Mast cell activation test : a new asset in the investigation of the chlorhexidine cross-sensitization profile

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Key messages

- Passively sensitized mast cells constitute an attractive tool to explore cross-reactivity between structurally similar compounds
- PHMB, but not Chlorhexidine, alexidine and octenidine, shows MRGPRX2 agonistic activity
- Mast cell activation tests can advance our insights in cross-reactivity patterns and MRGPRX2 agonistic activities.

Authors contributions

DG, JE, VSO have made substantial contributions to conception and design. JE and NM performed the experiments. JE, CM have been involved in acquisition of data, or analysis and interpretation of data. DGE, JE and VS been involved in drafting the manuscript or revising it critically for important intellectual content. All authors revised the manuscript, contributed with revisions and approved the final l paper.

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Abstract

Background: Insights into the IgE cross-sensitization and possible cross-reactivity patterns of sera reactive to chlorhexidine (CHX) are still incomplete and are likely to benefit from a functional exploration using a passive mast cells activation test (pMAT). Therefore, we want to study whether the pMAT with CHX-specific IgE (sIgE), enables to depict effector cell degranulation in response to alexidine (ALX), octenidine (OCT) and/or polyhexamethylene biguanide (PHMB) indicative of cross-reactivity between these compounds and CHX.

Methods: Serum of 10 CHX-allergic patients, 9 individuals with an isolated sIgE CHX and 5 healthy controls were included. Human cultured mast cells (MCs) were, before and after sensitization, challenged with CHX, ALX, OCT or PHMB. Degranulation was measured via quantification of up-regulation of CD63.

Results: MC responsiveness to ALX and OCT was demonstrable with 4/10 and 3/10 of the sera of CHX-allergic patients, respectively. Percentage of degranulation varied between 12-34% for ALX reactive MCs and between 4-22% for OCT reactive MCs. No reactivity to ALX or OCT was demonstrable when using sera obtained from individuals with an isolated sIgE CHX or from healthy controls. Unlike CHX, ALX and OCT, PHMB turned out to be a direct MC activator via occupation of MRGPRX2. PHMB-reactive sIgEs were demonstrable in some patients with an isolated sIgE CHX but were unable to trigger PHMB-induced degranulation in MRGPRX2 knock-down MCs.

Conclusion: MCs constitute an attractive tool to explore cross-reactivity between structurally similar compounds. Along with the identification of safe alternatives for the individual patient, the pMAT can advance our insights in sIgE cross-reactivity patterns including assessment of molecules not yet approved for human use.

Introduction

Since its first description in 1986 [1], IgE-mediated allergy to the biguanide antiseptic chlorhexidine (CHX), has evolved to a well-known condition [2]. Based upon the outcomes of specific IgE (sIgE) binding and hapten inhibition studies, basophil activation tests (BATs) and skin tests (STs), evidence has accumulated the CHX-sIgE antibody response to be polyclonal and to involve different reactivity patterns. Actually, it has been demonstrated that the CHX-sIgE reactivity profile extends beyond the immunodominant chlorophenyl endings, but also to encompass recognition of the biguanide and the hexamethylene components [3, 4]. Figure 1 shows that CHX is composed out of chlorophenyl, biguanide and hexamethylene structures.

Recently, we showed that MCs cultured from donor peripheral blood progenitors (PBCMCs) can be passively sensitized with patients' sera enabling determination of the clinical significance of CHX-reactive sIgE antibodies [5, 6]. The technique proved to be sensitive generating data from CHX-sIgE titres as low as 0.46 kUA/L. Encouraged by these promising findings, we anticipated that activation of passively sensitized PBCMCs (henceforth called pMAT) could constitute a useful instrument to further unveil the CHX-sIgE reactivity profile and a become a substitute for a more complex approach necessitating combinations of different *in vitro*, *ex vivo* and *in vivo* tests. In this study we want to analyze the capacity of sera from CHX- allergic patients and patients with an isolated sIgE CHX to activate MCs in response to the biguanide antiseptic alexidine (ALX), the bispyridine cationic antiseptic octenidine (OCT) and polyhexamethylene biguanide (PHMB; also known as polyhexanide). As shown in figure 1, ALX consists of two (2-ethylhexyl-) guanide units linked by a hexamethylene bridge, OCT has only the hexamethylene part in common with CHX and ALX, whereas PHMB shares both the biguanide and hexamethylene residues with CHX. The chlorophenyl endings are unique for CHX and constitute the immunodominant epitopes.

Materials and methods

Study population

In this study historical serum samples collected from patients between 2004 and 2020 were used. All patients were included through the outpatient clinics of Allergology and Immunology of the Antwerp University Hospital and the AZ Jan Palfijn Hospital in Ghent. Patients were enrolled if they experienced a suspected perioperative hypersensitivity reaction and had a complete diagnostic workup including STs for all potential causes. The perioperative hypersensitivity reactions were classified corresponding to a modified Ring and Messmer classification as used in the Sixth National Audit Project [7]. Participants gave written informed consent in accordance with the Declaration of Helsinki, and the study was approved by the Ethical Committee of the University Hospital of Antwerp (Belgium B300201316408).

Specific IgE measurements (ImmunoCAP)

Total and specific IgE to CHX (commercially available) and PHMB (research use only) were quantified with a FEIA ImmunoCAP technique (Thermo Fisher, Uppsala, Sweden). Results $\geq$ 0.35 kUA/L were considered positive. For the hapten inhibition experiments, sera with a positive sIgE PHMB were pre-incubated with 1000 µmol/L PHMB for 1 hour at room temperature.

Skin testing

Skin testing was performed with chlorhexidine digluconate. Skin prick tests (SPTs) are performed up to a maximal non irritative concentration of 5 mg/mL chlorhexidine digluconate, a positive control and a saline buffer solution as negative control. SPTs are read after 15 minutes and a wheal surrounded by flare equaling or exceeding 3 mm is considered positive. Intradermal tests (IDT)s are performed if SPTs are negative and were performed with

chlorhexidine digluconate 0.002 mg/mL. IDTs are considered positive when the wheal surrounded by flare equals or exceeds 5 mm or doubles as compared to the injection bleb.

Sera from patients and control individuals

Sera from 10 patients with a CHX-sIgE >0.35 kUA/L were selected (Table 1). All these patients had a history consistent with reactions to chlorhexidine and positive STs and/or dBATs with CHX confirmed the diagnosis of an IgE-mediated CHX allergy (CHX-allergy). Additionally, 9 sera with CHX-sIgE >0.35 kUA/L obtained from individuals who experienced perioperative hypersensitivity, but where clinical allergy to chlorhexidine could not be confirmed as they had negative STs and dBATs to CHX, were tested (isolated sIgE CHX). As shown in Table 1, in the majority of individuals with an isolated sIgE, the perioperative hypersensitivity reaction was attributable to alternative causes. Finally, 5 sera of healthy controls demonstrating a negative ST and sIgE to CHX were included (Table 1). Due to insufficient serum, in the pMAT ALX and OCT 10 sera from CHX-allergic and only 5 sera from individuals with an isolated sIgE CHX were used. In the pMAT PHMB using MRGPRX2-silenced MCs, only 3 sera from CHX-allergic and 7 from individuals with an isolated sIgE CHX were studied (indicated with ° in table 1).

In vitro culture of PBCMCs

Human PBCMCs were cultured as previously described [5, 8]. Briefly, peripheral blood mononuclear cells were isolated from 50 mL of fresh peripheral blood from healthy volunteers, and $CD34^+$ progenitor cells were enriched using the EasySep™ Human CD34 Selection Kit (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Isolated peripheral blood $CD34^+$ progenitor cells were cultured in a serum-free methylcellulose-based medium (MethoCult™ SF H4236; STEMCELL

Technologies) supplemented with penicillin (100 units/mL; Life Technologies, Waltham, MA, USA), streptomycin (100 mg/mL; Life Technologies), low-density lipoprotein (10 mg/mL; STEMCELL Technologies), 2-mercaptoethanol (55 mmol/L; Life Technologies), stem cell factor (100 ng/mL; Miltenyi Biotec, Bergisch Gladbach, Germany), interleukin-3 (100 ng/mL; PeproTech, Rocky Hill, NJ, USA), and interleukin-6 (50 ng/mL; Miltenyi Biotec) for 4-5 weeks. PBCMCs harbour a MRGPRX2^{+} and a MRGPRX2^{-} subpopulation, relevant to our further experiments (Suppl. Figure 1).

Direct mast cell activation test (MAT)

PBCMCs, defined as CD117^{+} and CD203c^{+} cells, were suspended in pre-warmed (37°C) Tyrode's buffer at a concentration of 5x10^{5} cells/mL. Next, 100 µL of the cells were stimulated with 100 µL Tyrode's buffer as a negative control, 100 µL substance P (74 µmol/L, Sigma-Aldrich, St Louis, MO, USA) as positive control for the MRGPRX2 pathway, 100 µL anti-FcεRI (2.5 µg/mL, Thermo Fisher Scientific) as a positive control for the IgE-pathway, 100 µL CHX (Sigma-Aldrich), 100 µL ALX (Sigma-Aldrich), 100 µL OCT (Thermo Fischer Scientific) or 100 µL PHMB (Boc Sciences, Shirley, NY, USA), for 3 and 20 min at 37°C. The final concentrations of CHX were based on previous dose finding experiments [5] and implied 0.028 and 2.8 µmol/L. The final concentrations of ALX and OCT were 2.8.10^{-6}, 2.8.10^{-4}, 2.8.10^{-2}, 2.8 µmol/L, as higher concentrations revealed to be toxic. The final concentrations of PHMB were 0.35, 0.7, 1.4, 2.8 and 5.6 µmol/L Subsequently, supernatants were removed by centrifugation (500 x g, 4°C, 5 min). Cells were stained with anti-human CD117-APC (clone 104D2, BD Biosciences, Erembodegem, Belgium), anti-human CD203c-PECy7 (clone NP4D6, eBioscience, San Diego, USA), anti-human MRGPRX2-PE (clone K125H4, BioLegend, San Diego, USA) and anti-human CD63-FITC (clone H5C6, BD Biosciences) for 20 min at 4°C. Next, cells were fixed with 1 mL Phosflow Lyse/Fix buffer (BD Biosciences) for 20 min. Reactions were stopped by placing the

cells on ice. Finally, cells were washed and resuspended in PBS with 0.1% sodium azide and measured using flow cytometry. Degranulation of PBCMCs was measured as surface upregulation of the lysosomal degranulation marker CD63.

Passive mast cell activation test (pMAT)

In the pMAT, degranulation of PBCMCs was measured by passively sensitizing the cells (5×10^5 cells/mL) with serum in a 1:1 ratio at 37°C in a humidified CO_2 incubator overnight. The sensitized cells were then centrifuged (500g; 5 min; 20°C) and the cell pellet was resuspended in pre-warmed Tyrode's buffer (Sigma-Aldrich) to 5×10^5 cells/mL. Then, 100 µL of the cells was pre-incubated with interleukin-33 (IL-33) (100 ng/mL) (PeproTech) for 20 min at 37°C, and the pre-incubated sensitized PBCMCs were stimulated with 100 µL Tyrode's buffer as a negative control, 100 µL anti-IgE, (1 µg/mL, BD Biosciences) as a positive control for the IgE-mediated activation after passively sensitization, 100 µL CHX, 100 µL ALX, 100 µL OCT or 100 µL PHMB, as described above.

MRGPRX2 silencing by Dicer-substrate small interfering RNA (DsiRNA) electroporation

PBCMCs at a concentration of $1x10^6$ cells/mL were washed twice in cold serum-free Opti-MEM I medium (Gibco Invitrogen, Grand Island, NY, USA) and resuspended in 200 µL of the same medium. Cells were transferred to a 4.0-mm electroporation cuvette (Cell Projects, Maidstone, UK), and 1 µM pool of two DsiRNA against *MRGPRX2* at a 1:1 ratio or a non–targeting control DsiRNA (Integrated DNA Technologies, Lowe, USA, Catalog #:51-01-14-03) were added to the cuvette (Duplex sequences: DsiRNA 1: 5'-GGCAUUCAGUGGUUCCUAAUAUUAT-3' and 3'-AACCGUAAGUCACCAAGGA UUAUAAUA-5', DsiRNA 2: 5'GUUACGUGUUCCA CAGAAUAAAATA-3' and 3'-UUCA AUGCACAAGGUGUCUUAUUUUAU-5'). A square wave protocol (500 V, 5 ms, 0 gap, 1 pulse)

was used to electroporate the cells (Gene Pulser Xcell™ device, Bio-Rad Laboratories, Hercules, California, USA). Immediately after electroporation, cells were transferred to 5 mL of IMDM medium (Thermo Fischer Scientific) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) (pre-heated at 37°C) and incubated for 20 min at 37°C and 5% CO_2. Thereafter, cells were centrifuged and transferred to IMDM medium with SCF and IL-6. Five days after electroporation, a repeated analysis of MRGPRX2 expression and functionality of the PBCMCs was performed as described above.

Flow cytometry

Flow cytometry was performed on a FACSCanto II™ flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA) equipped with three lasers (405, 488, and 633 nm). Correct compensation settings for antibodies conjugated with fluorochromes were performed using BD™ CompBeads (BD Biosciences). Flow cytometric data was analyzed using Kaluza Analysis 2.1 software (Beckman Coulter, Brea, CA, USA). A fluorescence minus one (FMO) was used to distinguish between positive and negative cells. MCs were gated out as CD117 and CD203c positive cells. At least 500 MCs were counted per sample. As previously described for CHX, the diagnostic threshold was set on ≥ 3 [6, 9, 10].

Statistical analysis

GraphPad Prism version 8 (GraphPad Software Inc., San Diego, CA, USA) was used for data analysis and paired Student's t-tests were performed. Results are expressed as mean ± SEM. A P-value of < 0.05 was considered significant.

Results

As shown in Figure 2 and table 1, all CHX-allergic patients demonstrated responsiveness in the pMAT with CHX. In contrast, using sera from individuals with an isolated sIgE CHX and healthy controls, no degranulation was demonstrable in response to CHX. In the pMAT, MC degranulation triggered by ALX and OCT was demonstrable with 4/10 (#2, 5, 7, 9) and 3/10 (#2, 5, 7) of the sera of CHX-allergic patients, respectively. For ALX, percentages of degranulating MCs varied between 12 and 34% for the corresponding concentration of 2.8 µmol/L. For OCT, percentages of degranulating MCs varied between 4 and 22% for the corresponding concentration of 2.8 µmol/L. The highest percentage degranulation was observed in the patient (#5), who had a history of hypersensitivity reactions to OCT and also demonstrated a positive skin prick test OCT with a 0.01% OCT solution (Octenisept®) with a wheal and surrounding flare of 4/15 mm. Skin prick tests with OCT in 5 healthy controls were negative. In contrast to CHX-allergic patients, using serum of 5 individuals with an isolated sIgE CHX or 5 healthy controls, no MC degranulation in response to ALX and OCT was demonstrable (Figure 2). A representative individual plot is shown in Suppl. Figure 2. MCs incubated directly with CHX, ALX and OCT without prior sensitization remained unresponsive to all three antiseptics (data not shown).

PHMB, in contrast to CHX, ALX and OCT, triggered direct degranulation without prior passive sensitization. This degranulation was strictly restricted to the MRGPRX2^{+} MC subpopulation (Figure 3) and was completely abolished by selective MRGPRX2 silencing (Figure 4). As MRGPRX2 silencing did not affect FcεRI signalling in targeted cells (Figure 4 and Suppl. Figure 3) [11, 12], we took the opportunity to analyse MC activating capacity of PHMB in a pMAT using MRGPRX2-silenced cells. As shown in Figure 5, MCs sensitized with sera of CHX-allergic patients and individuals with an isolated sIgE CHX failed to degranulate in response to PHMB.

However, sIgE against PHMB was detectable in 3 sera of individuals with an isolated sIgE CHX. Pre-incubation with PHMB resulted in an almost complete inhibition of the sIgE PHMB (72 and 81%) (n=2). Note that total IgE was significantly lower in CHX-allergic patients (191 kU/L (range: 65-582)) as compared to patients with an isolated sIgE CHX with an isolated sIgE CHX (2459 kU/L (range: 188-6079)) (p=0.002).

Discussion

This study shows that the application of donor PBCMCs in immediate drug hypersensitivity extends beyond their use as a diagnostic tool for sIgE-dependent reactions or as a method to safely identify MRGPRX2 agonists [5, 6, 11, 12]. Despite the limited number of experiments, our observations with dMATs and pMATs should be considered as pivotal and warranting further evaluation of MC-based approaches to explore potential cross-reactivity between compounds with structural similarity, and to assess the effector cell activating capacity of drug-reactive sIgE antibodies. Actually, we show that PBCMCs passively sensitized with CHX-sIgE containing sera from patients with a sIgE-mediated CHX allergy, can become responsive to ALX and OCT. As indicated by earlier hapten inhibition studies [3, 4], the explanation for these observations should likely be sought in the sensitization of the cells with functionally active CHX-sIgE antibodies that can cross-react with the biguanide and/or hexamethylene parts of ALX and OCT, as both these antiseptics lack the chlorophenyl structure unique to CHX. The reason for this polyclonality of the anti-CHX response could relate to the origin(s) and route(s) of sensitization, both uncertain in a majority of CHX allergic patients. Alternatively, the absence of MC responses to ALX and OCT using sera of individuals with an isolated sIgE CHX with positive CHX-sIgE but negative STs and dBATs CHX, may indicate that ALX and OCT are not at the origin of the clinically irrelevant CHX sensitization. Another peculiarity from our study relates to the unpredictability of the CHX cross-reactivity patterns. These profiles seem to vary between the CHX-allergic patients. As shown in table 1, in some CHX-allergic patients, MC reactivity was observed to three out of the four tested compounds, i.e. CHX, ALX and OCT. In others, MC responsiveness was less broad or even restricted to CHX alone.

On analyzing the PBCMC responses to PHMB we observed significant differences with CHX, ALX and OCT. In contrast to CHX, ALX and OCT, PHMB was found to trigger direct degranulation

of the donor PBCMCs. This degranulation appeared to be restricted to the MRGPRX2^{+} subpopulation and could be effectively abolished by selective silencing of the receptor. Moreover, we showed that MRGPRX2 silencing did not affect sIgE/FcεRI-signaling triggered by CHX. Therefore, we took the opportunity for additional pMAT experiments with PHMB in targeted PBCMCs. However, no sIgE/FcεRI-dependent degranulation in response to PHMB was demonstrable. Likely, the main reason for this observation relates to the absence of PHMB-sIgE in CHX-allergic patients. PHMB-sIgE was detectable in three individuals with an isolated sIgE CHX and an alternative explanation should be sought for this. Based upon the hapten inhibition studies, it is unlikely for the clinically irrelevant PHMB-sIgE results to result from non-specific binding to the solid phase. More likely, the incapacity of these PHMB sIgE antibodies to trigger MC degranulation is related to a low affinity or recognition of only a single epitope that does not allow cross-linking of sIgE/FcεRI complexes. Admittedly, these hypothesis needs further investigation as the numbers of patients in our experiments are low. Because of the discovery of PHMB to be a MRGPRX2 agonist, the PHMB sIgE-assay is of limited value. The main issue of a negative sIgE result is to discriminate between a false negative (overlooked an IgE-meditated reaction) or a true negative result that does not exclude a MRGPRX2 reaction. A positive test would require additional functional testing such as BAT or pMAT with MRGPRX2-silenced cells, as skin test are not discriminative between IgE- and MRGPRX2-mediated reactions.

To appreciate the significance of this novel application of the MAT, it is important to understand the limitations of currently applied methods as indicated in the introductory paragraph. A significant part of our knowledge about the CHX cross-reactivity profile stems from sIgE binding and hapten inhibition studies. However, this approach can be hindered by difficulties of coupling the drug to the solid phase or by the masking of relevant epitopes [4, 13].

311 Furthermore, results of sIgE inhibition studies are not always predictive for the clinical
312 outcome during subsequent exposure [5]. The reason for STs to be more predictive of the clinical
313 significance of cross-reactivity is obvious. STs necessitate cross-linking of membrane-bound
314 sIgE antibodies to start sIgE/FcεRI-signaling finally culminating in the exteriorization of
315 mediators triggering the wheal and flare responses. However, being an *in vivo* procedure, STs,
316 in particular intradermal tests, are restricted to compounds approved for human use and,
317 most importantly, a positive ST response does not necessarily indicate an sIgE-dependent
318 mechanism. A positive ST result might also reflect an irritant response, or nonspecific
319 histamine release by MRGPRX2 occupation, as this receptor is abundantly expressed by skin
320 MCs [14, 15]. In the light of these difficulties, readily accessible basophils rapidly became an
321 attractive alternative to explore functionally relevant cross-reactivity patterns [4, 16]. In dBATs,
322 patients' cells are directly incubated with antigen and activation/degranulation is appreciated
323 via quantification of mediator release or the upregulation of specific activation/degranulation
324 markers [17]. Although the dBAT has advanced our insights and changed paradigms about cross-
325 reactivity, it leaves us with some significant weaknesses. Direct BATs necessitate fresh viable
326 cells and 5-15% of the patients show an unpredictable basophilic non-responder status that
327 can only be depicted ad hoc [18]. In an attempt to solve these weaknesses, some groups have
328 invested in the pBAT. The pBAT uses stripped donor basophils that are sensitized with
329 patients' sera and subsequently incubated with relevant allergen. However, pBATs are highly
330 dependent on the donor, are technically challenging and their analytical sensitivity is generally
331 less sensitive than traditional dBATs [4]. Alternatively, pMAT circumvents the need for viable
332 patients' cells, solves the issue of non-responder status and has a high analytical sensitivity
333 which shows promise for future collaborative studies of chlorhexidine cross-reactivity
334 patterns. Admittedly, an important consideration about our study relates to the source

335 material of our technique, that is, primary human MCs cultured out of peripheral blood
336 progenitors from healthy donors. Such PBCMCs might be difficult to keep viable over longer
337 time periods and continuously require new cultures. However, to the best of our knowledge,
338 an attempt to passively sensitize immortal LAD2 cells, with drug-reactive sIgE antibodies, has
339 so far been unsuccessful [19].
340 One could argue that in the absence of a CHX challenge test, no absolute conclusion can be
341 drawn with respect to the cases demonstrating a positive CHX sIgE in isolation. However, given
342 the negative outcome of three different more functional tests, e.g. skin tests, BAT and pMAT,
343 we are quite confident that these isolated sIgE results are clinically irrelevant and frequently
344 result from nonspecific binding to the solid phase. To avoid misdiagnosis, an elevated sIgE
345 result should always be confirmed by a positive result in either skin tests, dBAT or pMAT. A
346 further perspective could be to consider a local validation of a CHX challenge protocol in
347 patients with an isolated sIgE CHX.In conclusion, this study provides encouraging evidence
348 that the pMAT, which uses PBCMCs passively sensitized with patients' sera, can benefit
349 functional exploration of sIgE cross-reactivity patterns for CHX. Although it is far to go from
350 this proof of concept to more systematic use, we think that collaborative studies involving
351 clinical centers and centralized experienced laboratories can ease promotion and
352 breakthrough of this attractive technique.
353

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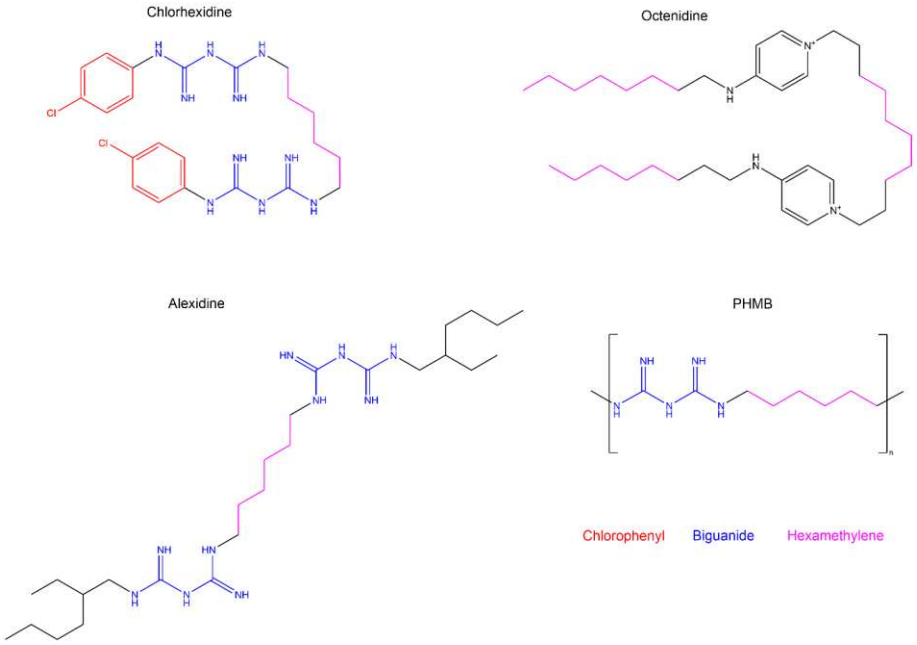
Tables and figures

P/C	Age (yr.)/sex	Delay (d.)	Severity grade	Total IgE (kU/L)	sIgE CHX† (kUA/L)	sIgE PHMB† (kUA/L)	ST CHX	SPT/IDT	ST concentration (mg/mL)	Culprit	dBAT CHX (%)	pMAT CHX‡ (%)	pMAT ALX (%)	pMAT OCT (%)
p/1	68/m	37	4	65	1.2	<0.35	+	SPT	0.5	CHX	47	13	0	0
p/2	41/m	161	2	68	10.3	<0.35	+	SPT	5	CHX	55	88	27	14
p/3	62/m	18	3	582	18.5	<0.35	+	SPT	5	CHX	64	68	2	2
p/4°	62/m	14	3	252	8.38	<0.35	+	IDT	0.002	CHX	43	71	0	0
p/5°	19/m	147	2	103	7.85	<0.35	+	SPT	0.5	CHX	57	74	15	22¶
p/6	52/m	35	4	66	1.14	<0.35	+	SPT	5	CHX	54	8	2	1
p/7°	68/f	455	1	232	0.46	<0.35	+	IDT	0.002	CHX	33	68	34	4
p/8	67/m	177	3	190	3.5	<0.35	+	IDT	0.002	CHX	ND	8	1	1
p/9	66/m	192	3	175	28.8	<0.35	+	SPT	5	CHX	ND	67	12	2
p/10	42/f	115	3	167	4.94	<0.35	+	SPT	5	CHX	ND	34	0	1
p/13	63/m	121	4	6079	24.8	4.41	-	IDT	0.002	ROCU	0	0	0	1
p/14°	44/f	72	4	2483	2.17	1.06	-	IDT	0.002	ROCU	1	1	0	1
p/15	51/f	153	3	832	32	<0.35	-	IDT	0.002	Unkown	1	1	1	2
p/16°	60/f	10	3	657	4.01	<0.35	-	IDT	0.002	ROCU	0	1	1	1
p/17°	51/f	36	3	188	7.07	1.17	-	IDT	0.002	ROCU	0	0	1	1
p/18°	77/m	44	2	4848	1.71	<0.35	-	IDT	0.002	Unkown	1	0	ND	ND
p/19°	54/f	114	4	815	6.8	<0.35	-	IDT	0.002	Unkown	0	0	ND	ND
p/20°	63/m	31	4	3014	30	<0.35	-	IDT	0.002	Unkown	2	0	ND	ND
p/21°	65/m	41	4	3217	11.1	<0.35	-	IDT	0.002	ROCU	0	1	ND	ND
c/1	49/f	NA	NA	4	<0.35	<0.35	-	IDT	0.002	NA	ND	0	0	0
c/2	38/m	NA	NA	511	<0.35	<0.35	-	IDT	0.002	NA	ND	1	0	0
c/3	50/f	NA	NA	85	<0.35	<0.35	-	IDT	0.002	NA	ND	0	0	0
c/4	56/m	NA	NA	19	<0.35	<0.35	-	IDT	0.002	NA	ND	0	0	0
c/5	23/f	NA	NA	5	<0.35	<0.35	-	IDT	0.002	NA	ND	1	0	0

Table 1: Patient characteristics, results of confirmatory testing and pMAT results. P, patient; C, control; yr., years; IgE, immunoglobulin E; sIgE, specific IgE; d., days between the index reaction and the confirmatory tests; ST, skin test; CHX, chlorhexidine; SPT, skin prick test; IDT, intradermal testing; pMAT, mast cell activation test after passive sensitization; M, male; F, female; NA, not applicable; ND, not determined. ¶ sera used in the pMAT PHMB after selectively silencing of MRGPRX2; †the threshold for sIgE positivity is set on 0.35 kUA/L; ‡ As previously described, the threshold for pMAT positivity is set on $\geq$3% [6]. Severity grade according to Garvey L.H. et al. [7].° The patient demonstrated also a positive skin test OCT. pMAT results were shown for PBCMCs activation with CHX, ALX or OCT at a final concentration of 2.8 µmol/L.



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412 **Figure 1: Structures of chlorhexidine, alexidine, octenidine and polyhexamethylene**
413 **biguanide (PHMB).**

414 Chlorhexidine contain a chlorophenyl, group, biguanide and hexamethylene group. Alexidine
415 contain a biguanide and hexamethylene group. Octenidine contains three hexamethylene
416 groups and PHMB consists of repeated elements of biguanide and hexamethylene groups. The
417 chlorophenyl group is displayed in red, the biguanide in blue and the hexamethylene group in
418 purple. Figure adapted from Mueller-Wirth et al.[4].

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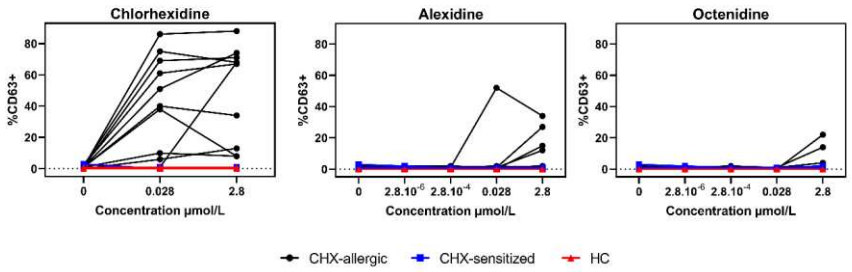


420

421 **Figure 2: Passive mast cell activation with chlorhexidine, alexidine and octenidine**

422 Cultured human-derived mast cells were activated with chlorhexidine, alexidine or octenidine
423 after passive sensitization of the cells with sera from 10 CHX-allergic patients with positive
424 CHX sIgE, skin test and direct basophil activation test/pMAT CHX (black lines: ● symbols), sera
425 from 5 patients sensitized to CHX (i.e. positive sIgE to CHX, but negative skin test and direct
426 basophil activation test (blue lines: ■ symbols) or with sera from 5 healthy controls (HC) (red
427 lines: ▲ symbols).

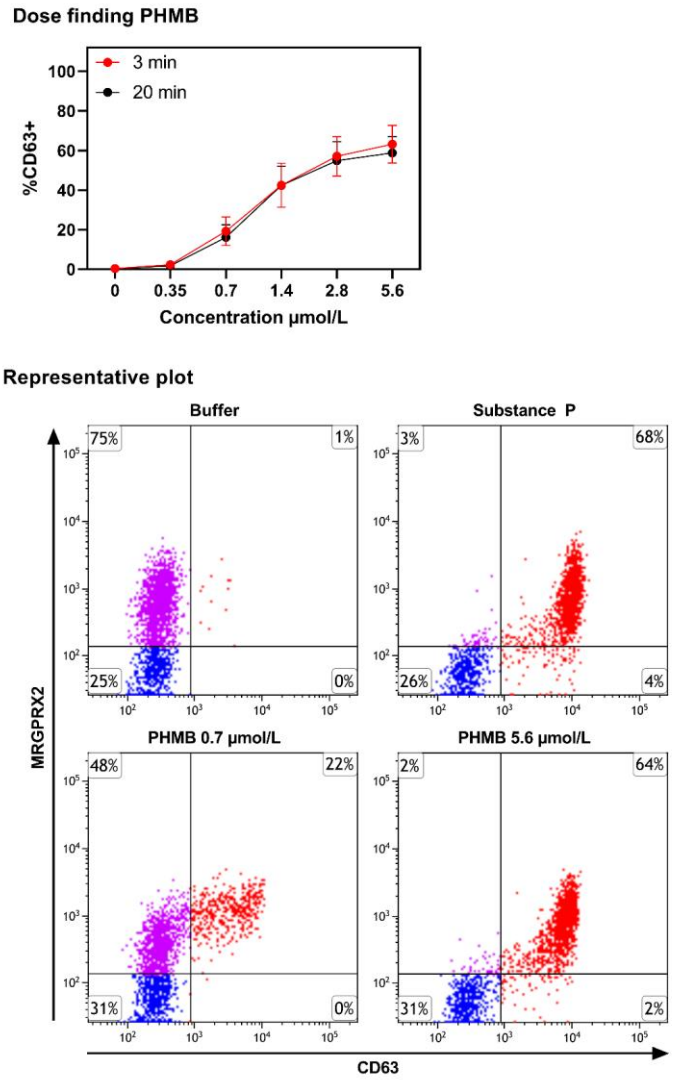


Figure 3: Direct mast cell activation test with polyhexamethylene biguanide (PHMB)

Cultured human-derived mast cells from 5 healthy donors were activated with PHMB 0.35, 0.7, 1.4, 2.8 or 5.6 µmol/L for 3 or 20 minutes (top). Representative plot of the direct mast cell activation test with PHMB or substance P (74 µmol/L) (bottom). Blue, CD203c positive; Purple, MRGPRX2 positive; Red, CD63 positive.

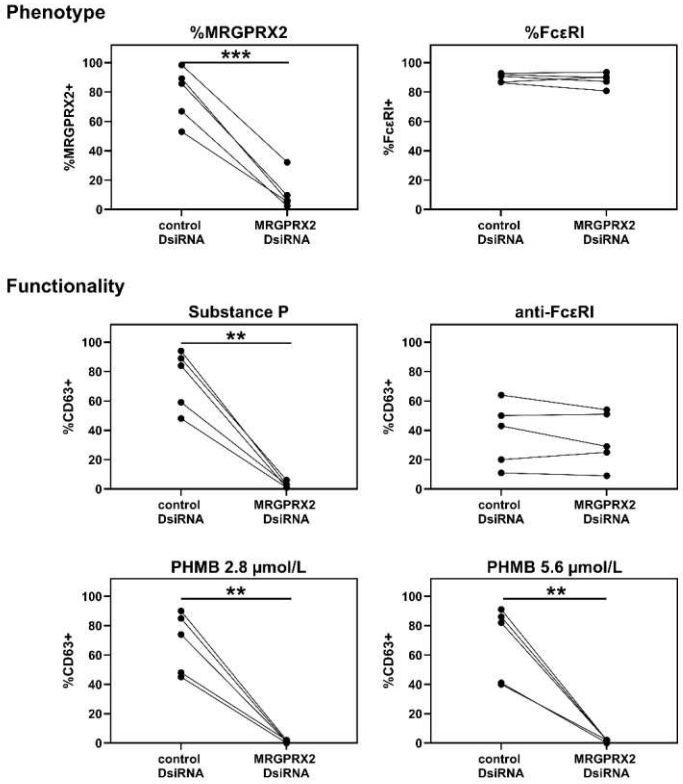


Figure 4: Effect of MRGPRX2 silencing on PBCMC phenotype and functionality

PBCMCs were electroporated with a negative control DsiRNA or a MRGPRX2-specific DsiRNA. Comparison of the surface expression of MRGPRX2 or FcεRI between PBCMC electroporated with non-targeting DsiRNA or DsiRNA specific for MRGPRX2 (top). Degranulation, i.e. CD63 up-regulation, after 3 min of incubation with substance P (74 µmol/L), the natural agonist of MRGPRX2, anti-FcεRI (2.5 µg/mL) or PHMB (2.8 and 5.6 µmol/L) (bottom). Results of CD63 measurements were expressed as the net value of percentages of positive cells, i.e. the percentage of $CD63^+$ cells in stimulated cells minus the percentage of $CD63^+$ cells in resting cells. Results from 5 different donor MC cultures. Paired student t-test, $p < 0.01$**, $p < 0.001$***, $p < 0.0001$***.

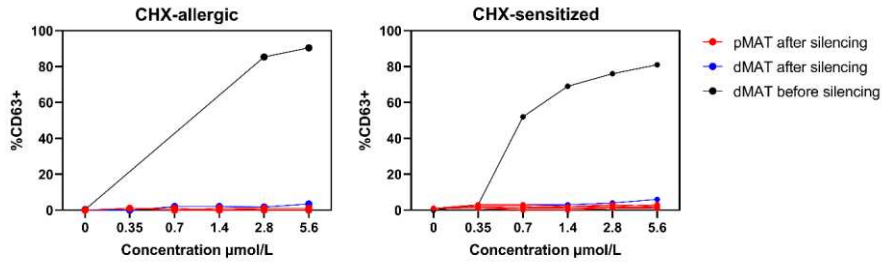
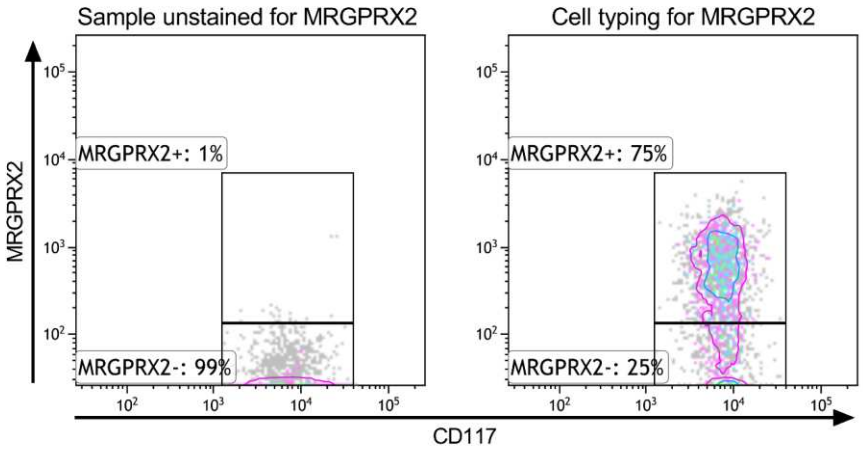


Figure 5: Mast cell activation test with polyhexamethylene biguanide (PHMB) before and after selectively silencing of MRGPRX2.

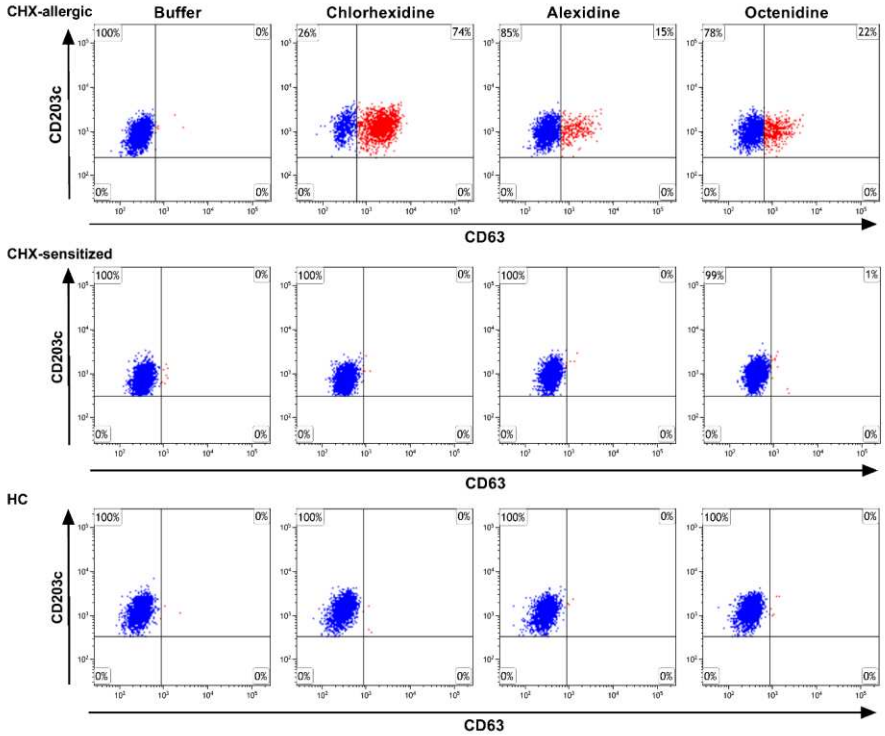
MRGPRX2-silenced cells were sensitized with sera from CHX-allergic patients (n=3,left) of CHX-sensitized patients (n=7,right), after sensitization cells were activated with PHMB (red lines). The same silenced cells were activated with PHMB without passive sensitization (blue line). PBCMCs from the same donor were activated with PHMB without selectively silencing of MRGPRX2 (black line).

Online repository figure



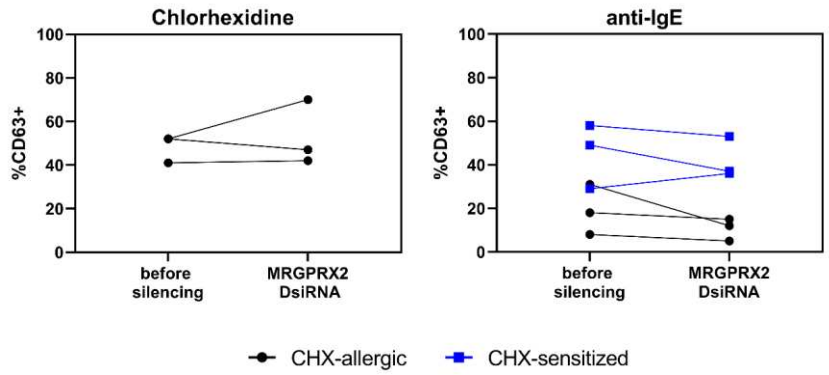
Supplementary figure 1: Representative plot for the MRGPRX2 expression on PBCMCs.

Peripheral blood cultured mast cells (PBCMCs) are defined as $CD117^{+}CD203c^{+}$ cells. PBCMCs harbour two subpopulations: cells with surface expression of MRGPRX2 ($MRGPRX2^{+}$) and cells without expression of MRGPRX2 ($MRGPRX2^{-}$).



Supplementary figure 2: Representative plot of the passive mast cell activation test with chlorhexidine, alexidine or octenidine

Cultured human mast cells were activated with chlorhexidine, alexidine or octenidine 2.8 µmol/L after passive sensitization of the cells with serum of a patient with established CHX allergy, a CHX-sensitized patient (isolated positive sIgE to CHX) and a healthy control (HC) with a negative sIgE and negative skin test to CHX. Blue, CD203c positive; Red, CD63 positive.



Supplementary figure 3: Passive mast cell activation test after MRGPRX2-silencing

Cultured donor PBCMCs were incubated with CHX (2.8 µmol/L) or anti-IgE (1 µg/mL) after passive sensitization of the cells with 3 sera from CHX-allergic (black lines, ● symbols) or with 3 sera of CHX-sensitized individuals (blue lines: ■ symbols). Results of CD63 measurements were expressed as the net value of percentages of positive cells, i.e. the percentage of $CD63^+$ cells in stimulated cells minus the percentage of $CD63^+$ cells in resting cells.