the effector mechanisms of IDHRs, benefit identification of antibody recognition sites, expand our understandings of desensitization and tolerance induction strategies, predict natural disease courses and prognosis.

536 **Table 1: Basophil activation tests vs. passive mast cell activation tests**

Characteristic	Basophil activation test (BAT)	Mast cell activation test (pMAT)
Source material(s)	Patients' whole blood Basophils	Donor MCs or MC lines Patients' sera/plasma
Non-responders	Up to 10-15% and unpredictable[(c)]	No (MC responsiveness can be verified in advance)[(f)]
Priming	IL-3 (detrimental for CD203c)[(d)]	IL-33[(g)]
Used readouts	CD63, CD107a, CD203c, avidin, DAO[(e)]	CD63, CD107a, CD203c, avidin, DAO
Early analyses post index event possible?[(a)]	Yes	Yes
Deferred testing possible?[(b)]	No	Yes
Batch testing	No	Yes[(h)]

[(a)] BATs, and likely also pMATs, can be performed early after the index reaction (unpublished data). However, if the test is negative it should be repeated.

[(b)] Although BATs can be performed up to 24 hours after sampling, it is recommended to perform analysis within 4 hours after collection (2). In contrast, patients' sera or plasma that are used in the pMAT can easily be stored and shipped and thereby enable deferred analyses.

[(c)] Up to 10-15% of the patients have non-responsive basophils that do not react to positive control nor allergen. In these patients the BAT does not advance diagnosis. Note that this unresponsiveness cannot be determined in advance. It is an ad hoc determination. In non-responders in the BAT, the pMAT can be a valuable adjunct (11).

[(d)] IL-3 priming induces expression of the ectoenzyme CD203c without exposition to allergen and can therefore blunt the responsiveness of the cells to allergen. This does not apply to the lysosomal associated membrane protein (LAMP) 3 or CD63.

[(e)] For a detailed description of the characteristics of the different readout families the reader is referred elsewhere (2) and figure 1.

[(f)] In contrast to BAT, that uses patients' basophils, the pMAT uses a MC cell line or cultured donor cells. Responsiveness of these cells can readily be evaluated before starting the analyses.

[(g)] IL-33 has been shown to boost MC responsiveness to sIgE/FcεRI cross-linking but not MRGPRX2-dependent activation (15, 22).

[(h)] pMAT allows simultaneous testing of several patients' sera or plasma using the same MC line or donor MC culture.

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538 **Table 2: humanized MC lines used for the detection of IgE-mediated activation**

Humanized cell line	FcεRI chain expressed	Allergen	Read out	Reference
RBL SX-38	α, β[1], γ	Peanut, grass pollen, Timothy grass	Radiolabeled serotonin β-hexosaminidase	(49, 67) (47, 51)
RS-ATL8	α, β, γ	Wide variety (e.g *Holcus lantanus,* grass pollen)	Chemiluminescence (Luciferase)	(68, 70, 71)
RBL NFAT DsRed	α[2]	Wide variety of allergens	Chemiluminescence (DsRed)	(69, 71)
RBL-T8	α[2]	House dust mite	Calcium Influx (flow cytometric)	(65)
RBL-48	α[2]	House dust mite, latex, ragweed, cedar pollen	Histamine release β-hexosaminidase surface plasmon resonance imaging	(48, 52, 66)
RBL-hEla-2B12	α[2]	House dust mite	β-hexosaminidase	(50)

[1]Ali et al showed loss of transfected β-chain in RBL SX-38 (126)

[2]Expression of the human α chain is sufficient to confer reactivity toward human IgE (65)

DsRed: *Discosoma sp.* red fluorescent protein

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Table 3: Overview of human mast cell lines and their use in the diagnose of an IgE-dependent allergy

	FcεRI	SCF dependence	Mutation in KIT receptor	Used in diagnosis of IgE-dependent allergy	Read out	Allergen	Reference
HMC1	No	No	Yes	No	/	/	
LUVA	Yes*	No	No	No	/	/	
LAD-2	Yes	Yes	No	Yes	Flow cytometry (CD63)	Peanut	(11, 58)
ROSA $^{kit\ wt}$	Yes	Yes	No	No	/	/	
ROSA $^{kit\ D816V}$	Yes	No	Yes	No	/	/	
iPSC-MC	Yes	Yes	No	Yes	Histamine β-hexosaminidase	Cedar pollen, Mite, House dust	(39)

* The expression of FcεRI on LUVA cells is sometimes contradictorily.

542 **Table 4: MRGPRX2-expressing cell lines used to explore the MRGPRX2-activating potential of drugs**

Cell line	Read out	Opiates	NMBA	(fluoro)quinolones	Antibiotics	Miscellaneous	Reference
CHO-K1-MRGPRX2	Calcium	+ Morphine	ND	ND	ND	ND	Akuzawa(127)
LAD-2 HEK-293	Histamine release Cytokine release Calcium β-hexosaminidase	+ Morphine	+ Rocu + Atra - Succ	+ Moxi + Cipro + Levo + Olfoxacin	ND	+Octreotide + Mastoparan	McNeil(40)
LAD-2	Calcium β-hexosaminidase	ND	+ Atra	+ Cipro	ND	ND	Azimi(41)
HEK-293 LAD-2	Calcium β-hexosaminidase	ND	ND	ND	+ Vancomycin	ND	Azimi(42)
LAD-2 HEK-293 HTLA	Calcium β-hexosaminidase	+ Morphine	- Rocu - Atra	- Moxi - Cipro - Levo - Olfoxacin	ND	- Octreotide + Mastoparan	Lansu(43)
LAD-2	Calcium Histamine release	ND	ND	+Moxi +Cipro +Norfloxacin +Lomefloxacin	ND	ND	Han(82)
LAD-2	β-hexosaminidase Flow cytometry (CD63)	+ Morphine	- Rocu + Cisatra	ND	- Vancomycin - Teicoplanin - Amoxicillin	ND	Navines-Ferrer(44)
LAD-2	β-hexosaminidase Histamine release TNF-α Calcium		+Cisatra +Mivacurim				Che(128, 129)
LAD-2 HEK-293	Calcium β-hexosaminidase Histamine release	ND	ND	+ Moxi +Levo +Sparfloxacin +Gatifloxacin +Enoxacin	ND	ND	Liu(77)

				+Norfloxacin +Lomefloxacin +Pefloxain +Fleroxacin			
LAD-2 HEK-293	β-hexosaminidase Histamine release TNF-α Calcium	+ Morphine	ND	ND	ND	ND	Zhang(130)
LAD-2	β-hexosaminidase	ND	+Atra	ND	ND	ND	Shtessel(131)
LAD-2	Calcium β-hexosaminidase CCL2 release	ND	+/-Rocu +Atra	ND	ND	ND	Fernandopulle(80)
LAD-2	β-hexosaminidase	ND	+ Rocu	ND	ND	ND	Chompunud(81)

+, MCs activation/degranulation; -, no MCs activation/degranulation; ND, not determined; NMBA, neuromuscular blocking agent; rocu, rocuronium; atra, atracurium; cisatra, cisatracurium; moxi, moxifloxacine; Cipro, ciprofloxacin; Levo, levofloxacin; Succ, Succinylcholine

Table 5: Primary human mast cell protocols

Source	Progenitors	Duration (weeks)	Cytokines	Serum factor	Medium	FcεRI	Tryptase (%)°	Chymase (%)°	Reference
PB	$CD34^+$	6	SCF, IL-3	Autologous serum	RPMI	+	100	< 3	Rottem (132)
PB	$CD34^+$	10	SCF, IL-3, IL-6	Serum free	StemPro-34	ND	+	ND	Kirshenbaum (133, 134)
PB	$CD34^+$	12	SCF, IL-3, IL-6	FBS	Methylcellulose	ND	100	10	Wang (23)
PB	$CD34^+$	8	SCF, IL-3, IL-6	FBS	Methylcellulose	+	ND	ND	Saito (135)
PB	$CD34^+$	9	SCF, IL-3, IL-6, IL-9	Serum-free	StemSpan	+	99	95	Lappaleinen (24)
PB	$CD133^+$	7	SCF, IL-3, IL-6	FBS	StemSpan	+	80	ND	Holm (25)
PB	$CD34^+$	8	SCF, IL-3, IL-6	Serum free	StemPro-34	ND	ND	ND	Radringer (87)
PB	$CD113^+$	7	SCF, IL-3, IL-6	Serum free	StemSpan	+	100	< 2	Frandsen (26)
PB	$CD133^+$	8	SCF, IL-6	Serum free	IMDM + GlutaMAX	+	+	-	Gaudenzio (136)
PB	$CD34^+$	3	SCF, IL-3, LDL	Serum free	Methylcellulose	+	+	4-20	Schmetzer (27)
PB	$CD133^+$	8	SCF, IL-3, IL-6, IL-4	Serum free	StemSpan	+	ND	ND	Hoffman (137)
PB	$CD34^+$	9	SCF, IL-3, IL-6	Serum free	StemSpan-IMDM	+	95	95	* Joulia (19)
PB	$CD34^+$	6	SCF, IL-3, IL-6	Serum free	StemPro-34 medium	+	ND	ND	Yin (21)
PB	$CD34^+$	6	SCF, IL-3, IL-6, IL-9, IL-4	Serum free	IMDM	+	100	71	** Tam (28)
PB	$CD34^+$	4	SCF, IL-3, LDL	Serum free	Methylcellulose	+	96	90	*, **,*** Cop (54)

PB	CD133+	7	SCF, IL-3, IL-6	FBS	StemSpan/Collagen Matrix	+	100	97	Derakhshan (29)
PB	CD34+	5	SCF, IL-3, IL-6	Serum free	StemSpan	ND	ND	ND	Luo (138)
PB	CD117	8	SCF, Il-3, IL-6, LDL	Serum free	StemSpan-IMDM	+	+	+	* Bahri (10, 139)
CB	CD34+	34	SCF, IL-3	Serum free	IMDM	ND	ND	ND	Durand (140)
CB	CD34+	10	SCF, IL-3, IL-6, PGE2	FBS	IBL media I	+	99	18	Saito (30)
CB	CD34+	8	SCF, IL-3, IL-6	FBS	Alfa-medium	ND	+	ND	Kempuraj (141)
CB	CD133+	12	SCF, IL-3, IL-6	Serum free	StemSpan	+	83	36	Dahl (31)
CB	CD34+	8	SCF, IL-6	Serum free	QSBF60	+	95	9	Yamaguchi (32)
CB	CD133+	7	SCF, IL-3, IL-6	FBS	StemSpan	+	75	ND	Anderson (33)
CB	CD133+	8	SCF, IL-3, IL-6	FBS	StemSpan	ND	ND	ND	Radringer (87)
BM	CD34+	6	SCF, IL-3	Autologous serum	RPMI	+	100	< 3	Rottem (132)
BM	CD34+	12	SCF, IL-6	Human serum	Media III	+	100	100	Shimizu (142)
BM	CD34+	10	SCF, IL-3, IL-6	Serum free	StemPro-34	ND	+	ND	Kirshenbaum (133, 134)

PB, peripheral blood; CB, cord blood; BM, bone marrow; SCF, stem cell factor; IL, interleukine; FBS, fetal bovine serum; IMDM, Iscove's Modified Dulbecco's Medium ;ND, not determined; °, if the number of tryptase or chymase positive cells is not mentioned, it is designated as positive (+) or negative (-); * culture used for pMAT; ** cultured used for aMAT; *** cultured used for dMAT

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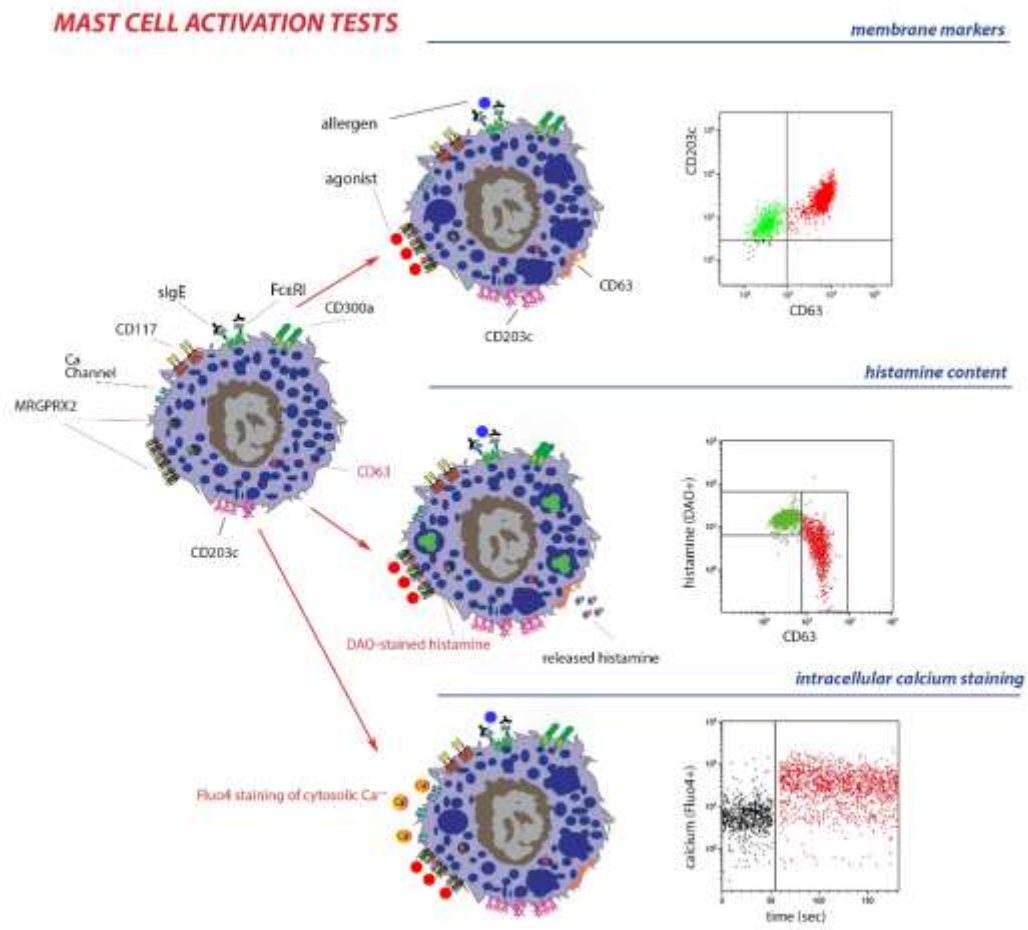


553

554 Figure 1: Mast cell Identification and activation/degranulation markers. Mast cells
555 express various molecules that can be used in isolation or in combination to identify
556 the cells and measure their activation/degranulation status by flow cytometry (FCM) in
557 a technique designated as the MAT. Mast cell activation/degranulation can occur via
558 IgE/FcεRI-dependent and IgE/FcεRI-independent pathways. Individual cell
559 activation/degranulation can be measured by FCM on 5 levels: (1) via appearance or
560 upregulation of surface markers (such as CD63, CD107a, CD63, and avidin binding
561 of membrane-associated exteriorized anionic proteoglycans released from granules);
562 (2) Newly synthesized chemokines such CCL4 or CXCL8 can be trapped and
563 measured intracellularly; (3) phosphotyrosine staining, a parameter associated to cell
564 protein kinase function, can also be used to study MCs activation; (4) via changes in
565 mediator content (such as decrease of histamine); or (5) via increased intracellular
566 calcium staining. Intracellular molecules are denoted in pink.

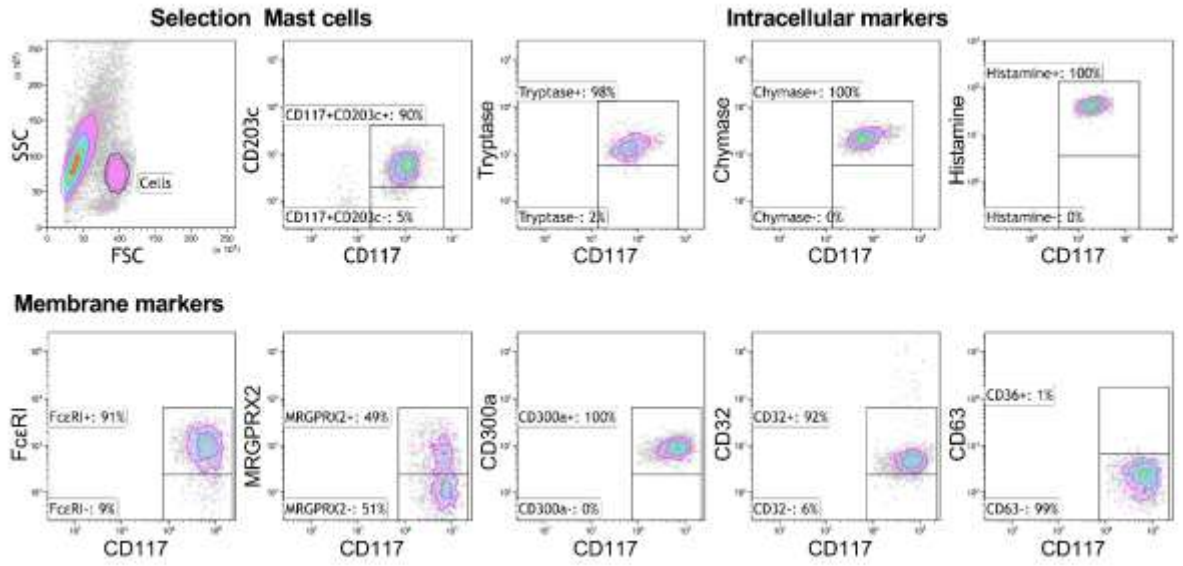


Figure 2: Representative plot of flow cytometric phenotyping of peripheral blood cultured human mast cells. Cells were selected based on side scatter (SSC) and forward scatter (FSC). MC were defined as CD117 and CD203c positive. These MC uniformly express FcεRI, CD300a and CD32. MRGPRX2 Is partially expressed on MC creating two clearly subpopulations $MRGPRX2^{+}$ and $MRGPRX2^{-}$. CD63 is barely expressed on resting MC. The MCs contain also tryptase, chymase and histamine (DAO).

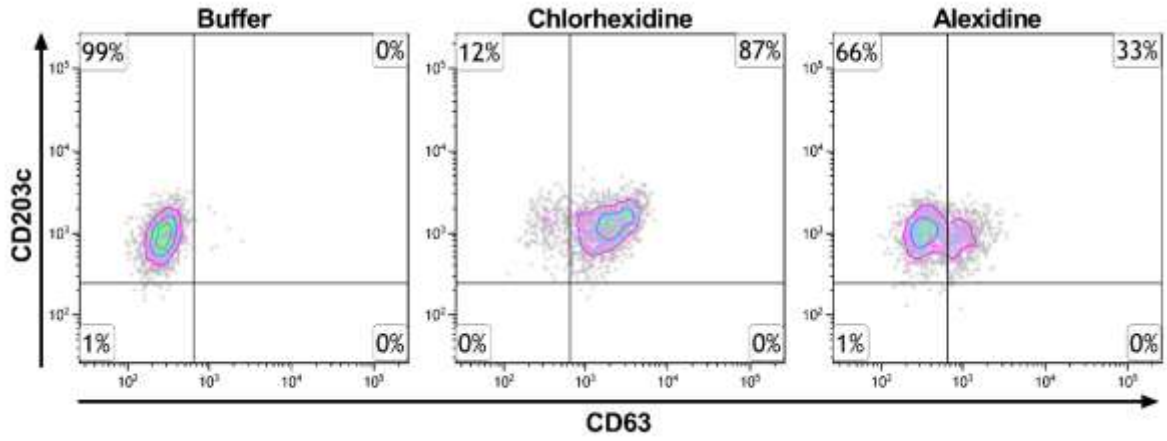
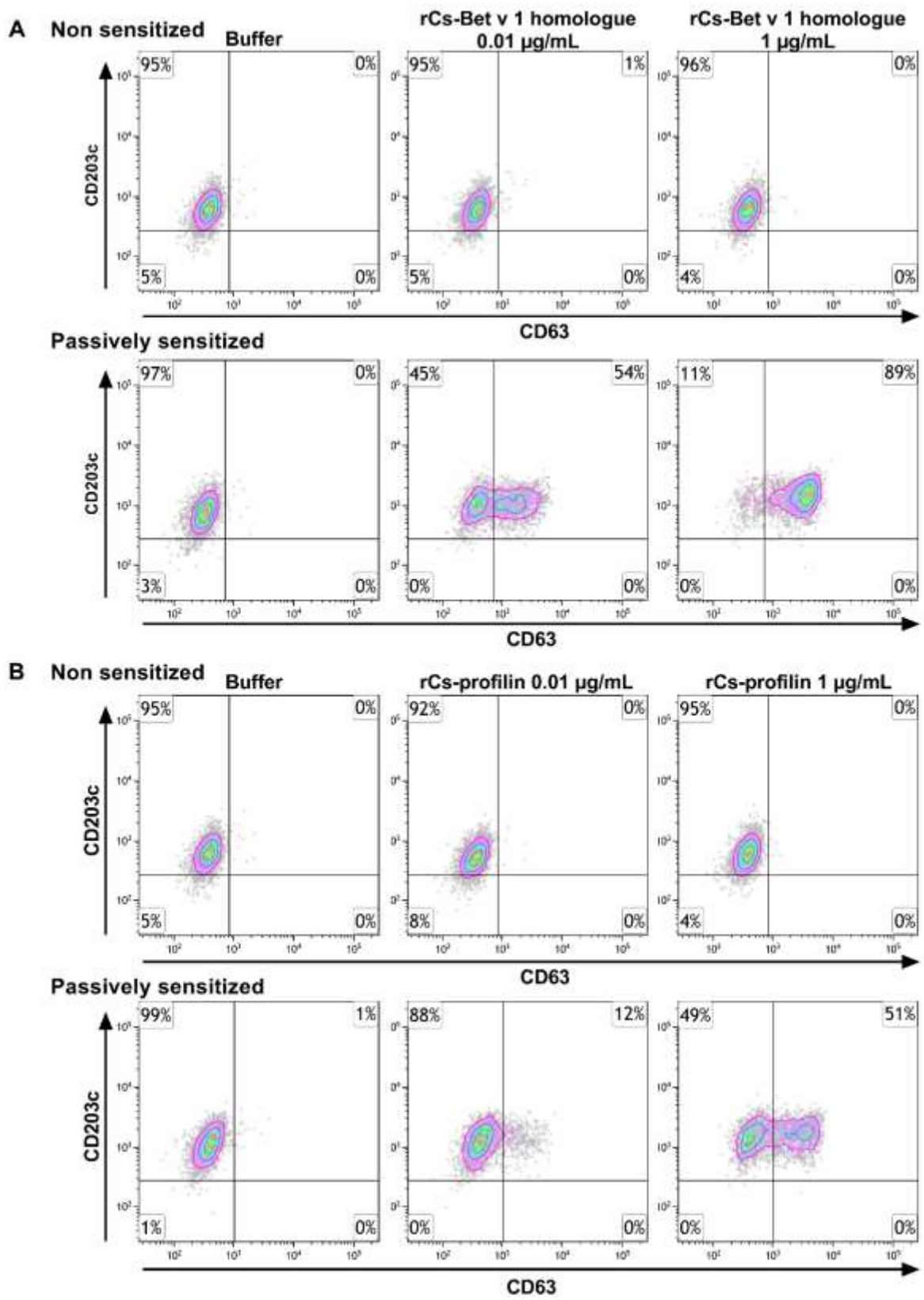


Figure 3: Representative plot of utilization of the indirect mast cell activation test to study cross-reactivity between structural homologues. MCs were passively sensitized with serum of a patient with documented CHX-allergy (sIgE: 0.46 kUa/L, FEIA ImmunoCAP, Thermo Fisher together with positive STs and basophil activation tests CHX). MCs are stimulated with CHX (2.8 µM) or with a structural homologue alexidine (2.8 µM).



583

584 Figure 4: Representative plot of a mast cell activation test with recombinant
585 homologue of the major birch pollen allergen Bet v 1 from Cannabis sativa (rCs-Bet v
586 1 homologue) **(A)** or cannabis sativa profiling **(B)**. Unlike cells not passively sensitized

with a rCs-Bet v 1 or Cs-profilin reactive serum (top), passively sensitized show a dose-dependent degranulation to rCs-Bet v 1 or Cs-profilin.

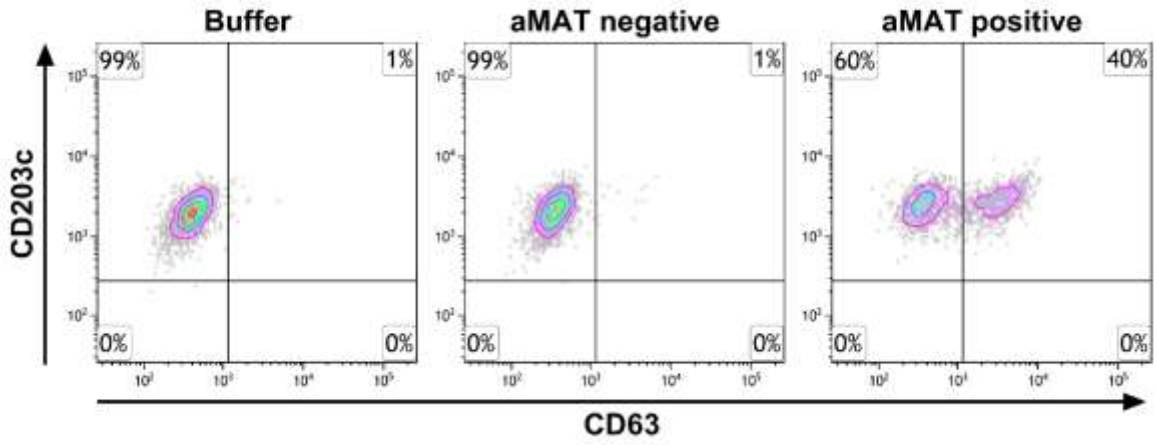


Figure 5: Representative plots of an autoimmune mast cell activation test (aMAT) for suspected autoimmune chronic spontaneous urticaria. After incubation with control serum there is no up-regulation of CD63 (aMAT: negative). After incubation of cells with serum of a patient, 40% of the mast cells up-regulate expression of CD63.

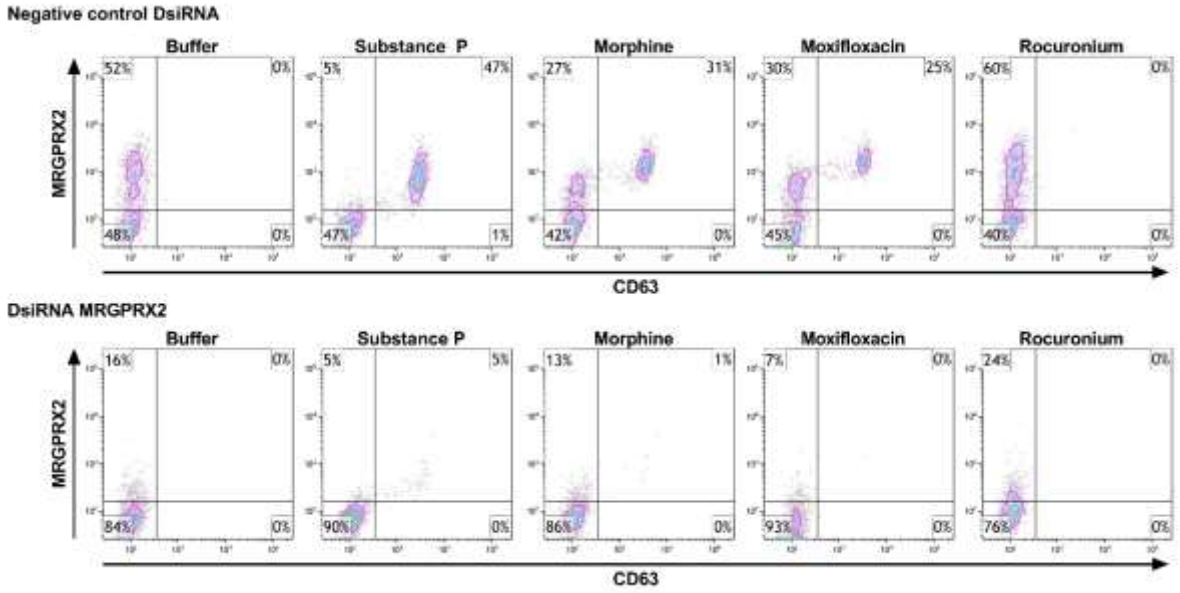
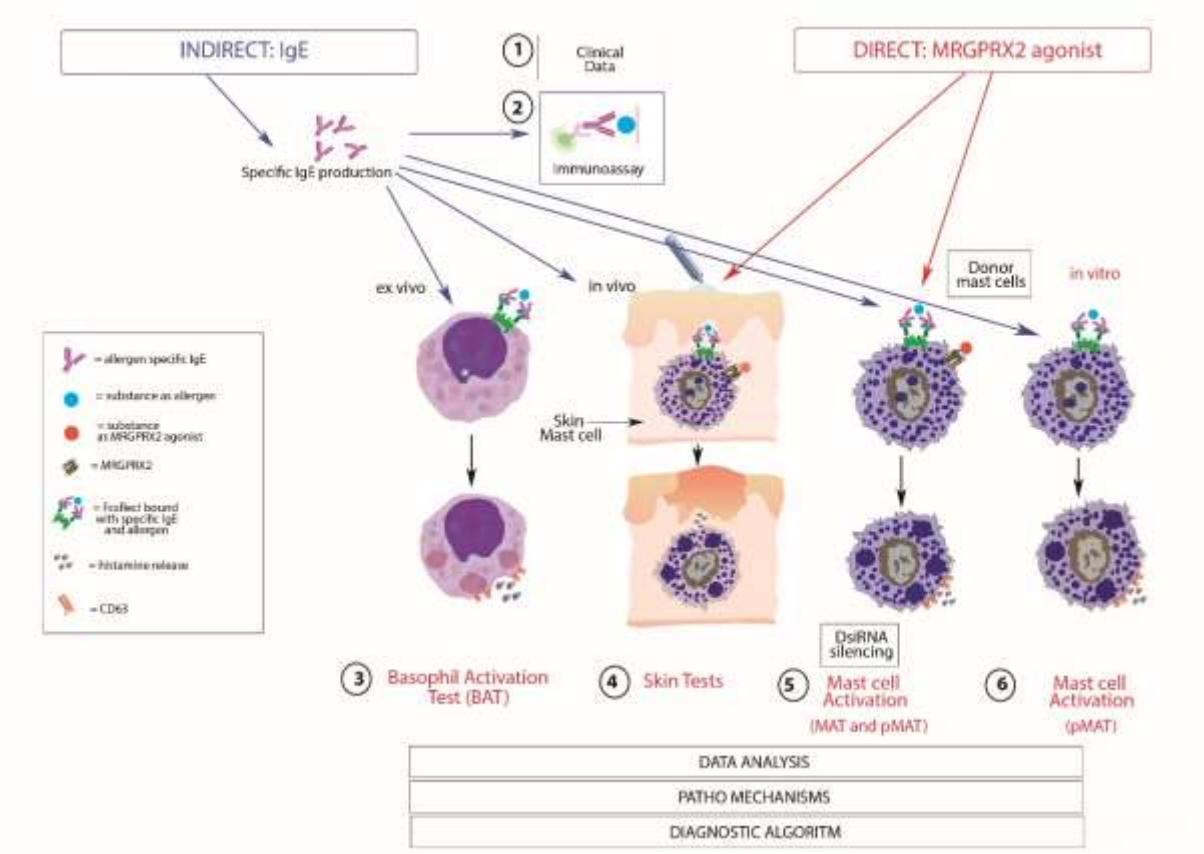


Figure 6: Direct mast cell (MC) activation by MRGPRX2 agonists substance P (natural ligand), the opiate morphine and the fluoroquinolone moxifloxacin. Note that, unlike reported by McNeil (40), rocuronium does not induce degranulation of primary human MCs. Silencing of MRGPRX2-dependent degranulation by DsiRNA (55).



603

604 Figure 7: exploring IgE- and MRGPRX2-dependent immediate drug hypersensitivity.
605 Proposal for an integrated approach using skin tests, IgE-immunoassays, basophil
606 activation tests, direct/passive mast cell activation tests, and selective MRGPRX2
607 silencing to deepen pathomechanistic insights in IgE- and MRGPRX2-dependent
608 immediate drug hypersensitivity. Note that starting from $CD34^{+ve}$ progenitors, it is
609 possible to obtain a $MRGPRX2^{+ve}$ and $MRGPRX2^{-ve}$ population that can be studied
610 separately using multicolor flow cytometry. Ultimately, this approach could advance
611 development of novel diagnostic.

612

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