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Abstract

Background

Mechanisms that govern priming and degranulation of human mast cells (MCs) remain elusive. Besides, most of our knowledge is based on experiments from which data only reflect an average of all stimulated cells. This study aims at investigating the effects of pro-inflammatory cytokines IL-6, IL-33 and TNF-α on IgE-dependent and IgE-independent activation of individual MCs.

Methods

MCs were derived from $CD34^+$ progenitors isolated from 50mL whole blood from 4 healthy controls and 5 birch pollen allergic patients. Passively sensitized MCs were pre-incubated with IL-6, IL-33 or TNF-α and stimulated with anti-IgE/birch pollen allergen or substance P, the latter being a ligand for the G-protein coupled MRGPRX2-receptor. Activation, *i.e.* up-regulation of CD203c, and anaphylactic degranulation, *i.e.* appearance of CD63, were measured using flow cytometry.

Results

Pre-incubation with IL-33 demonstrated up-regulated CD203c density without degranulation. Subsequent IgE-dependent stimulation (anti-IgE/birch pollen allergen) resulted in higher appearance of CD63 as compared to cells without pre-incubation, indicating IL-33 to exert a priming effect (P=0.04). IL-6 only increased allergen-specific responses but to a lesser extent than IL-33. Combination of IL-33/IL-6 had a synergistic effect, demonstrating more degranulation in response to allergen. TNF-α had no effect on IgE-mediated activation, nor synergistic effects with IL-33. Stimulation with substance P resulted in degranulation that could not be enhanced by pre-incubation.

Conclusions

In conclusion, IL-33, and in a lesser extent IL-6, prime individual MCs for subsequent IgE-mediated activation. Moreover, this priming effect is synergistic. In contrast, none of the cytokines had a priming effect on MRGPRX2-mediated activation of MCs.

Introduction

Mast cells (MCs) play a critical contribution in innate and adaptive immune responses. Along with their physiological functions MCs also exert an important effector function in detrimental immunepathological reactions such as IgE-dependent allergies and autoimmunity (1–3). The mechanisms regulating MCs degranulation are controlled by complex cross-talks between various activating and inhibitory signals that vary according to the different stimulation pathways (IgE vs. non-IgE) and conditions (e.g. the presence/absence of priming factors) (4–8). However, the mechanisms that culminate in priming and degranulation of MCs remain incompletely understood. Studies in rodent MCs have revealed that MCs biology is influenced by pro-inflammatory cytokines, such as interleukin-6 (IL-6), IL-33 and tumor necrosis factor-alpha (TNF-α) (9–12). Actually, it has been shown that IL-6 and IL-33 promote IgE-dependent degranulation as measured by release of cytokines and/or mediators in the supernatant (13–15). Besides, IL-33 has been shown to increase cytokine and chemokine production after IgE-independent stimulation via IgG immune complexes (16). In contrast, data about the effect of these cytokines on human MCs are scarce and have mainly been gathered using mediator release assays in the supernatant of cultured human MCs (17–23). Results from these assays only reflect the average of all stimulated cells and do not allow to obtain data from individual cells. Recently, we developed a flow cytometric assay that enables to study degranulation patterns of individual basophils (7,24,25) and individual cultured human MCs (26). The most important goal of this assay is that it allows to simultaneously analyze the immunophenotype and histamine release. The aim of the present study is to investigate, in a human in vitro system, the effects of pre-incubation with IL-6, IL-33 and TNF-α on IgE-dependent (anti-IgE/birch pollen allergen) and IgE-independent (substance P) MCs activation. For this purpose, anaphylactic degranulation of MCs is analyzed by flow cytometry (24-27).

Materials and methods

In vitro culture of human MCs

Human MCs were cultured as described elsewhere (26). Briefly, peripheral blood mononuclear cells were isolated from 50 mL whole blood. $CD34^+$ progenitor cells were enriched using the EasySep Human CD34 Selection Kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's instructions. Isolated $CD34^+$ 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, USA), streptomycin (100 µg/mL) (Life Technologies), low-density lipoprotein (LDL, 10 µg/mL) (Stemcell Technologies), 2-mercaptoethanol (55 µmol/L) (Life Technologies), stem cell factor (SCF, 100 ng/mL) (Miltenyi Biotec, Bergisch Gladbach, Germany) and interleukin-3 (IL-3, 100 ng/mL) (PeproTech, Rocky Hill, USA) during 3-5 weeks. Participants gave a written informed consent as approved by the Ethical Committee of the University Hospital of Antwerp (Belgium B300201525454).

Sera from birch pollen allergic patients and healthy control individuals

Sera and cells were derived from the same donor individuals. Sera from five birch pollen allergic patients were included. Diagnosis of birch pollen allergy was based on an evocative history of rhinoconjunctivitis and/or asthma during spring and confirmed by a positive specific IgE (sIgE) value (> 0.10 kUA/L) for recombinant Bet v 1 (rBet v 1), the major allergen from birch pollen (*Betula verrucosa*) quantified by ImmunoCAP FEIA (Thermo Fisher scientific, Uppsala, Sweden). Individuals who answered negative on a standardized questionnaire on medical history including allergic, infectious and inflammatory diseases, and intake of medication, were included as healthy control individuals (n = 4). The study was conducted outside tree pollen season and patients were free of medication. Demographic data and IgE results of patients and healthy controls are displayed in table 1.

Flow cytometric analysis

Immunophenotyping

Cultured human MCs were immunophenotyped as described elsewhere (26). Briefly, human MCs were stained for surface makers with monoclonal anti-human CD45-peridinin chlorophyll (CD45-PerCP) (BioLegend, San Diego, USA), anti-human CD117-phycoerythrin (CD117-PE) (BD Biosciences) or anti-human CD117-allophycocyanin (CD117-APC) (BD Biosciences) when in combination with anti-human FcεRI-phycoerythrin (FcεRI-PE) (eBioscience, San Diego, USA), anti-human CD203c-allophycocyanin (CD203c-APC) (Pharmingen, BD Biosciences, Erembodegem, Belgium), and anti-human CD63-fluorescein isothiocyanate (CD63-FITC) (BD Biosciences). Cultured mature human MCs were defined as $CD45^+$, $CD117^+$ and $CD203c^+$ cells.

IgE-mediated activation

IgE-mediated function of mature human MCs was evaluated by passively sensitizing the cells with autologous or allogenic serum during 30 minutes at 37°C in a humidified CO_2-incubator. Next, cells were centrifuged (200 g, 5 minutes, 20°C) and the cell pellet was resuspended in pre-warmed (37°C) Tyrode's buffer (Sigma-Aldrich) at a concentration of 0.5 x 10^6 cells/mL. Subsequently, 100 µL of the cells were pre-incubated with cytokines IL-6 (50 ng/mL), IL-33 (100 ng/mL), TNF-α (5 ng/mL) or a combination of IL-33 with IL-6 or TNF-α for 20 minutes on 37°C. Next, these pre-incubated MCs were stimulated with 100 µL Tyrode's buffer as a negative control, 100 µL of 2 µg/mL monoclonal anti-IgE (BD Biosciences) as a positive control or 100 µL of 0.1 µg/mL rBet v 1 as allergen during 20 minutes at 37°C. Reactions were stopped by chilling on ice and spinning for 5 minutes (4 °C, 200 g). The supernatant was removed and cells were stained with monoclonal anti-human CD45-PerCP, CD203c-APC, CD117-PE and CD63-FITC for 20 minutes at 4°C. Finally, cells were washed and resuspended in PBS with 0.1% sodium azide and measured. Appearance of CD63 indicates anaphylactic degranulation of MCs (25,27).

Non-IgE mediated activation with substance P

Cultured human MCs (N = 10), pre-incubated with IL-6, IL-33 and TNF-α as described above, were incubated with 100 µL Tyrode's buffer or 100 µL of an optimal end concentration of 74 µM substance P (Sigma-Aldrich) during 20 minutes at 37°C. Reactions were stopped by chilling on ice and spinning for 5 minutes (4 °C, 200 g). The supernatant was removed and cells were stained with monoclonal anti-human CD45-PerCP, CD203c-APC, CD117-PE and CD63-FITC for 20 minutes at 4°C. Finally, cells were washed and resuspended in PBS with 0.1% sodium azide and measured.

Flow cytometric analysis

Flow cytometric analysis was performed on a FACSCanto II flow cytometer (BD Immunocytometry Systems, San Jose, CA) equipped with three lasers (405 nm, 488 nm and 633 nm). Correct compensation settings for antibodies conjugated with fluorochromes were performed using BD CompBeads (BD Biosciences). Flow cytometric data were analyzed using Kaluza Analysis 1.5 software (Beckman Coulter, USA). Unstained samples were used to set a marker between positive and negative cells according to the 99^{th} percentile. A fluorescence minus one (FMO) was used to set a marker between positive and negative cells for degranulation marker CD63. The percentages refer to the net

number of cells positive for each parameter (test condition minus blanco). Density measurements were performed using standardized fluorospheres (SPHERO Ultra Rainbow Calibration particles, Spherotech, Lake Forest, IL, USA) as described by the manufacturer. Briefly, the measured median fluorescence intensity (MFI) was converted to a calibrated value using linear regression. Results were expressed as the Molecules of Equivalent Specific Fluorochrome PE per cell (MESF-PE/cell) for FcεRI density or Molecules of Equivalent Specific Fluorochrome APC per cell (MESF-APC/cell) for CD203c density.

Statistical analysis

IBM SPSS Statistics version 24 software was used for data analysis, non-parametric statistical analysis was performed. Results are expressed as median and range. A P-value of < 0.05 was considered significant.

Results

Immunophenotyping

A representative sample for immunophenotyping of the cells is illustrated in figure 1. Cultured mature human MCs, defined as cells expressing CD45, CD117 and CD203c, were gated for analysis. MCs cultured out of peripheral blood from healthy control individuals showed a similar phenotype as compared to birch pollen allergic donors, viz. their mature CD117-positive mast cells express CD203c and FcεRI with similar densities. Actually, expression of CD203c was 233 (208 – 266) (MESF-APC/cell) on MCs from healthy control individuals (Figure 1E), and 206 (180 – 265) MESF-APC/cell on MCs from birch pollen allergic donors (Figure 1G). Expression of FcεRI was 484 (420 – 538) (MESF-PE/cell) on MCs from healthy control individuals (Figure 1F), and 415 (330 – 448) MESF-PE/cell on MCs from birch pollen allergic donors (Figure 1H). There was no expression of CD63 by resting cells, nor in healthy control individuals nor in birch pollen allergic patients.

Effect of cytokines

As demonstrated in figure 2, pre-incubation with IL-33 resulted in a unimodal increase of CD203c density (Wilcoxon signed rank test: $P = 0.04$), without appearance of CD63 on the cell membrane. IL-6 and TNF- α did not cause an up-regulation of CD203c, nor an appearance of CD63.

IgE-mediated activation

As compared to stimulation with anti-IgE (Figure 3B) or specific birch pollen allergen (rBet v 1) (Figure 3C), pre-incubation with IL-33 of MCs from birch pollen allergic patients, passively sensitized with autologous serum, resulted in an increased density of CD203c, with a higher appearance of CD63 (Figure 3E-F) (Wilcoxon signed rank test: P = 0.04). IL-6 demonstrated only allergen-specific responses with up-regulated CD203c density and higher expression of CD63 (Figure 3H) (Wilcoxon: P = 0.04). Simultaneous pre-incubation of the cells with IL-33 and IL-6 synergistically augmented appearance of CD63 in response to rBet v 1 stimulation (Figure 3H) (Wilcoxon: P = 0.04). Lastly, TNF-α had no effect on IgE-mediated activation, nor had it a synergistic effect in combination with IL-33.

No significant up-regulation of CD203c density, nor appearance of CD63 was obtained from pre-incubation of MCs from healthy donors, passively sensitized with autologous serum, and stimulated with anti-IgE/rBet v 1. This lack of activation in cultures derived from healthy control individuals is probably due to the low total IgE values in their sera (table 1). In contrast, when healthy donor cells were incubated with allogenic sera from birch pollen allergic patients, the cells could be activated with rBet v 1, resulting in appearance of CD63.

MRGPRX2-dependent activation with substance P

Stimulation of human MCs with substance P resulted in significant appearance of CD63 as shown in figure 4 (Wilcoxon: P = 0.005) (Figure 4B, E). Pre-incubation of the cells with IL-6, IL-33 or TNF-α did not enhance expression of CD63 as compared to single stimulation with substance P (Figure 4D-E).

Discussion

As already exemplified in the introductory paragraph, data on the priming effect of pro-inflammatory cytokines such as epithelium-derived IL-33 and monocyte/macrophage-derived IL-6 and TNF-α on cultured human mast cells remain scarce and are mainly cultured out of poorly defined buffy coats. Besides, our current knowledge is mainly based upon data gathered by mediator release tests from which the results merely represent the average of all stimulated cells. In this study we sought to investigate the effect of pre-incubation with IL-6, IL-33 and TNF-α on both IgE- and MRGPRX2-dependent stimulation of individual human mast cells. Therefore, we took advantage of our flow cytometric technique that allows to combine immunophenotyping and quantification of degranulation by

individual cells to study the effect of these pro-inflammatory cytokines on IgE- and MRGPRX2-dependent activation of cultured mast cells that, as already reported in the Journal, express a connective tissue phenotype (MC_{TC}) (26).

From our findings it emerges that short pre-incubation with these pro-inflammatory cytokines exert distinct effects that vary according to the stimulation route. In a first set of experiments we focused on IgE-dependent activation by anti-IgE and birch pollen allergen of MCs that were passively sensitized with patients' sera containing specific IgE antibodies to the major birch pollen allergen Bet v 1. The most relevant findings in this set of experiments relate to IL-33. Actually, incubation with IL-33 did not elicit anaphylactic degranulation by itself. However, pre-incubation with IL-33 had a significant priming effect with an enhanced responsiveness of pre-incubated cells towards subsequent IgE/FcεRI cross-linking by both anti-IgE and specific birch pollen allergen. These findings are in line with the priming effect of IL-3 on basophils, another effector cell of the IgE-mediated allergic reaction (5,28). Moreover, our findings parallel the recent findings by Joulia et al. (29), who demonstrated that also longer pre-incubation for one hour with IL-33 potentiates human MCs responsiveness towards stimulation with anti-IgE or anti-DNP. Here we extended the knowledge about the priming effect of IL-33 in an allergen-specific model. Furthermore, we observed that also IL-6 had a priming effect on allergen-specific stimulation of the cells but the effect was less pronounced than for IL-33. Moreover, we could demonstrate a synergistic enhancing of co-incubation with IL-33 and IL-6. The clinical relevance of this observation has probably to be sought in the fact that "invading" allergens might trigger activation of epithelium and macrophages with the release of IL-33 and IL-6, respectively (30,31). As a matter of facts, priming of tissue resident MCs by these cytokines could contribute to more pronounced acute reactions and further skewing to Th2 responses, essential for IgE-mediated allergies (32,33). Lastly, TNF-α did not influence activation through IgE/FcεRI cross-linking.

In a second set of experiments we sought to investigate the influence of IL-6, IL-33 and TNF-α on IgE-independent degranulation by stimulation of the cells with substance P that acts through the surface-bound G-protein coupled MRGPRX2-receptor (34,35). Our study demonstrates that stimulation of MCs with substance P resulted in anaphylactic degranulation by appearance of degranulation marker CD63 on the single cell level and demonstrates that this anaphylactic degranulation could not be potentiated with IL-6, IL-33 or TNF-α. Although at first glance this observation might differ from the findings by Theoharides et al. (21), it should be noted that different techniques were used to evaluate activation. Actually, Theoharides et al. (21) demonstrated that longer pre-incubation with

IL-33 increased cytosolic calcium and release of VEGF in the supernatant by LAD2 cells and human cord blood derived MCs in response to substance P. Therefore, it is anticipated that the readouts used by Theorahides et al. (21) might dissociate from anaphylactic reaction, as revealed by the appearance of the lysosome associated membrane protein LAMP 3 (CD63).

In essence, it is demonstrated that IL-33, and in a lesser extent also IL-6, exert a priming effect on IgE-mediated degranulation of individual MCs. Moreover, the priming effect of these pro-inflammatory cytokines is synergistic. In contrast, none of the cytokines had a priming effect on MRGPRX2-activation of the cells by substance P.

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Tables

	Male/Female	Age (years)	Total IgE (kU/L)	sIgE Bet v 1 (kUA/L)
Birch pollen allergic patients	1/4	30 (26 – 43)	237 (27 – 1720)	13 (3 – 61)
Healthy control individuals	0/4	28 (26 – 52)	18 (4 – 40)	0 (<0.10)

Table 1. **Demographics and IgE data.**

Figure legends

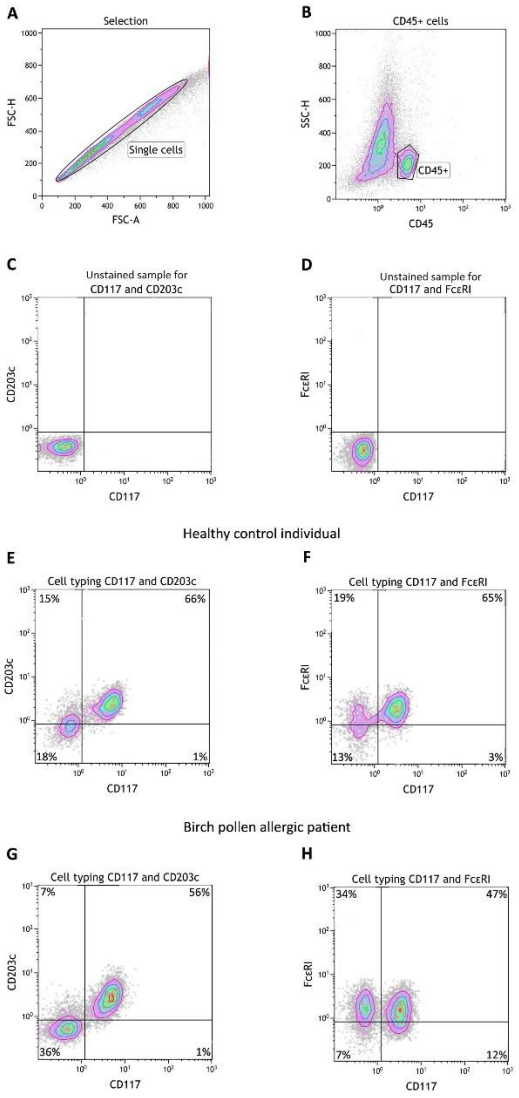

Figure 1. **Phenotypical characteristics of mast cells cultured from peripheral blood of healthy control individuals or birch pollen allergic donors.**

Selection of single cells for analysis based on FSC-H (forward scatter-Height) and FSC-A (forward scatter-Area) **(A)**. $CD45^+$ cells were gated for further analysis **(B)**. Unstained sample for lineage markers CD117 and CD203c **(C)**. Unstained sample for lineage markers CD117 and FcεRI **(D)**. Expression of $CD117^+$ and $CD203c^+$ on the cell membrane of mast cells cultured from peripheral blood of healthy control individuals **(E)** or birch pollen allergic patients **(G)**. Expression of $CD117^+$ and the high affinity IgE-receptor, FcεRI, on the cell membrane of mast cells cultured from peripheral blood of healthy control individuals **(F)** or birch pollen allergic patients **(H)**. Mature mast cells are defined as cells expressing $CD117^+$ and $CD203c^+$, and also constitutively express FcεRI. The percentages refer to the number of cells positive for each marker.

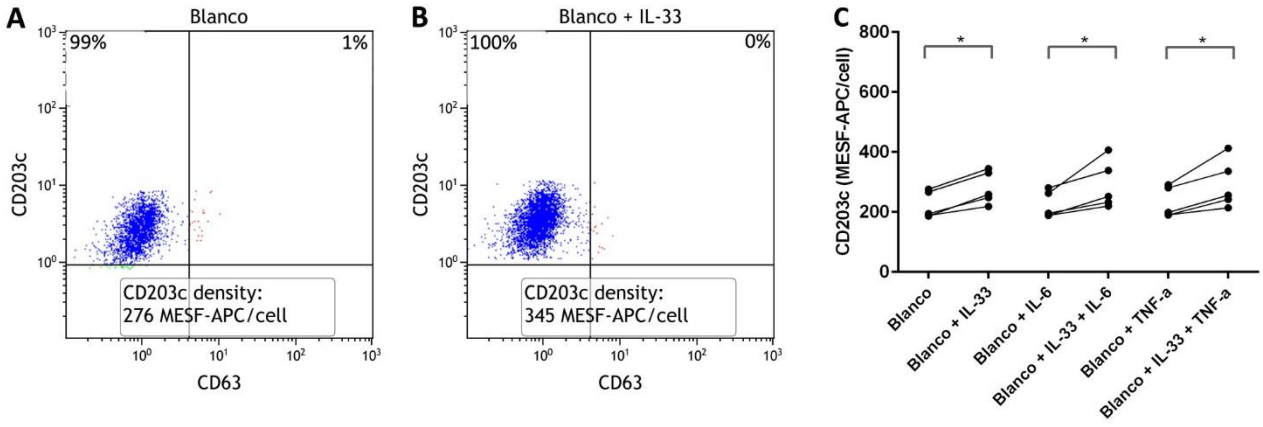

Figure 2. **Effect of pre-incubation with pro-inflammatory cytokine IL-33 on *in vitro* cultured human mast cells.**

(A) MCs without pre-incubation with IL-33. **(B)** MCs pre-incubated with IL-33. **(C)** Comparison of pre-incubation with IL-33, IL-6 and/or TNF-α. Pre-incubation with IL-33 results in an increase of CD203c density, expressed as the Molecules of Equivalent Specific Fluorochrome APC per cell (MESF-APC/cell). **(*)** P values < 0.05. P values were calculated using Wilcoxon signed rank test. The connecting lines represent corresponding values from the same human MCs culture (N = 5).

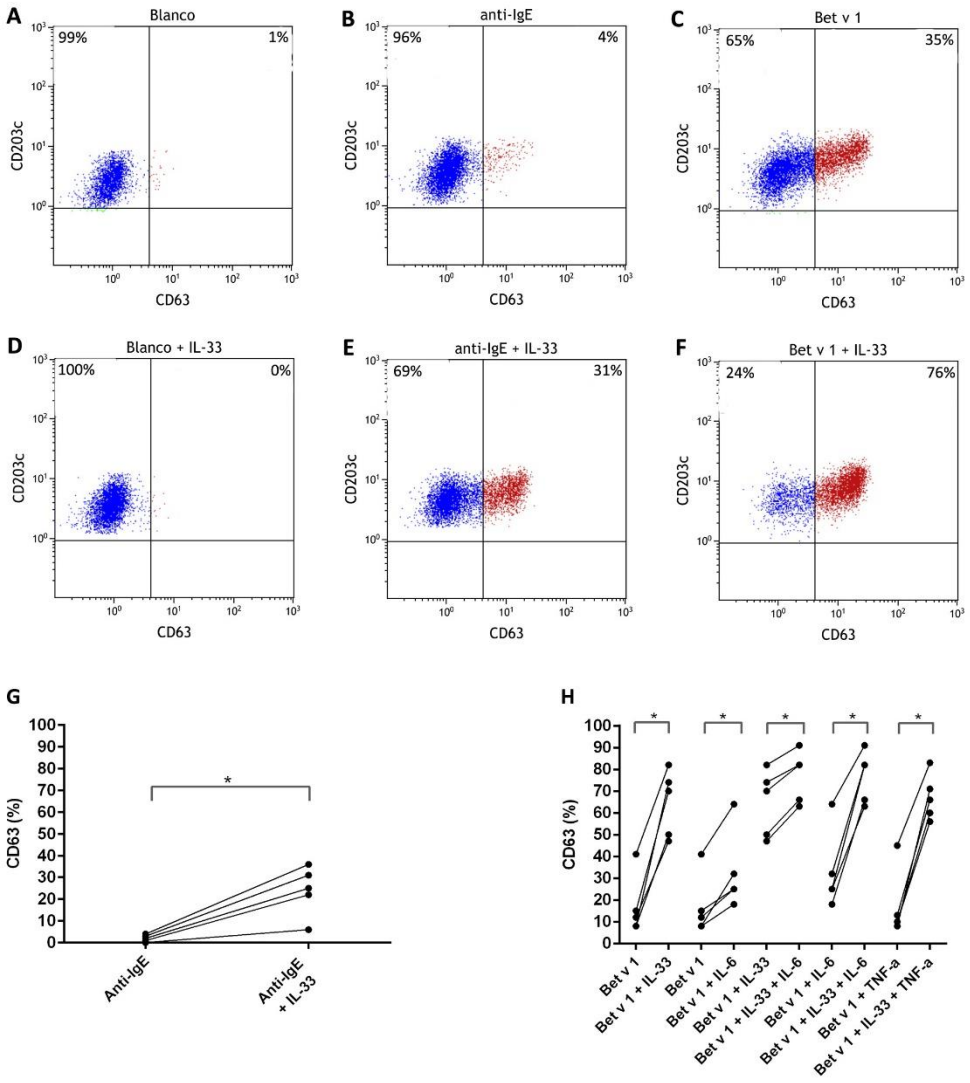

Figure 3. **Effect of pre-incubation with pro-inflammatory cytokines on IgE/FcεRI-dependent activation of *in vitro* cultured human mast cells.**

(A) MCs without pre-incubation and without IgE-mediated stimulation. **(B)** Stimulation with anti-IgE of passively sensitized MCs without pre-incubation. **(C)** Stimulation with recombinant birch pollen allergen (rBet v 1) of passively sensitized MCs without pre-incubation. **(D)** MCs pre-incubated with IL-33 without IgE-mediated stimulation. **(E)** MCs pre-incubated with IL-33 and stimulated with the positive control anti-IgE. **(F)** MCs pre-incubated with IL-33 and stimulated with rBet v 1. **(G)** Pre-incubation with IL-33 resulted in a significant higher net expression of CD63 after stimulation with anti-IgE as compared to cells stimulated but not pre-incubated. **(H)** Pre-incubation with IL-33 or IL-6 resulted in a significant higher expression of CD63 after stimulation with rBet v 1. Simultaneous pre-incubation of the cells with IL-33 and IL-6 synergistically enhanced degranulation. **(*)** $P < 0.05$ (Wilcoxon signed rank test). The connecting lines represent corresponding values from the same human MCs culture (N = 5).

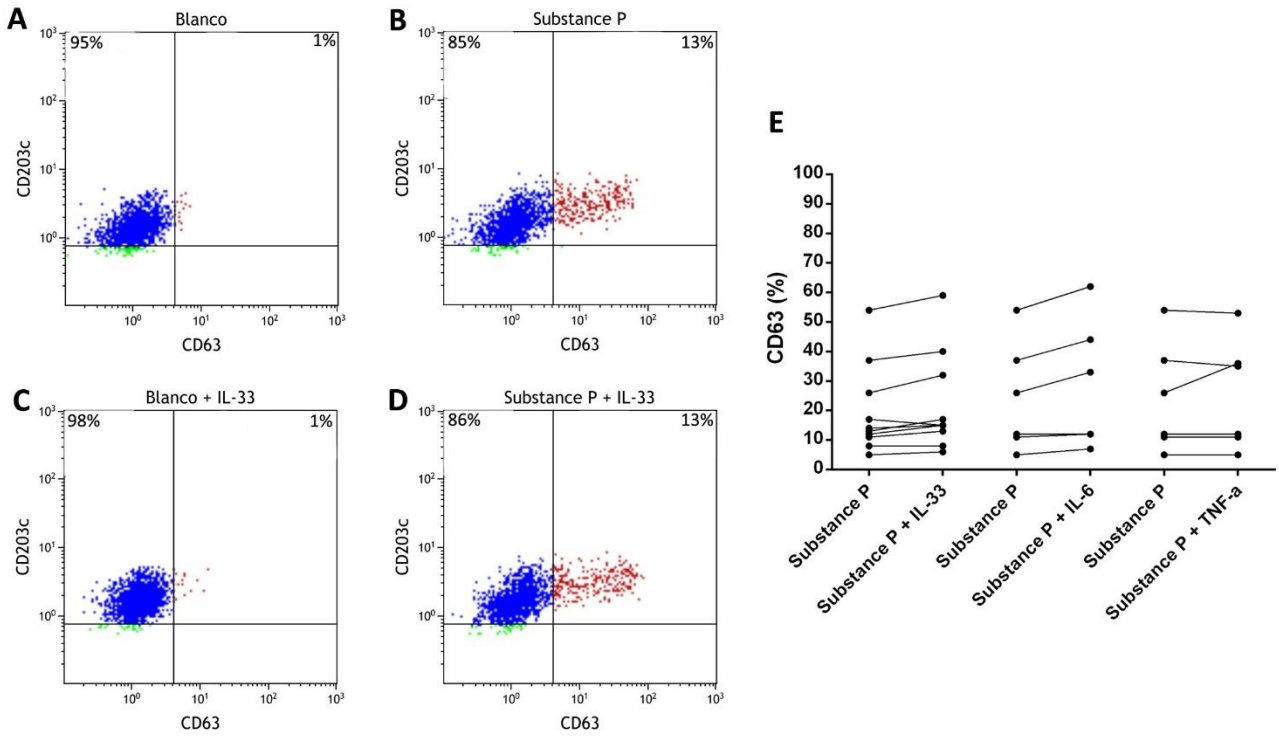

Figure 4. **Effect of pre-incubation with pro-inflammatory cytokines on IgE-independent activation with substance P of *in vitro* cultured human mast cells.**

(A) MCs without cytokine pre-incubation and without stimulation. **(B)** Stimulation with substance P of MCs without pre-incubation. **(C)** MCs pre-incubated with IL-33 without stimulation. **(D)** MCs pre-incubated with IL-33 and stimulated with substance P. **(E)** Pre-incubation of the cells with IL-33, IL-6 or TNF-α did not significantly enhance expression of CD63 as compared to stimulation with substance P alone. The connecting lines represent corresponding values from the same human MCs culture.