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Article type : Letter

FcεRI- and MRGPRX2-evoked degranulation responses are fully additive in human skin mast cells

To the Editor,

Mast cell (MC) activation by FcεRI-aggregation is the root of hypersensitivity reactions. However, the recent discovery of MRGPRX2 (*Mas*-Related G Protein-Coupled Receptor-X2) as a major IgE-independent stimulation pathway has also attracted much interest ¹⁻⁴. Indeed, MRGPRX2 is activated by a plethora of ligands, which can be constantly present in tissues, but the low-affinity nature of MRGPRX2 (requiring micromolar agonist concentrations) likely impedes its permanent stimulation; it is therefore controversial whether clinically significant reactions can be triggered by MRGPRX2 (alone) ^{2,3}. Combinatorial scenarios are an interesting option, but the sequels following concurrent activation of FcεRI and MRGPRX2 remain unresolved.

We tested this possibility in skin MCs stimulated by FcεRI-aggregation or the MRGPRX2 agonists compound 48/80 (c48/80), Substance P (SP), or codeine, individually or jointly. MRGPRX2 agonists were also combined. Degranulation was assessed by histamine or β-hexosaminidase release. Activated MCs were quantified by CD107a exteriorization (detailed methods can be found in supplementary material).

Using cultured skin-derived MCs, more responsive to FcεRI-aggregation than to MRGPRX2 ligation ⁵, we found that histamine release on combinatorial stimulation surpassed the one elicited by each receptor individually (Fig. 1a). Still more, joint stimulation of the routes was fully additive since measured release by the two stimuli was at least equal to the calculated secretion when

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both were applied separately (i.e. sum of “stimulus 1+stimulus 2”) (Fig. 1b). Results were the same for β -hexosaminidase (Suppl. Fig.1).

For CD107a upregulation, barely any increase was noted after 2 min in cells stimulated via the Fc ϵ RI-route, while MRGPRX2 ligation led to exteriorization at this point (Fig. 1c and Suppl. Fig. 2a). Conversely, Fc ϵ RI-aggregation resulted in a well-separated CD107a⁺ population after 30 min (Fig. 1e and Suppl. Fig. 2b). Differential kinetics were as expected, since signaling by MRGPRX2 is more rapid^{1,3,6}. Importantly, the proportions of CD107⁺ cells were additive on combined stimulation (Fig. 1d, f).

In *ex vivo* skin MCs, simultaneous activation of MRGPRX2 and Fc ϵ RI likewise elevated the response over each stimulus alone (Fig. 2a-c). Additivity was ascertained by contrasting the sum of individual stimuli against the measured response on joint stimulation (Fig. 2d-f). Activating the routes at different stimulus concentrations revealed additivity also at suboptimal conditions, even trending towards synergism (Suppl. Fig. 4a-d).

Collectively, in skin MCs, signals from the two receptor systems unite to produce strong and indeed additive responses, a finding of substantial clinical relevance. For example, allergens reportedly contain stimuli activating both receptors (e.g. hymenoptera venoms); moreover, drug classes (opiates, antibiotics), that activate MRGPRX2, can also elicit IgE responses in susceptible individuals. When co-activated, the anaphylactic threshold may be more readily exceeded.

We tested whether low concentrations of MRGPRX2 agonists can amplify each other. Indeed, SP and codeine at suboptimal concentrations resulted in increased responsiveness over each individual stimulus (Suppl. Fig. 3a). In contrast, joining SP and codeine at saturating concentrations did not enhance degranulation *vis-à-vis* each substance (Suppl. Fig. 3b)^{6,7}. Accordingly, the sum was equivalent to the measured value in the suboptimal setting (Suppl. Fig. 3c), but far above under optimal conditions (Suppl. Fig. 3d).

Collectively, we demonstrate that IgE-dependent and -independent pathways unite to boost exocytosis of human skin MCs. The same degree of mediator release and clinical response may thus be achieved by strong activation of Fc ϵ RI or MRGPRX2 individually or by weaker activation of the two simultaneously, the latter likely the common scenario *in vivo*.

Multiple combinations of elicitors are conceivable, also encompassing low levels of *different* MRGPRX2 agonists, which likewise amplify each other. Considering the wide repertoire of ligands, MRGPRX2 may plausibly contribute to anaphylactic reactions despite its low affinity. The study has also implications for urticaria, mastocytosis, and atopic dermatitis.

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Short title: MRGPRX2- and FcεRI-responses are additive

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Keywords

Mast cell, MRGPRX2, pseudo-allergy, drug allergy, anaphylaxis, FcεRI, IgE receptor, Substance P, opiates, skin

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Disclosure of potential conflict of interest

The authors declare they have no relevant conflicts of interest.

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Figure Legends

Figure 1. Simultaneous activation of FcεRI and MRGPRX2 results in cumulative histamine release and the proportion of CD107a⁺ cells

a) Net histamine secretion in cultured skin-derived MCs stimulated by IgER-CL (cross-linking, AER-37, 0.1 µg/ml), c48/80 (10 µg/ml), SP (30 µM), or codeine (100 µg/ml) individually or jointly. b) Comparison of the calculated release triggered by two individual stimuli (sum) with the measured (meas.) release on concomitant activation. Sum – the two routes are activated in separate wells and the resultant histamine releases are added up to give the theoretical additivity. Mean±SEM, n=10 independent experiments. Net percentage of CD107a⁺ cells at c) 2 min, e) 30 min. d, f) Comparison of the calculated with the measured proportion of CD107⁺ cells at d) 2 min, f) 30 min. Mean±SEM, n=9-11. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns—not significant.

Figure 2. FcεRI- and MRGPRX2-elicited degranulation responses are fully additive in skin MCs *ex vivo*

Net histamine release triggered in *ex vivo* skin MCs by a) IgER-CL (29C6, 0.5 µg/ml), c48/80 (10 µg/ml) or IgER-CL + c48/80. b) IgER-CL, SP (30 µM) or IgER-CL + SP. c) IgER-CL, codeine (100 µg/ml) or IgER-CL + codeine. d-e) Comparison of the calculated net release triggered by two individual stimuli (sum) with the measured (meas.) release on concomitant activation. The data are the mean±SEM of n=6-12 individual MC preparations. **p < 0.01, ***p < 0.001, ****p < 0.0001.

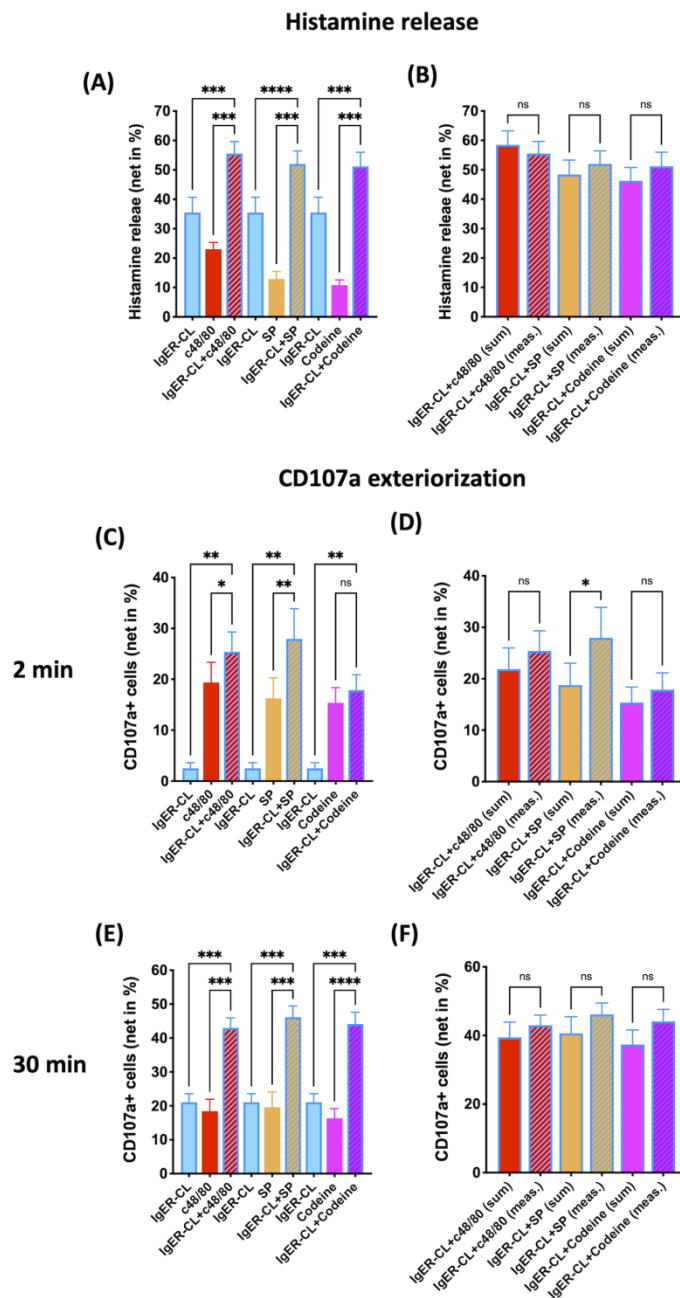


Figure 1_Babina et al.

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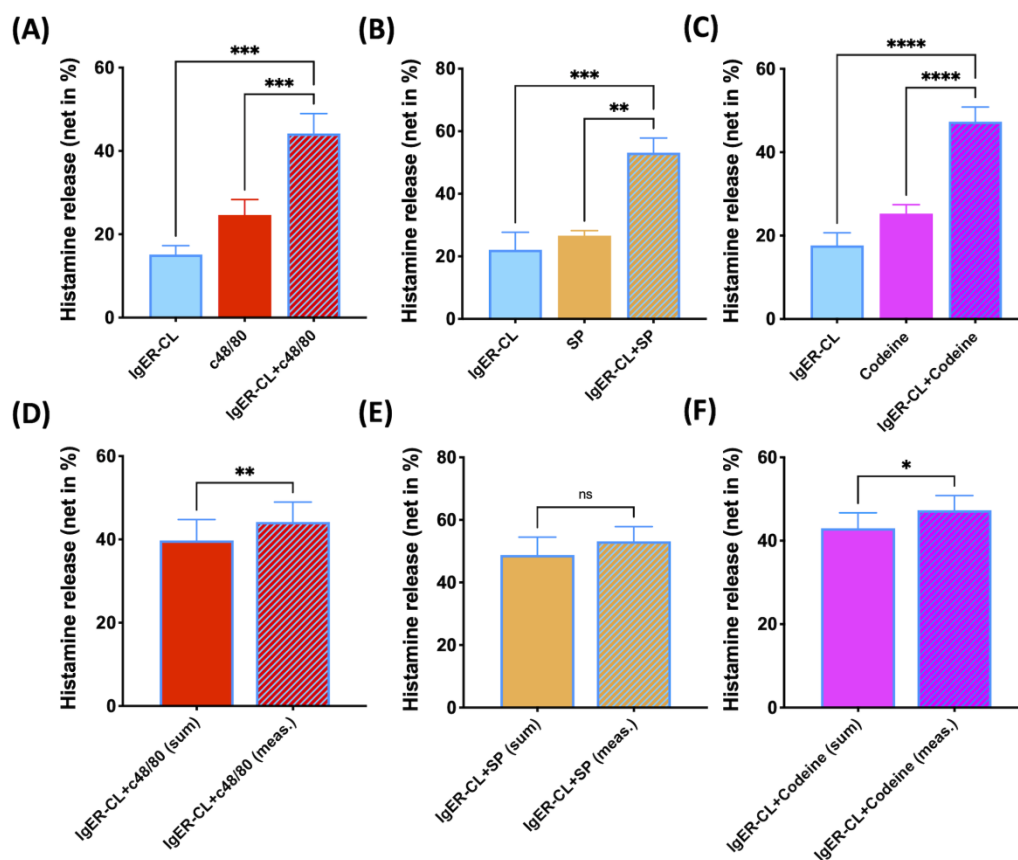
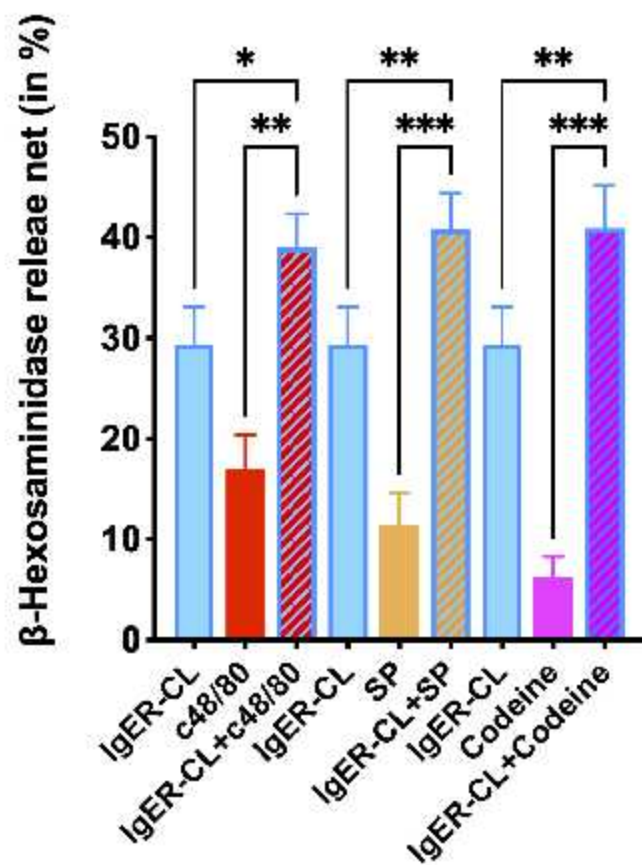
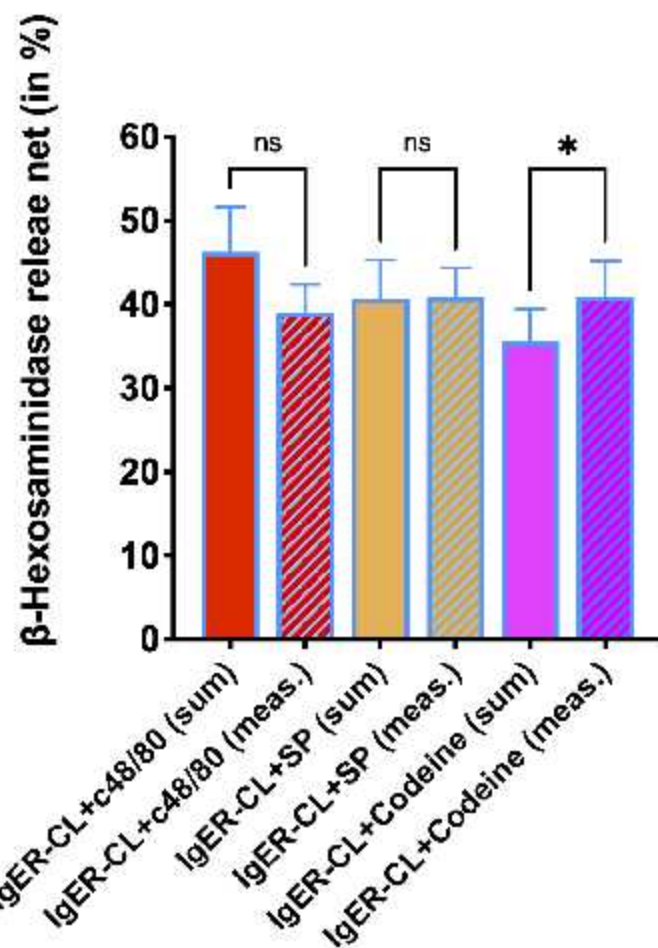
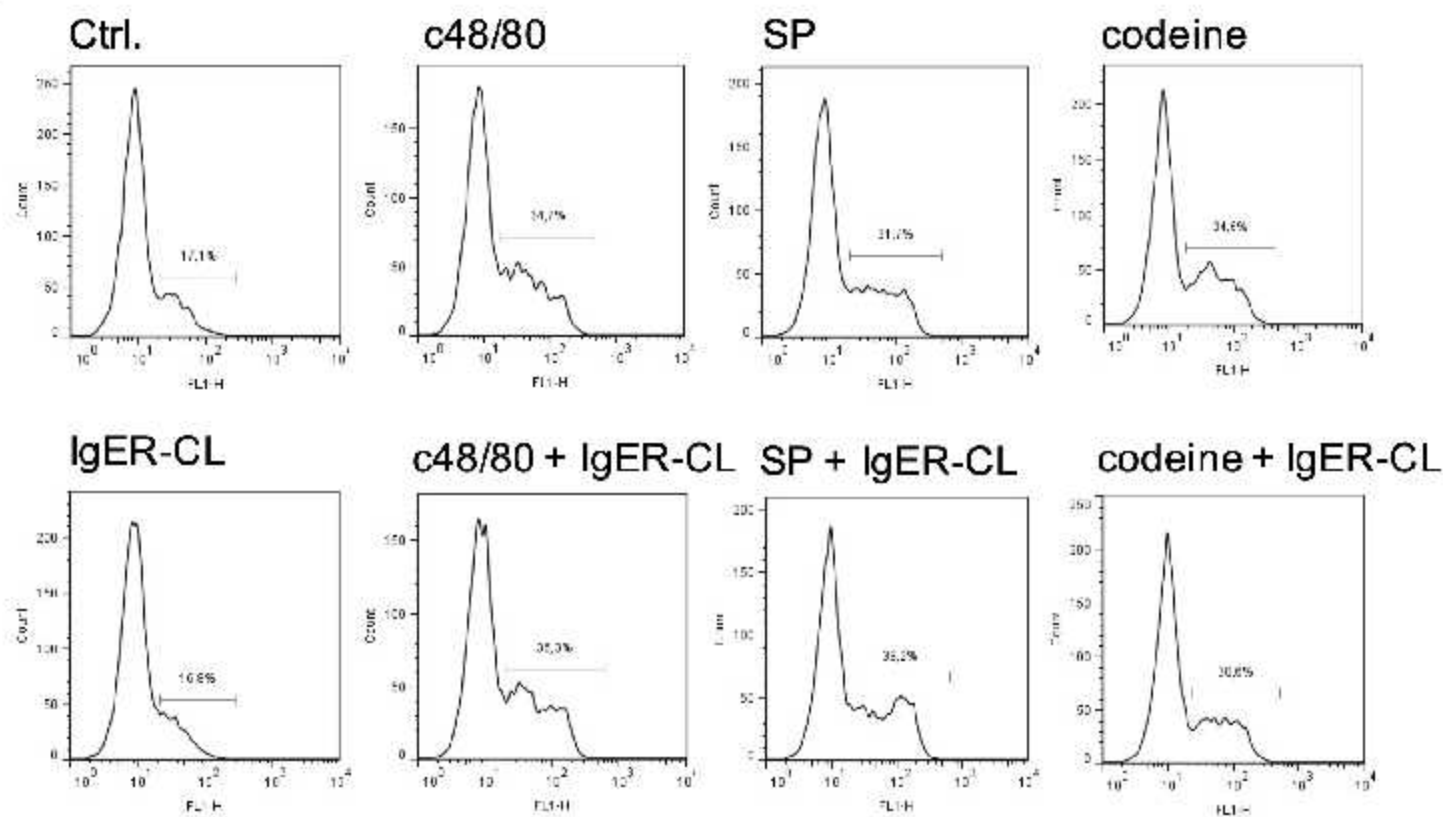


Figure 2_Babina et al.

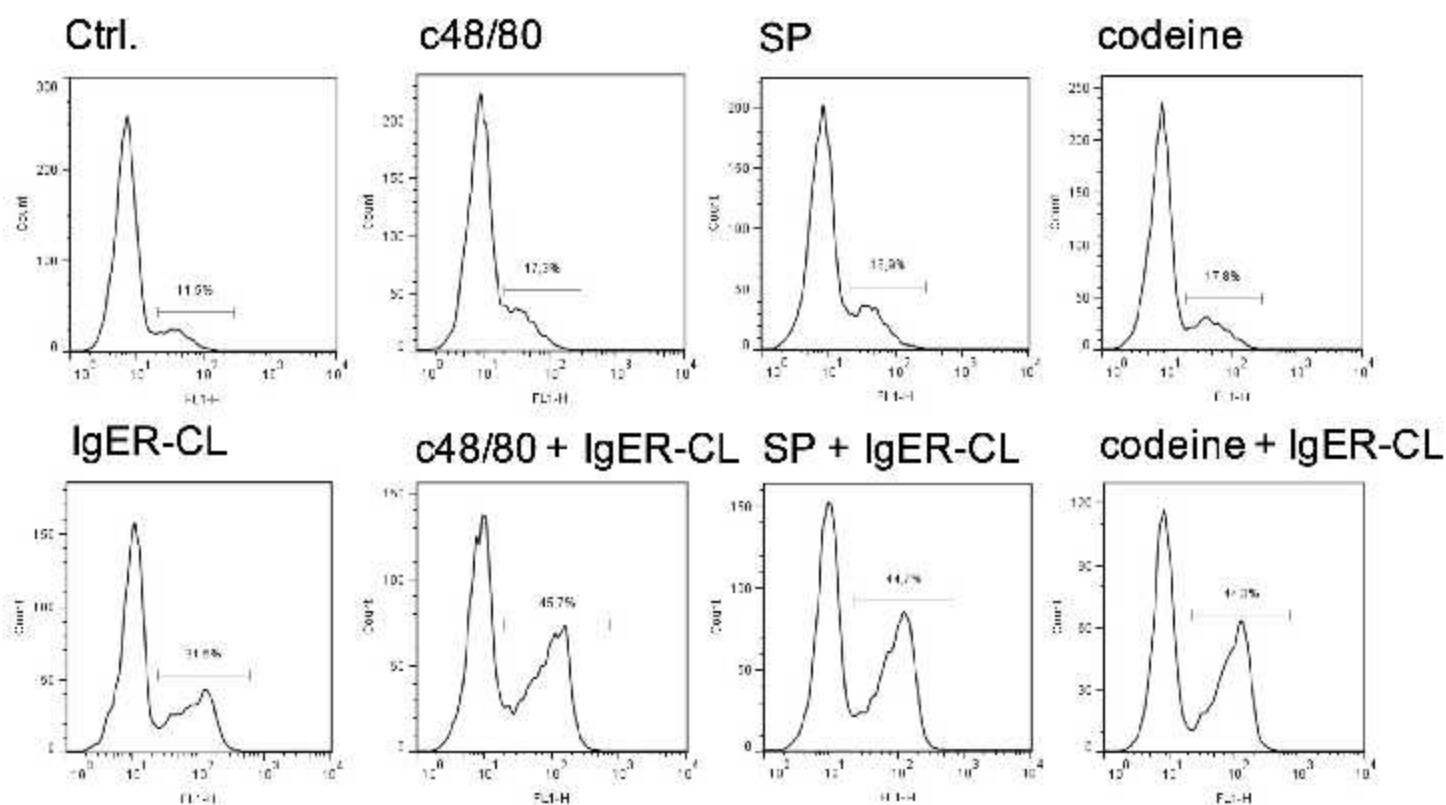
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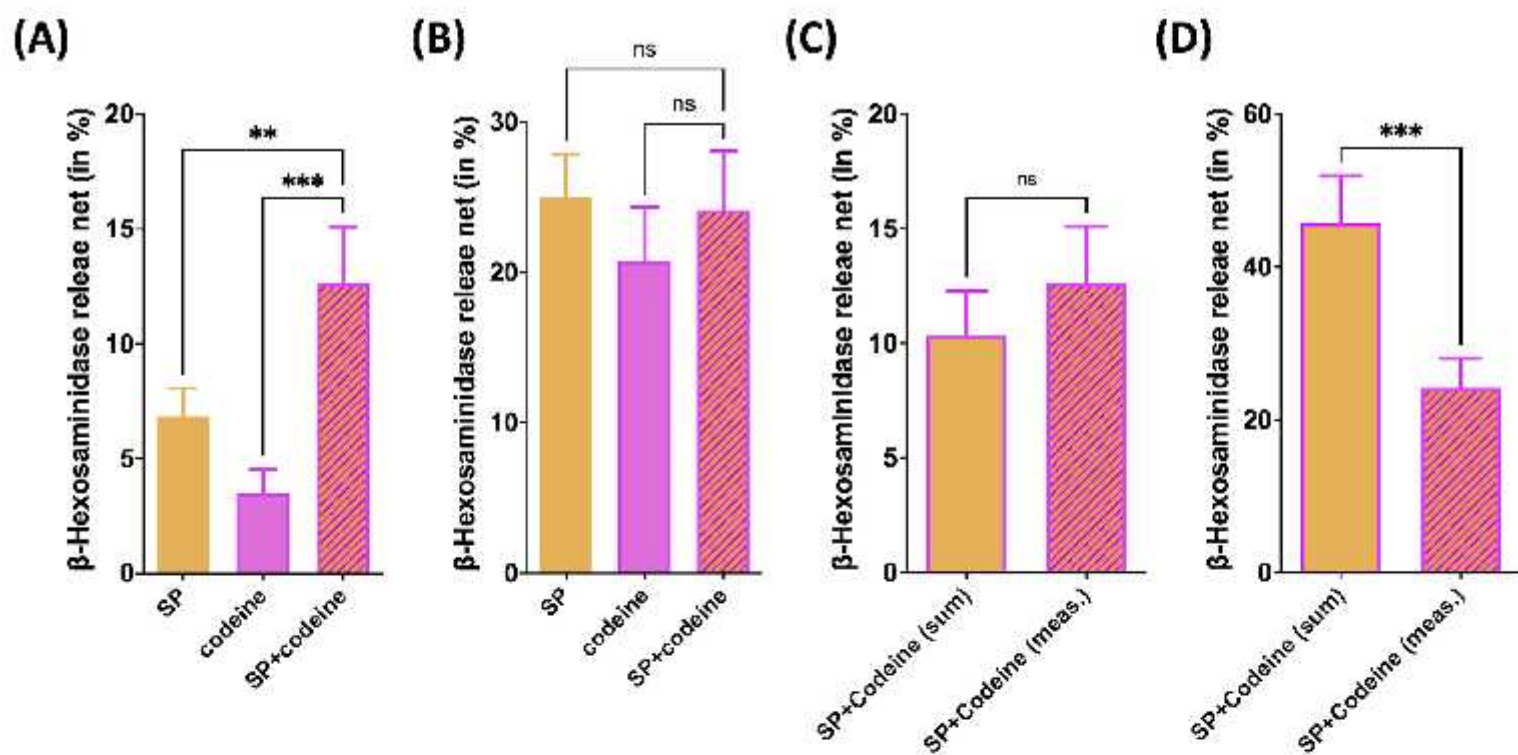
(A)**(B)**

(A) 2 min

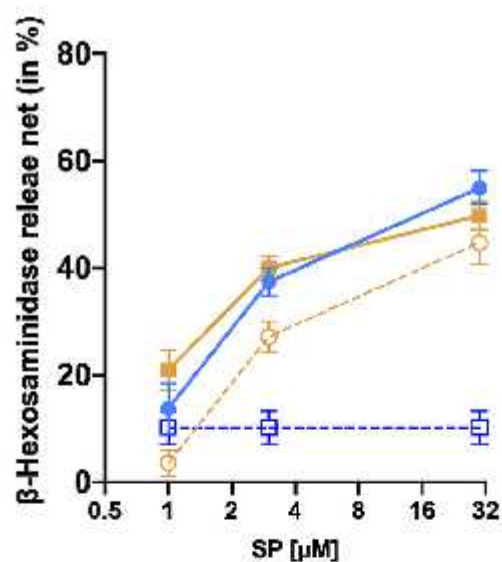
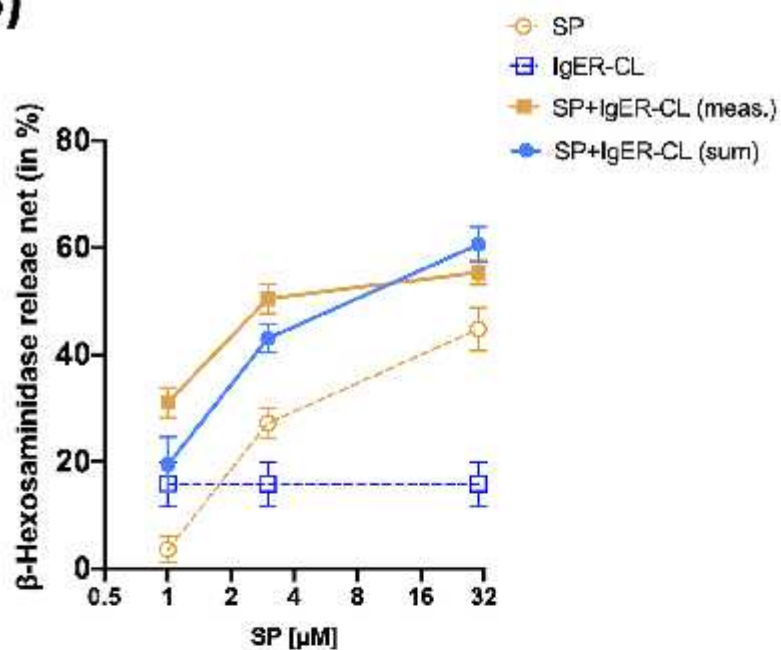
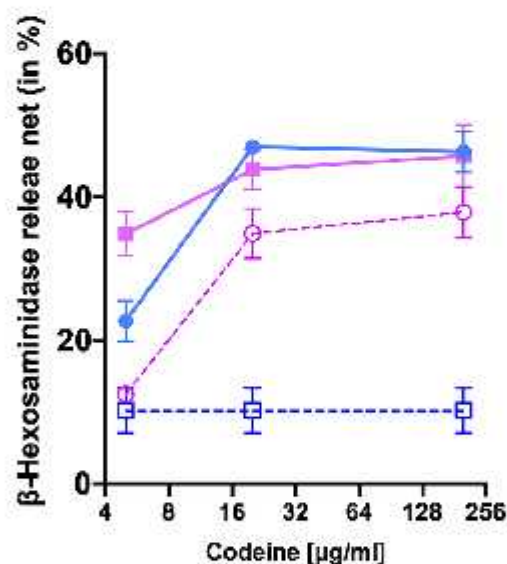
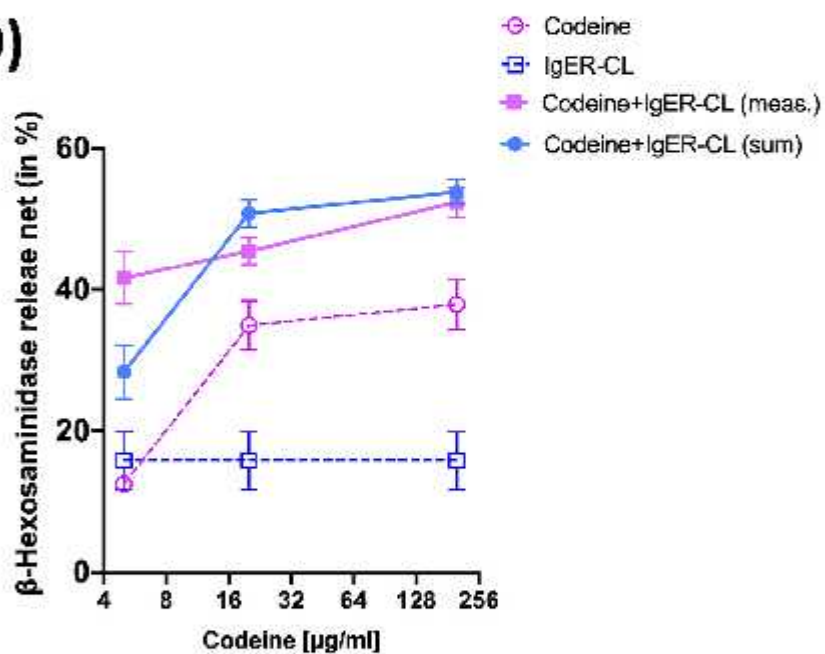


(B) 30 min





Suppl. Figure 3_Babina et al.

(A)**(B)****(C)****(D)**

1 **LETTER TO EDITOR – ONLINE SUPPLEMENT**

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3 **FcεRI- and MRGPRX2-evoked degranulation responses are fully additive in human**
4 **skin mast cells**

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17 **Short title: MRGPRX2- and FcεRI-responses are additive**

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SUPPLEMENTARY METHODS AND MATERIALS

Skin samples

Donor skins, which otherwise would be disposed of, were obtained from circumcisions and provided to the study authors anonymously, as described ^{1,2}. Informed consent of the patients or their legal guardians was obtained. The study was approved by the Ethics Committee of the Charité Universitätsmedizin Berlin (protocol code EA1/204/10, 9 March 2018) and experiments were conducted according to the Declaration of Helsinki Principles.

MC purification and culture

MCs were isolated from skin samples, as described ^{1,3}. Briefly, skin was cut into strips and treated with 3.5 U/ml dispase (BD Biosciences, Heidelberg, Germany) at 4°C overnight. After removal of the epidermis, the dermis was chopped and digested with 1.5 mg/ml collagenase type 1 (Worthington, Lakewood, NJ, USA), 0.75 mg/ml hyaluronidase type 1-S (Sigma, Deisenhofen, Germany), and 10 µg/ml DNase I (Roche, Basel, Switzerland) at 37 °C in a shaking water bath for 75 min. The remaining tissue was filtered, and the dermal cell suspension was labelled with anti-human c-Kit microbeads to purify MCs by an Auto-MACS device (both from Miltenyi Biotec, Bergisch Gladbach, Germany). MC purity always exceeded 95%, as assessed by acidic toluidine-blue staining and flow cytometry (described below). Skin MCs were either used *ex vivo* (within 18 h after purification) or cultured in Basal Iscove medium, supplemented with 10% FCS (Biochrom, Berlin, Germany), 100 ng/ml SCF (Peprotech, Rocky Hill, NJ, USA) and 10 ng/ml IL-4 (Peprotech) for 2-4 weeks, exactly as described ⁴⁻⁶

Histamine release assay (HRA)

The HRA was performed according to a method routinely employed in our laboratory ^{1,7}. In brief, MCs were stimulated in PAG-CM buffer (Piperazine-N,N-bis[2-ethanesulfonic acid]-Albumin-Glucose buffer containing 3 mM CaCl₂ and 1.5 mM MgCl₂, pH 7.4) for 30 min at

37°C. FcεRI-aggregation was induced by 0.5 µg/ml 29C6 (kind gift from Dr. Hakimi, Hoffmann La Roche, Nutley, NJ, USA) for *ex vivo* skin MCs, or 0.1 µg/ml AER-37 (eBioscience, San Diego, CA, USA) for cultured skin MCs. AER-37 cannot be used for *ex vivo* skin MCs since it binds inefficiently to the FcεRI/IgE complex and preferentially recognizes empty FcεRI. Activation of MRGPRX2 was by 10 µg/ml c48/80 (Sigma, Steinheim, Germany), 30 µM SP (Bachem, Budendorf, Switzerland) or 100 µg/ml codeine (solution prepared by the Charité pharmacy, Berlin, Germany). Cells without any stimulus served to detect spontaneous release. The simultaneous activation of FcεRI and MRGPRX2 was achieved by combining stimuli at the same concentrations as above. Histamine in the supernatants and complete cellular histamine content were measured by an automated fluorescence method (Alliance Instruments, Salzburg, Austria). All determinations were performed in triplicate. Net histamine release (%) was calculated as [(stimulated release–spontaneous release)/complete histamine in the MC preparation]x100.

β-hexosaminidase release assay

Suspensions of skin MCs were washed twice and resuspended at 5×10^5 cells/ml in PAG-CM buffer, then 100 µl aliquots were seeded into 96-well-plates with either saturated concentrations (the same concentrations as in HRA) or suboptimal concentrations of the stimuli. Combining SP and codeine under suboptimal concentrations required the performance of dose responses for each culture separately due to variable expression and function of MRGPRX2 across cultures^{6,8}. SP was employed at 2.5, 5, 7.5, or 15 µM and codeine at 5, 7.5, or 30 µg/ml prior to the actual experiment. The lowest concentrations giving a signal above noise (without approaching saturation) were then selected as suboptimal for the combined treatments.

For *ex vivo* MCs (which respond more vigorously to MRGPRX2 agonists), SP was used at 1 or 3 µM (no reaction/suboptimal) or 30 µM (optimal), while codeine was used at 5 or 20 µg/ml (suboptimal) or 200 µg/ml (optimal). MRGPRX2 agonists at the above concentrations were

also combined with the FcεRI-antibody 29C6 at 0.1 µg/ml (suboptimal) and 0.5 µg/ml (optimal).

After incubation for 60 min, supernatants were collected by centrifugation at 500 g, 4 °C for 3 min, and the pelleted MCs rapidly frozen at -80 °C. After thawing, aliquots of 50 µl of 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MUG) (Sigma-Aldrich, Munich, Germany) solution at 5 µM in citrate buffer (pH 4.5) were mixed with the same volume of supernatant or lysate and incubated for 60 min at 37 °C to measure the level of secreted and cell-bound β-hexosaminidase. The reaction was stopped by adding 100 mM sodium carbonate buffer (pH 10.7). Liberated 4-MUG was determined fluorometrically at an emission wavelength of 460 nm after excitation at 355 nm. Percent β-hexosaminidase release was calculated as: [fluorescence intensity supernatant / (fluorescence intensity supernatant + fluorescence intensity lysate)] × 100. Net release was calculated by subtracting spontaneous release, as in the histamine release assay above.

Flow cytometry

MC purity was assessed as described (Babina et al., 2020). Anti-human c-KIT-PE (clone REA787, Miltenyi-Biotec, Bergisch Gladbach, Germany) was used at 3 ng/ml, PE-labelled mouse IgG1k (clone P3.6.2.8.1, eBioscience, San Diego, CA, USA) served as isotype control. Anti-human FcεRIα-FITC (clone AER-37, eBioscience) was used at 0.5 µg/ml, mouse IgG2b, κ-FITC (clone eBMG2b, eBioscience) served as isotype control.

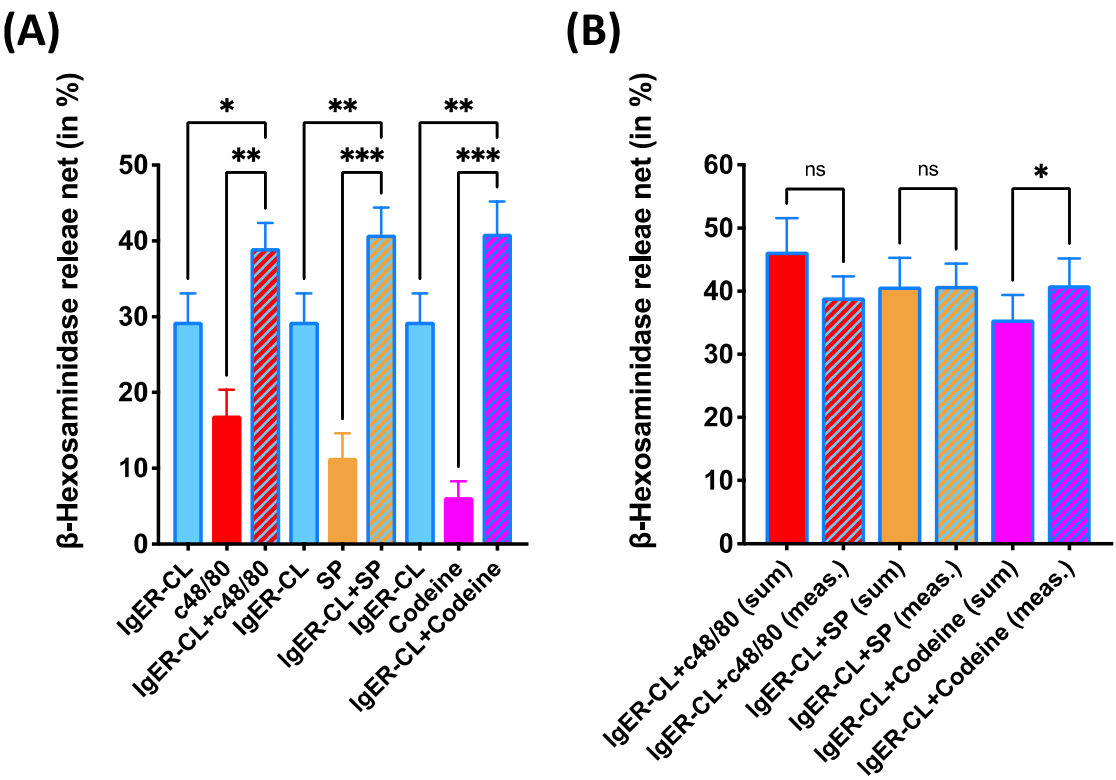
Representative dot plots of purity assessment can be found in Fig. S1.

Flow-cytometric detection of CD107a cell surface expression was as described^{9,10}. In brief, MCs were stimulated for 2 or 30 min with 0.1 µg/ml AER-37, 10 µg/ml c48/80, 30 µM SP, 100 µg/ml codeine, or control without stimulus. For the concomitant activation of AER-37 with either c48/80 or SP or codeine the same concentrations as above were used. The reaction was stopped by ice-cold 4% PFA and incubation for 15 min. After washing, the cells were incubated with 10 µl of anti-human CD107a-FITC antibody (LAMP-1) (BioLegend, San

Diego, CA, USA) together with 10 µl of human AB-serum for 30 min at 4 °C. After washing, CD107a expression was detected on the Facscalibur (BD Biosciences, San Jose, CA, USA). Flow cytometric results were analyzed with the FlowJo analysis software (FlowJo LLC, Ashland, OR, USA).

Statistical analysis

Statistical analyses were performed using PRISM 9 (GraphPad Software, La Jolla, CA, USA). For differences between paired data within two groups, the paired t-test (normally distributed) or Wilcoxon matched-pairs signed rank test (not normally distributed) was employed. For multi-group paired data comparisons, the RM one-way ANOVA with Šídák's multiple comparisons test (normally distributed) or Friedman test with Dunn's multiple comparisons test (not normally distributed) was applied. To determine additivity, measured values (simultaneous addition of stimuli) were compared with calculated values (sum of two individual stimuli) of the same experiment. $p < 0.05$ was considered statistically significant.



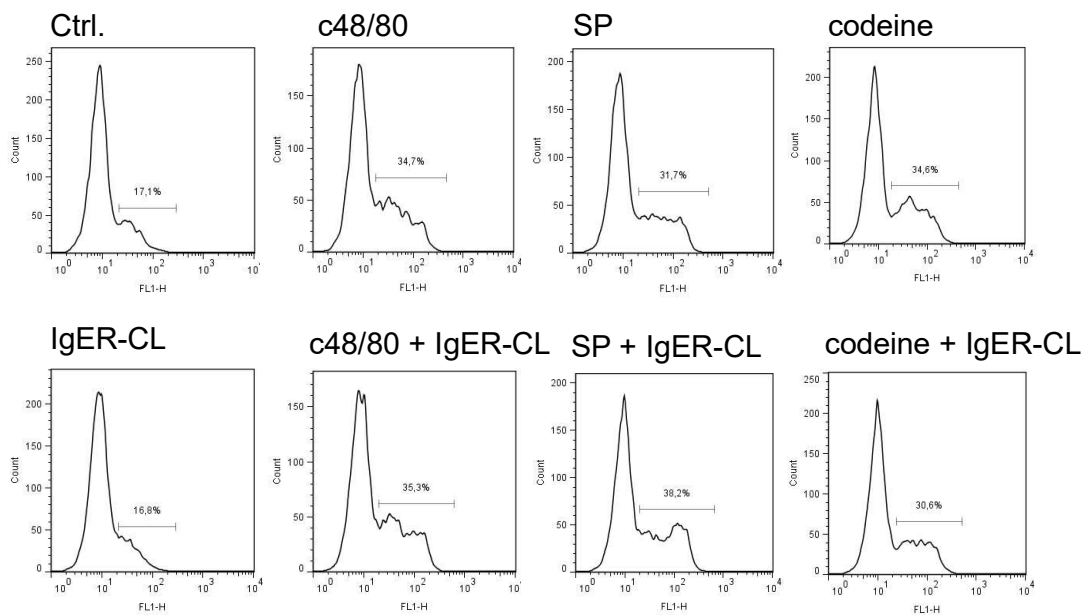
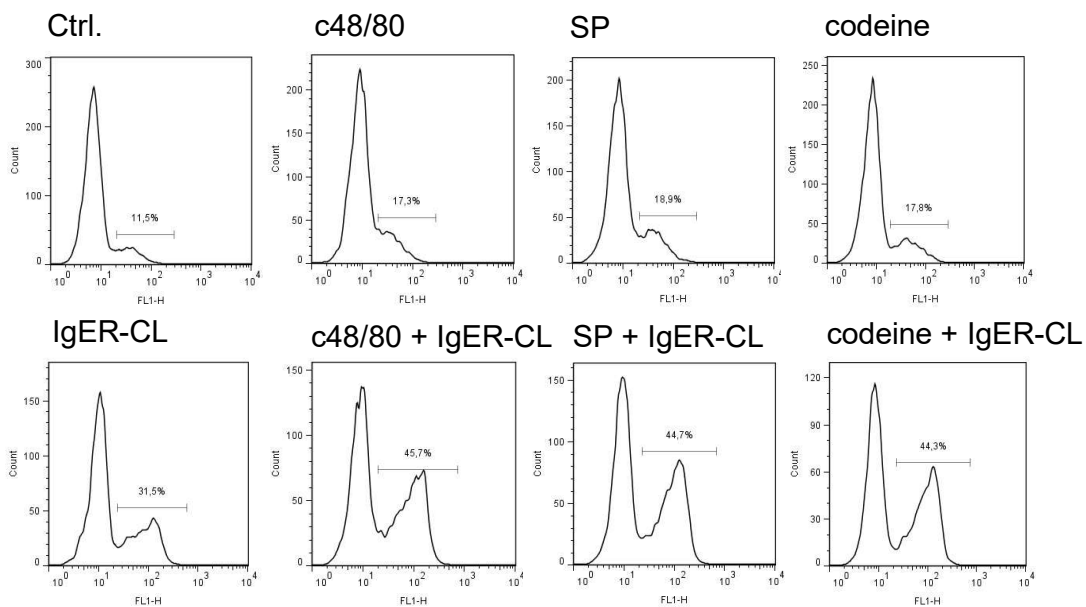
Supplementary Figure 1. β-hexosaminidase release concurrently triggered via FcεRI and MRGPRX2 is additive

Cultured MCs were stimulated as in figure 1, and β-hexosaminidase release was determined.

A) Net β-hexosaminidase release triggered by the indicated stimuli individually versus jointly.

B) Comparison of the calculated net release triggered by two individual stimuli (sum) with the measured (meas.) release on concomitant activation. The data are the mean±SEM, n=8

independent cultures. * p < 0.05, ** p < 0.01, *** p < 0.001, ns: not significant.

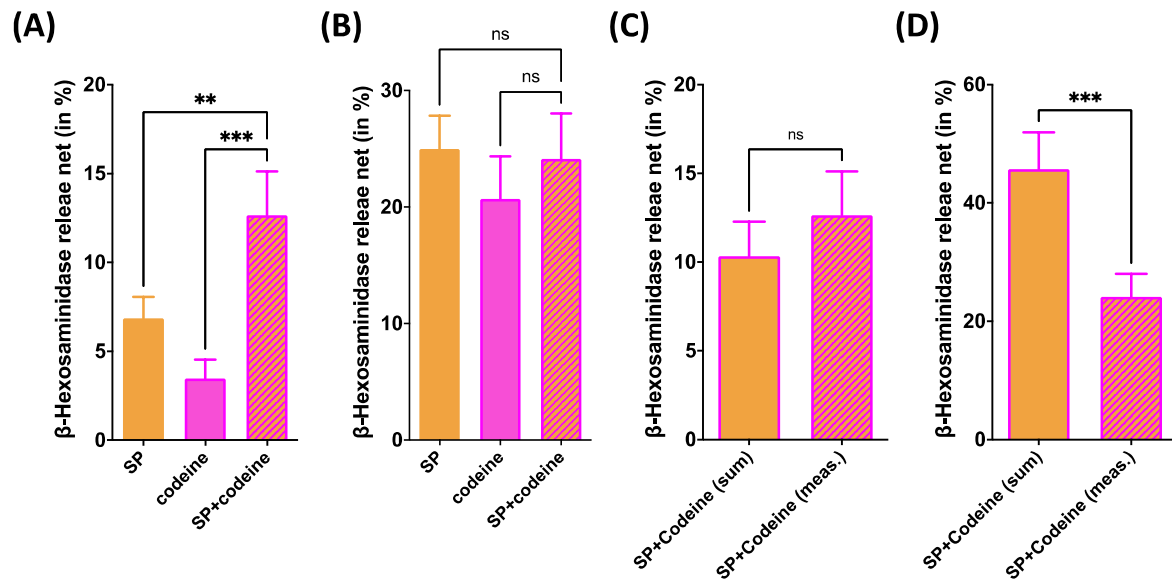
(A) 2 min**(B) 30 min**

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135 **Supplementary figure 2. FcεRI- and MRGPRX2-driven stimuli add up to enhance the**
 136 **proportion of CD107a⁺ cells**

137 Representative histograms illustrating the findings of main Fig. 1c-e.

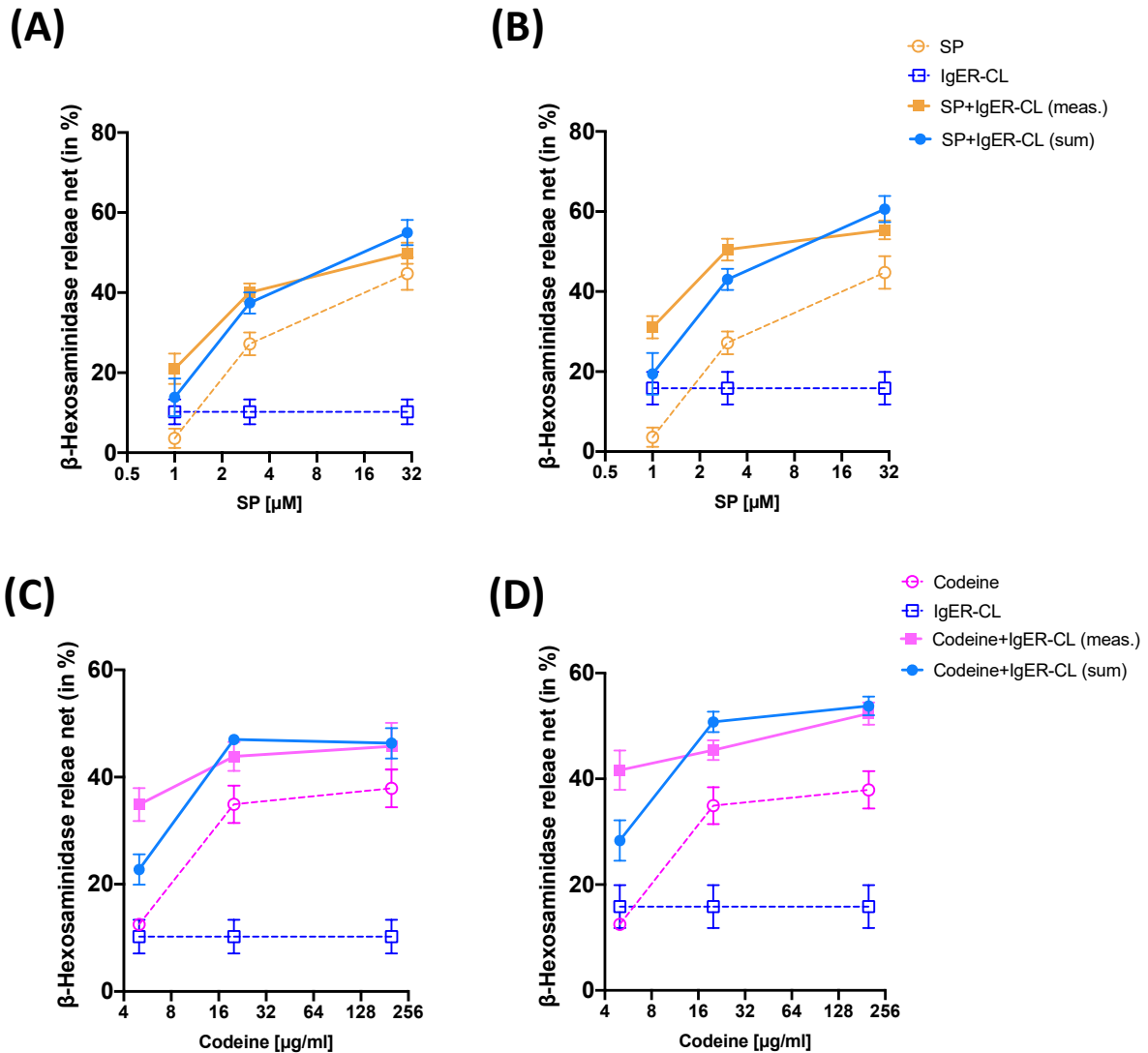
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141 **Suppl. Figure 3. Endogenous and exogenous agonists of MRGPRX2 can have**
 142 **cumulative effects**

143 A) Cultured MCs were activated by SP and codeine either individually or simultaneously
 144 under suboptimal conditions. The determination of suboptimal concentrations for each
 145 stimulus and culture is described in Supplementary Methods. The net β -hexosaminidase
 146 release was determined. B) MCs were activated with SP (30 μ M), codeine (100 μ g/ml) or
 147 SP+codeine at optimal concentrations. C, D) Comparison of the calculated net release
 148 triggered by the individual stimuli (sum) with the measured (meas.) release on concomitant
 149 activation. C) suboptimal setting (corresponding to A), D) optimal setting (corresponding to
 150 B); note that at suboptimal concentrations the two agonists give additive effects
 151 (sum $\approx$ meas.). The data are presented as mean $\pm$ SEM with n=9. ** p < 0.01, *** p < 0.001, ns:
 152 not significant.



Suppl. Figure 4. IgE-aggregation and MRGPRX2 agonists at suboptimal concentrations can induce cumulative degranulation in *ex vivo* skin MCs

Ex vivo skin MCs were activated by MRGPRX2 agonists (SP and codeine) and FcεRI-antibody 29C6 either individually or in combination at no reaction/suboptimal or optimal concentrations, the net β-hexosaminidase release was determined. Measured release (meas.) on combined activation was plotted in comparison with the calculated net release triggered by the individual stimuli (summed up). A, B) MCs were activated by SP at 1, 3 or 30 μM with or without FcεRI-antibody 29C6 at A) 0.1 μg/ml and B) 0.5 μg/ml or 29C6 alone. C, D) Release was induced by codeine (at 5, 20 or 200 μg/ml) with or without FcεRI-antibody 29C6 at A) 0.1 μg/ml and B) 0.5 μg/ml or 29C6 alone. The data are presented as mean±SEM

with n=5. Note that at low concentrations of MRGPRX2 agonists, there is a trend towards synergism (i.e. measured > sum), though it did not reach statistical significance.

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