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Behavioural analysis of individual human mast cells.

Functionele analyse van individuele humane mastcellen.

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Promotoren: Prof. Dr. D.G. Ebo, Prof. Dr. L.S. De Clerck

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"It always seems impossible until it is done"

- Nelson Mandela

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List of abbreviations

7-AAD	7-aminoactinomycin D	***MC_{TC}***	Connective tissue mast cells
ADR	Adverse drug reactions	***MC_T***	Mucosal mast cells
AF700	Alexa Fluor 700	***MESF***	Molecules of equivalent specific fluorochrome
APC	Allophycocyanin	***MFI***	Median fluorescence intensity
BAT	Basophil activation test	***MHC***	Median histamine release per cell
CD	Cluster of differentiation	***MRG***	Mas-related gene
CO_2	Carbon dioxide	***NMBA***	Neuromuscular blocking agent
COX	Cyclooxygenase	***NSAIDs***	Non-steroidal anti-inflammatory drugs
Cre	Cre-recombinase	***PAF***	Platelet activating factor
DAO	Diamine oxidase	***PAMPS***	Pathogen-associated molecular patterns
DHR	Drug hypersensitivity reactions	***PBCMC***	Peripheral blood cultured mast cells
DNP	Dinitrophenyl	***PBS***	Phosphate buffered saline
DPT	Drug provocation test	***PBS-TX***	Phosphate buffered saline with Triton-X-100
E-NPP-3	Ecto-nucleotide pyrophosphatase/ phosphodiesterase CD203c	***PE***	Phycoerythrin
FcεRI	High-affinity IgE-receptor	***PECy7***	Phycoerythrin cyanine 7
FEIA	Fluorescent enzyme immune assay	***PerCP***	Peridinin chlorophyll
FITC	Fluorescein isothio cyanate	***PFA***	Paraformaldehyde
FMO	Fluorescence minus one	***PRR***	Pattern recognition receptor
FSC-H/A	Forward scatter-height/area	***rBet v 1***	Recombinant birch pollen allergen (betula verrucosa)
GPCR	G protein-coupled receptor	***Rs***	Spearman's rank correlation coefficients
HuMC	Human mast cells	***SCF***	Stem cell factor
IgE/M/G	Immunoglobulin E/M/G	***SSC-H/A***	Side scatter-height/area
IDHR	Immediate drug hypersensitivity reactions	***sIgE***	Specific immunoglobulin-E
IDT	Intradermal tests	***SPT***	Skin prick tests
IL	Interleukin	***ST***	Skin test
IMDM	Iscove's modified dulbecco's medium	***STAT5***	Signal transducer and activator of transcription 5
LAMP	Lysosome associated molecular protein	***Th2***	T helper type 2 cells
LDL	Low-density lipoprotein	***THIQ***	Tetrahydroisoquinoline
LT	Leukotriene	***TNF-α***	Tumor necrosis factor-alpha
MAPK	Mitogen-activated protein kinase		
MCPC	Mast cell progenitor cells		
MCs	Mast cells		

Chapter 1: Introduction and aims of the thesis

Introduction

Mast cells (MCs) are long living, multifunctional and granulated tissue-resident immune cells involved in several health and disease conditions. Because of their widespread distribution and ability to interact with the microenvironment, MCs play an important protective role and participate in homeostasis, wound healing, immune tolerance and defense against pathogens. On the other hand, as outlined at the end of this chapter, MCs can also be implicated in various auto-inflammatory diseases, auto-immunity and allergic reactions (1–7). Mast cells can be activated by a myriad of immunoglobulin E (IgE)-dependent and IgE-independent mechanisms. However, the exact mechanisms that govern MCs activation and degranulation remain largely elusive, mainly because of their difficult accessibility.

Mast cell characteristics

MCs can be characterized as cells of 5 – 10 µm with a single-lobed nucleus and containing many small granules. Mast cells show a significant heterogeneity. Depending on their localization and as a result of influences from the local microenvironment human MCs are traditionally classified into two main subtypes: connective tissue mast cells (MC_{TC}) and mucosal mast cells (MC_T). MC_{TC} contain both tryptase and chymase in their granules and settle in general in connective tissue, such as the skin and lymph nodes, as well as in smooth muscle. In contrast, MC_T characteristically only contain tryptase in their granules and reside in mucosal tissue, such as the lungs. These subtypes also significantly differ in their response to endogenous and exogenous stimuli provoking degranulation (8–12).

In general MCs can be characterized based on their cell surface markers and granule content as shown in table 1. Beside these main characteristics, MCs also express various adhesion molecules, cell-cell recognition receptors, cytokine and chemokine receptors, and several virus-binding sites. Traditionally, MCs are phenotyped as $CD117^+/CD203c^+$ cells that also express the high affinity receptor for IgE (FcεRI) (2,6,11,13–16). It has recently been demonstrated that MRGPRX2, a Mas-related gene (MRG) receptor that belongs to the G protein-coupled receptor (GPCR) family, is highly expressed on the cell surface of MC_{TC}, while only little expression is reported on MC_T cells (1,17–20). The importance of this receptor in the context of this thesis is outlined further.

Table 1: The main characteristics of mast cells.		
Phenotypical markers	CD45 CD117 FcεRI CD203c	leukocyte common antigen c-kit or stem cell factor receptor high affinity IgE-receptor ecto-nucleotide pyrophosphatase/ phosphodiesterase-3 (E-NPP-3)
Preformed granule content	Histamine β-hexosaminidase TNF Tryptase Chymase Carboxypeptidase A Cathepsin G	MC_T and MC_{TC}, best known biogenic amine MC_T and MC_{TC}, best known lysosomal enzyme MC_T and MC_{TC}, tumor necrosis factor MC_T and MC_{TC}, protease MC_{TC}, protease MC_{TC}, protease MC_{TC}, protease
Abbreviations: CD = cluster of differentiation, MC_{TC} = connective tissue mast cells, MC_T = mucosal mast cells, TNF = tumor necrosis factor		

Mast cell activation

MCs fulfil their protective or pathological role through the immediate release of inflammatory mediators. Mast cell mediators can be divided into three classes: 1) preformed mediators (*e.g.* histamine, β-hexosaminidase and several proteases), which are stored in mast cell granules; 2) new formed or lipid mediators (*e.g.* eicosanoids such as prostaglandin D2 or E2 and leukotrienes LTC4 or LTB4), which are derived from membrane lipids and; 3) *de novo*-synthesized mediators (*e.g.* cytokines, chemokines and growth factors), which are synthesized following transcriptional activation depending on the type of stimuli and receptor involved in MCs activation (6,11). Traditionally, MCs activation is subdivided in an IgE-dependent way and an IgE-independent way of activation.

IgE-dependent activation pathway

Tissue resident MCs and circulating basophils are often considered the primary effector cells in allergic reactions. Both MCs subtypes, MC_{TC} and MC_T, can be activated by IgE-allergen cross-linking of the FcεRI. There are two phases in the basic process of allergy: the sensitization phase and the effector phase. Sensitization begins with the exposure of allergens to antigen presenting cells, such as dendritic cells, and presentation of these allergens to naïve T cells. Next, under influence of interleukin (IL)-4 and IL-13 released by Th2 cells, B cells undergo an isotype switch from IgM to IgE and produce allergen-specific IgE (sIgE) antibodies. These sIgE antibodies are able to circulate freely but will also attach to high-affinity IgE-receptors (FcεRI), which are present on the membrane of MCs and basophils. The effector phase begins with each new exposure to the sensitizing allergen that results in cross-linking

of membrane-bound IgE/FcεRI complexes on sensitized MCs or basophils. These activated cells subsequently release their pro-inflammatory cytokines and/or mediators, thereby causing the clinical manifestations of the allergic reaction (Figure 1) (4).

IgE-independent activation pathway

MCs can also be activated via several IgE-independent pathways, mainly in response to invasive pathogens that trigger degranulation via activation of complement with generation of anaphylatoxins or through pattern recognition receptors (PRR) on their cell membrane. Moreover, MCs might contribute to the initiation and propagation of chronic inflammatory diseases as they are responsive to various inflammatory products such as IgG, cytokines, chemokines and others, or they can be directly activated by exogenous stimuli such as physical factors (heat and cold), various drugs, *etc.* (1,2,19,21). MC_{TC} and MC_T can differ in their response to several IgE-independent stimuli, for example only MC_{TC} respond to complement (*e.g.* anaphylatoxins C3a and C5a), compound 48/80, the neuropeptide substance P and opiate drugs (*e.g.* morphine), while platelet activating factor (PAF) only stimulates MC_T cells (1,20,22). In this thesis we will focus on MRGPRX2-mediated activation as a representative for IgE-independent activation of MCs, as occupation of this receptor can be involved in many diseases and conditions. Typical activators of MRGPRX2 are the neuropeptide substance P, cortistatin, somatostatin, neuropeptide FF, oxytocin and a number of peptidergic drugs capable of eliciting anaphylactic events (Figure 1) (1,10,18,20,23,24).

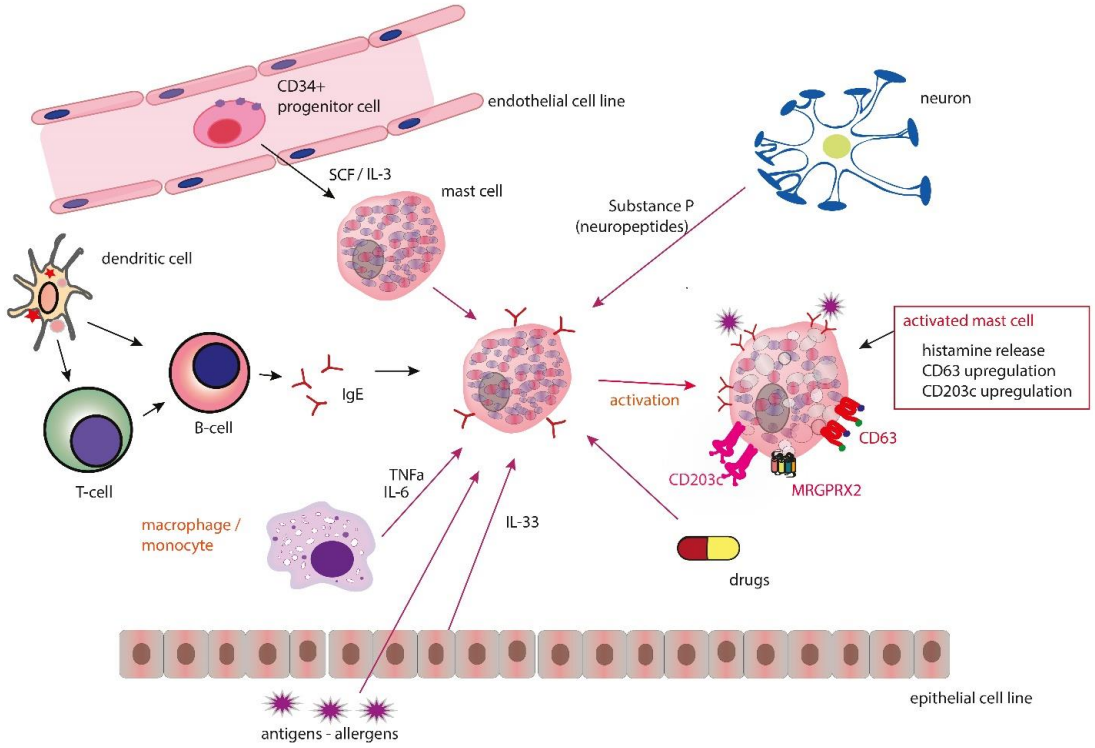

Figure 1: Overview of mast cell activation.

Mast cells originate from $CD34^+$ progenitor cells in the bone marrow. These progenitor cells leave the bone marrow and migrate via the circulation to the tissues, where they undergo a tissue-specific differentiation to mature mast cells under influence of stem cell factor (SCF) and interleukin (IL)-3. Next, these mature mast cells can be stimulated by a variety of IgE-independent stimuli such as substance P or various drugs through the MRGPRX2-receptor on their cell membrane. Besides, mast cells can be activated in an IgE-dependent way by antigens/allergens through cross-linking of membrane bound IgE on the high affinity IgE-receptor (FcεRI) of sensitized mast cells. In this regard, mast cell function can be influenced by a variety of cytokines such as epithelium-derived IL-33 or monocyte/macrophage-derived IL-6 and tumor necrosis factor-alpha (TNFa).

Mast cell models

Since tissue-resident mast cells are difficult to access and present only in small numbers, they are not readily available for extensive *ex vivo* and *in vitro* analysis. Isolation of mature cells from tissues poses several limitations, such as a low yield and possible disruption of normal cell phenotype or cell interactions due to the applied isolation process (12,25). Therefore, several alternative methods have been developed to unravel the functions and interactions of MCs with other immune cells (25,26).

Hitherto, MCs studies have predominantly been conducted on murine MCs, mainly including *in vivo* MCs (deficiency) models, the murine MCs line (RBL-2H3) and MCs derived from the bone marrow of different types of rats or mice (11,26–30). Most commonly used *in vivo* mouse models are deficient in mature MCs due to loss of function mutations in c-kit, for example the $WBB6F_1$-Kit $^{W/W-v}$ and C57BL/6-Kit$^{W-sh/W-sh}$ mice (31–33). However, because of c-kit's important function in the development of other cell types, such as hematopoietic stem and progenitor cells, melanocytes and others, several other abnormalities occur in these models and caution should be called upon interpreting the results (11,30,34,35). This has led to the more recent development of transgenic c-kit-independent MCs deficient mouse models. Most commonly used models are Cre-mediated strains in which Cre-recombinase (Cre) is expressed under the control of mast cell specific or associated promoters, for example the Mcpt5;Cre-R-DTA mice, Cpa3$^{Cre/+}$ or 'Cre-Master' mice and Cpa3-Cre;Mcl-1$^{fl/fl}$ or 'Hello Kitty' mice amongst others (11,30,35–39). Although these Cre-mediated strains display less abnormalities than the c-kit-dependent models, they demonstrate limitations due to, for example, the expression of Cre-controlling promotors in other cells than MCs during chronic inflammatory conditions or the initiation of compensatory mechanisms (30).

Of particular interest for this thesis is expression of the MRGPRX2-receptor on resting MCs, as this receptor seems to be involved in different pathologies and conditions. In humans there are four MRGPRX genes (MRGPRX1 – X4), while the mouse genome contains many more: MrgprA (A1-A10), MrgprB (B1-B5, B8), MrgprC (C11), MrgprD, MrgprE, MrgprF, MrgprG and MrgprH genes. The murine MRG receptors share only 45-64% amino acid sequence identity with their human orthologues, which could render extrapolation of murine data to humans difficult. For example, it has been proven that the affinity of mouse MrgprB2 for the neurokinin substance P and various drugs is significantly lower than the affinity of the human MRGPRX2-receptor (17,18,20). Taken together, these findings emphasize the need for representative human MCs models.

One way to gather phenotypic and functional data on human MCs implies the study of specific MCs lines, such as the HMC-1, LAD2, LUVA and ROSA cell line. However, these cell lines also display several important limitations that might impede extrapolation to human MCs. Firstly, HMC-1 cells are mast cell leukemia cells with an activating mutation in c-kit, making these cells independent of stem cell factor (SCF) for their growth. Moreover, as these HMC-1 cells only poorly express the FcεRI and lack well-formed granulates, these cells are unsuited to investigate IgE-mediated activation (26,30,40). Secondly, LAD2 cells are derived from a patient with mastocytosis, express FcεRI and degranulate well in response to antigen but demonstrate a relatively slow growth rate and express very low levels of tryptase and chymase, making LAD2 cells only intermediately differentiated MCs (26,30,41). Thirdly, LUVA cells are derived from peripheral blood $CD34^+$ cells obtained from a donor with aspirin exacerbated respiratory disease. These cells display a weak expression of FcεRI and only demonstrate a modest release of β-hexosaminidase upon IgE/FcεRI cross-linking (42). Recently, a fourth human MCs line was reported, the ROSA cell line. This cell line consists of two variants: 1) $ROSA^{KIT}$, a SCF-dependent human MCs line cultured from normal human cord blood; and 2) $ROSA^{KIT\ D816V}$, a SCF-independent variant, which is produced by stably expressing the c-kit activating mutation D816V in $ROSA^{KIT}$ cells. Both ROSA cell lines express CD117, CD203c and a functional FcεRI, and contain tryptase in their granules. However, they lack chymase. Furthermore, it was reported that the responsiveness of $ROSA^{KIT\ D816V}$ cells to IgE-dependent stimuli is weak as compared to normal MCs or $ROSA^{KIT}$ cells (43).

An alternative means to study human MCs are cultures from $CD34^+$ progenitor cells obtained from various sources. In this regard, bone marrow, where MCs originate, is not an easily accessible source and cord blood derived MCs are often still immature with a lower expression of FcεRI and CD203c, contain less histamine and are less dedicated for IgE-mediated activation (25,26,40,44,45). On the other hand, peripheral blood derived MCs are more mature and functionally active and compare best to mature tissue resident MCs, making these peripheral blood derived cultured MCs (PBCMC) probably the preferred method to study MCs function (25,26,46–49). However, we have to keep in mind that these culture methods are often expensive and time consuming, and can generate variable phenotypes depending on the used culture conditions (11,12,45). In this regard, it is known that the survival, differentiation and function of MCs is strictly regulated by signals from their environment, for example the c-kit ligand SCF and IL-3 are known to promote survival, proliferation and differentiation of *in vitro* cultured MCs (49–51). However, the majority of existing PBCMC methods also use pro-inflammatory cytokines, such as IL-6 or IL-9, to assure differentiation of progenitor cells into mature PBCMC (46,47,49,52). For some experiments these culture conditions might be deleterious as they may cause a “pro-inflammatory” impregnation and thus limit their use for investigating certain auto-immune

diseases, such as rheumatoid arthritis, where these pro-inflammatory cytokines are of importance in the pathology of the disease.

Recently, Schmetzer *et al.* (2014) have introduced a fast and easy method to produce mature MCs (48). Briefly, they isolated progenitor cells from stem cell concentrates discarded from the blood bank and promoted differentiation using merely SCF and IL-3 to generate mature MCs after only 3 weeks of culture. However, these PBCMC remain insufficiently characterized. PBCMC were defined as cells positive for CD117 and FcεRI, contained both tryptase and chymase and were proven to be functional for IgE-mediated stimulation with anti-FcεRI or IgE/anti-IgE. Although these findings are promising, phenotypical and functional analyses needs to be elaborated (*e.g.* expression of lineage-specific CD203c, MRGPRX2, allergen-specific activation and IgE-independent activation) and confirmed on the single cell level.

Functional analysis

MCs function is predominantly based on the release of mediators (histamine, proteases, β-hexosaminidase, *etc*.) from their granules into the environment. Traditionally, this degranulation is evaluated by quantifying these mediators in the supernatant before and after stimulation with the test compound (53–55). Unfortunately, the results of these tests merely represent an average of all stimulated cells and do not provide information on individual cells or cell subpopulations with heterogeneous responses. With the purpose of studying MCs activation on a single cell level, several activation markers have been identified on the cell surface of these cells, such as CD63 and CD203c amongst others. CD63, a membrane protein of the lysosome associated molecular protein (LAMP) tetraspanin family, is not expressed by resting MCs and appearance of this activation marker is closely associated with release of mediators during degranulation (54,56–59). Lineage specific CD203c, an ectonucleotide pyrophosphatase/phosphodiesterase family member (E-NPP-3), is already expressed on the cell surface of quiescent MCs and basophils and is up-regulated after stimulation. It has been suggested that CD203c merely mirrors early activation processes, which may not involve complete degranulation of the cells (56,58,60).

Recently, our research group has developed a new flow cytometric technique to combine immunophenotyping with quantification of intracellular histamine and its release of individual basophils, called the HistaFlow® (56). In this technique the intracellular content of histamine is quantified using the affinity of diamine oxidase (DAO) for its substrate histamine (61,62). By coupling the histaminase DAO to fluorochromes, intracellular histamine content and consequently release of

histamine can be measured by flow cytometry at a single cell level (56). However, at the beginning of this thesis, no technique allowing simultaneous analysis of surface markers and release of histamine for the individual cell had been described for MCs.

Mast cell priming

As illustrated in figure 1, MCs function can be influenced by various intrinsic and microenvironmental factors that affect either expression or functionality of surface receptors and/or signaling molecules involved in these responses. For example, since MCs express receptors for pro-inflammatory cytokines IL-33, IL-6 and TNF-α amongst others, they can be stimulated by these cytokines which might influence their function (11,12,63–65).

Epithelium-derived IL-33 is released from keratinocytes after various triggers, such as allergens, pathogens, tissue damage or exogenous proteases. It has been shown that IL-33 can influence MCs behaviour, *viz.* short-term exposure of MCs to IL-33 causes an enhancing effect on individual MCs activation (66), while long-term exposure (>72 hours) of MCs to IL-33 causes a substantial reduction in IgE-mediated activation of these cells (57). Besides, IL-33 can boost MCs activation by several IgE-independent stimuli, such as anaphylatoxin C5a (68), IgG immune complexes (69,70) or substance P (71,72) by increasing cytokine and chemokine production, calcium influx or release of mediators.

Additionally, IL-6 and TNF-α are mainly released by monocytes, macrophages and MCs. In this context, IL-6 is known to stimulate B cell growth, antibody production, effector T cell development, acute phase responses and inflammation (73–75). TNF-α is known to promote cellular differentiation and survival, upregulate adhesion molecules, stimulate production of inflammatory cytokines and chemokines, and assist recruitment of inflammatory cells (65,76). Furthermore, long-term exposure of MCs to IL-6 has been reported to promote maturation and enhance IgE-mediated signaling, degranulation and cytokine production (73). However, less is known about the effect of TNF-α on MCs activation. Only a few studies investigated this matter and these studies reported conflicting results. Two studies demonstrated that TNF-α inhibited activation of rat MCs by reducing release of histamine in the supernatant (77,78), while another study observed that recombinant TNF-α directly stimulated foreskin derived MCs to release histamine and tryptase in the supernatant (79).

Mast cell related diseases

As reviewed by da Silva *et al.* (2014) MCs have been implicated in various auto-inflammatory diseases, auto-immunity and allergic reactions (11). Briefly, MCs are involved in mastocytosis, a rare clonal mast cell disorder characterized by clonal accumulation and abnormal reactivity of MCs in one or more organs (80,81). MCs hyperplasia and/or increase in MCs products has been observed at sites of tissue inflammation in cardiovascular disease and several autoimmune diseases, such as bullous pemphigoid, multiple sclerosis and rheumatoid arthritis (82–84). The involvement of MCs in cancer is contradictory and both promotion of and protection against tumor growth have been observed (85,86). Finally, MCs play an important role in allergic inflammation by release of their mediators, initiating the immediate acute phase of the allergic reaction. Next, they are responsible for the recruitment of inflammatory cells to the site of inflammation, initiating an acquired immune response and contributing to the development of a chronic phase associated with persistent inflammation, tissue remodeling and fibrosis as seen in asthma, allergic rhinitis and atopic dermatitis (87–89).

Adverse drug reactions

Adverse drug reactions (ADR) are any noxious, unintended and undesired effects of a drug that occur at doses used for prevention, diagnosis or treatment. ADR can be classified as displayed in table 2 (90).

Table 2: Adverse drug reactions

Type A reactions (80%): Due to pharmacological actions of the drug	• drug-induced toxicity • side effects • secondary effects • drug interactions	• predictable • common
Type B reactions (20%): Due to drug hypersensitivity	• drug intolerance • idiosyncratic reactions • drug hypersensitivity reactions (DHR) → allergic DHR: immune mediated → non-allergic DHR: not immune mediated	• unpredictable • uncommon

Abbreviations: DHR = drug hypersensitivity reactions; Adapted from (90).

Drug hypersensitivity reactions (DHR) can occur immediately after exposure to the drug or can display a late onset pattern in which case DHR occur more than 72 hours after drug administration. Late onset DHR are usually T-cell-mediated and generally lack the rapidly evolving life-threatening features that are typical of anaphylaxis.

Immediate drug hypersensitivity reactions

This thesis focuses on immediate drug hypersensitivity reactions (IDHR). IDHR can be allergic (immune mediated) or non-allergic (not immune mediated). Allergic IDHR can be subdivided into 3 types of reactions: type I reactions, which occur through IgE/FcεRI-cross-linking; type II reactions, which are cytotoxic; and type III reactions, which are immunecomplex-mediated reactions. Type I reactions are typically characterized by urticaria, angioedema, rhinitis, conjunctivitis, bronchospasm, gastrointestinal symptoms and/or anaphylaxis, while type II and type III reactions are clinically manifested as hemolytic anemia and serum sickness syndrome (90–92). Non-allergic IDHR are more heterogeneous and can be caused by non-specific immediate histamine releasers (*e.g.* opiates) or inhibition of the cyclooxygenase (COX) pathway (*e.g.* non-steroidal anti-inflammatory drugs (NSAIDs)), or as recently demonstrated by McNeil *et al.* (2015) and Lansu *et al.* (2017) through ligation of the MRGPRX2-receptor by tetrahydroisoquinoline (THIQ)-motif containing drugs (*e.g.* moxifloxacin) (18,23,90,92).

Diagnosis of immediate drug hypersensitivity reactions

Evaluation of the clinical history

Diagnosis of IDHR usually starts with a thorough clinical history. History taking includes the symptomatology, the chronology of the symptoms (*e.g.* time-of-onset of symptoms, effect of stopping treatment), dosage of peak tryptase, other medications taken and the medical background of the patient (*e.g.* previous allergies, medical conditions that can be aggravated by the intake of certain drugs) (91,92).

Drug provocation tests

Drug provocation tests (DPT) are considered the gold standard for the identification of the drug provoking a DHR. However, DPT involve a significant risk of severe, life-threatening reactions and might be contraindicated. Moreover, these tests are not absolutely predictive (*e.g.* full dose provocations are not always possible) and have consequently not entered mainstream clinical practice (91–93).

Skin testing

In general, skin prick tests (SPT) and intradermal tests (IDT) are the most readily available means for confirming or excluding an IgE-dependent mechanism. SPT are performed on the ventral part of the forearm with histamine as a positive control to evaluate skin test reactivity, a saline buffer solution as a negative control to exclude cutaneous hyper-reactivity and the involved drugs. After 15 minutes, wheal reaction is assessed and considered positive when exceeding 3 mm with a flare. IDT are undertaken when SPT are negative and should be performed with the intravenously injectable form of the drug whenever possible. IDT responses are considered positive when the wheal equals or exceeds 5 mm or is doubled as compared to the injection bleb of 0.2 mL. Unfortunately, the sensitivity and predictive values for skin tests vary depending on the culprit drug and the clinical presentation (91,92,94).

Quantification of sIgE antibodies

Drug specific IgE (sIgE) measurement is performed through a fluorescent enzyme immune assay (FEIA) (*e.g.* ImmunoCAP) according to the manufacturer's instructions. These allergen-specific IgE assays are commercially available for penicilloyl G, penicilloyl V, ampicilloyl, amoxicilloyl, morphine and many other drugs (91,93). Other sIgE assays are available for research purposes only, such as rocuronium (93). Unfortunately, the large majority of these available tests have not yet been clinically validated and hence should never be used as a means to rule out drug allergy, but may help to circumvent pointless or dangerous oral challenges.

Cellular tests

The symptoms of IDHR result from mediators released by MCs and basophils. However, this mediator release does not necessarily involve IgE/FcεRI-crosslinking and thus cannot be depicted by traditional sIgE assays. Additional cellular tests might be more informative, because these tests can also demonstrate IDHR independent from IgE. As already mentioned earlier, traditional mediator release tests measure the extracellular content of mediators in the supernatant, merely representing an average of all degranulated cells. Hence, these assays do not provide information on individual cells or cell subpopulations with heterogeneous responses. The basophil activation test (BAT) relies on a flow cytometric analysis of alterations in specific activation (*e.g.* CD203c) and degranulation (*e.g.* CD63) markers on the cell membrane and allows for quantification on a single-cell level by use of specific monoclonal antibodies conjugated with different fluorochromes. As reviewed elsewhere, it appears that BAT is being rapidly incorporated in the diagnosis of IDHR and has mainly been applied to

document allergy to neuromuscular blocking agents (NMBA), antibiotics, aspirin and other NSAIDs (95). Recently, our research group provided the proof-of-concept that histamine and its release can also be studied by multicolor flow cytometry on a single cell level by an enzyme affinity method called HistaFlow® (56). However, for the time being, the clinical and scientific application of HistaFlow® remains restricted to a proof-of-concept in birch pollen allergy (56) and a follow-up study in wasp venom immunotherapy (96). Therefore **in chapter 4 of this thesis**, the HistaFlow® technique is verified as an instrument to quantify IgE-mediated basophil responses to drugs, which are believed to be less potent basophil activators than large proteinaceous allergens. Finally **in chapter 5 of this thesis**, non-allergic IDHR is investigated by use of MCs, which are known to express the MRGPRX2-receptor, since MRGPRX2 is mentioned as one of the possible IgE-independent mechanisms through which IDHR might occur. Hopefully, use of our single cell approach to study these reactions will help to further unravel the mechanisms underlying both allergic and non-allergic IDHR.

Aims of the thesis

The overarching aim of this thesis is to develop a simplified human mast cell culture generating sufficient cells enabling precise phenotypical and functional analysis of individual cells.

For this purpose, the first aim of this thesis was to develop and validate mast cell culture protocols **(Chapter 2)** starting from small volumes of peripheral blood from individual patients and appropriate control individuals **(Chapter 3)**.

Because of mast cell heterogeneity, the second goal of this work was to characterize the cells both phenotypically and functionally. Functional analysis explored IgE-dependent **(Chapter 2 and 3)** and IgE-independent **(Chapter 3 and 5)** stimulation of the cells.

The third aim of this dissertation was to identify the priming effect of the epithelium-derived cytokine IL-33 and monocyte/macrophage-derived cytokines IL-6 and TNF-α **(Chapter 3)**. These cytokines are involved in immune pathological mechanisms and their effect can effectively be blocked by targeted therapies.

The fourth goal of this thesis was to apply our PBCMC to help unravel the mechanisms and endotypes underlying immediate drug hypersensitivity reactions (IDHR). Therefore, we firstly aimed at verifying the applicability of our flow cytometric techniques to analyze allergic IDHR in a pilot study using readily available basophils **(Chapter 4)**. Secondly, we aimed at applying our PBCMC model to non-allergic IDHR and examine whether the human MRGPRX2-receptor might be involved in the underlying mechanisms of IDHR **(Chapter 5)**.

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Chapter 2: Phenotypic and functional characterization of in vitro cultured human mast cells

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Abstract

Background. Mast cell progenitor cells, derived from $CD34^+$ hematopoietic stem cells, enter the circulation and subsequently mucosal or connective tissues where they mature to mast cells. Upon activation, mast cells increase the expression of activation markers, *e.g.* CD63, and release histamine amongst other mediators. Traditionally, release of these mediators is quantified using assays measuring their extracellular concentration in the supernatant of stimulated cells.

Methods. Human mast cells were cultured from peripheral blood, phenotypically characterized, passively sensitized with allogenic IgE antibodies and finally stimulated by anti-IgE that cross-links IgE/FcεRI complexes. Alterations in the number of cells positive for CD63 and release of histamine were quantified simultaneously by flow cytometry.

Results. In culture, two distinct $CD45^+$ cell populations were identified: $CD117^+CD203c^{+hi}$ and $CD117^-CD203c^{+low}$ cells. Both populations showed positivity for FcεRI, tryptase and chymase, and contained histamine. Activation resulted in a significant increase of cells positive for $CD63^+$ up to 21% (range: 11–39) for $CD117^+CD203c^{+hi}$ cells (P = 0.005), and 27% (18–55) $CD63^+$ for $CD117^-CD203c^{+low}$ cells (P = 0.02). Baseline histamine content was higher for $CD117^+CD203c^{+hi}$ cells than for $CD117^-CD203c^{+low}$ cells, respectively 994 (695–6815) Molecules of Equivalent Specific Fluorochrome V500 per cell (MESF-V500/cell) and 797 (629–4978) MESF-V500/cell (P = 0.02). After activation, $CD117^+CD203c^{+hi}$ cells showed significant histamine release of 578 (366–1521) MESF-V500/cell, whilst $CD117^-CD203c^{+low}$ cells resulted in 310 (217–366) MESF-V500/cell histamine release.

Conclusion. This study discloses that culturing HuMC from $CD34^+$ progenitors yields 2 phenotypically distinct cell populations that display a greatly similar response upon cross-linking of IgE/FcεRI complexes.

Introduction

Mast cell progenitor cells (MCPC), derived from $CD34^+$ hematopoietic stem cells, enter the circulation and subsequently mature to mast cells after migration into the mucosal or connective tissues (1-3). Unlike MCPC, mature tissue-resident mast cells do not express CD34, demonstrate a higher level of CD117 (c-kit, mast/stem cell growth factor receptor), express the high affinity IgE receptor (FcεRI) and the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP-3) CD203c (3-5). Mature mast cells also contain granules loaded with mediators like histamine, heparin and cytokines and depending on the tissue in which they mature, tryptase (mucosal mast cells) or both tryptase and chymase (connective tissue mast cells) (6-8). Mast cells can be activated, either IgE-mediated, *i.e.* by IgE/FcεRI cross-linking, or non-IgE-mediated (*e.g.* through cytokines and growth factors, anaphylatoxins, immune complexes and physical triggers) (9). Upon activation, mast cells exhibit an increased membrane expression of activation markers such as CD63, CD203c and CD107, and release various mediators such as histamine, tryptase, prostaglandins, leukotrienes and cytokines (10,11). Traditionally, release of these mediators is quantified measuring their extracellular concentration in the supernatant of all stimulated cells (10,12).

Unlike peripheral blood basophils, mast cells are tissue-resident cells that are present only in small numbers and are thus poorly accessible for *ex vivo* and *in vitro* analyses. Therefore, studies on human mast cells (HuMC) have mainly focused on mast cell cultures (13-18). Previously, we provided a proof-of-concept that histamine content and release by individual basophils can be studied using multi-colour flow cytometry in a technique designated as HistaFlow® (19). Moreover, as the technique allows a simultaneous immunophenotyping of the cells, it was shown that the technique enables to study the functional behaviour of different subpopulations and individual cells (19,20).

Here we sought to investigate whether this technique could also be applicable for functional analysis of *in vitro* cultured HuMC.

Materials and methods

In vitro culture of HuMC

HuMC were cultured according to Schmetzer *et al*. (2014) and Wang *et al*. (2006) with modifications (12,21). Briefly, buffy coat cell concentrates were isolated from 500 mL healthy donor blood (supplied by Red Cross Donor Centre, Belgium). Next, peripheral blood mononuclear cells were isolated from these buffy coats using Histopaque-1077 (Sigma-Aldrich, St. Louis, Missouri, USA). $CD34^+$ cells were enriched using the EasySep Human CD34 Selection Kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's instructions. Isolated $CD34^+$ progenitor cells with a minimal purity of 80% 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). Cells were plated into a 6-well plate at a density of 1 to 2 x 10^5 cells/mL and kept at 37°C in a humidified CO_2-incubator for 14 days. At day 3, 7 and 10 cells were nourished by adding 300 µL Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies) containing penicillin (100 units/mL), streptomycin (100 µg/mL), 1% Insulin-Transferrin-Selenium (Life Technologies), 0.1% Bovine Serum Albumin (Sigma-Aldrich), SCF (20 ng/mL) and IL-3 (20 ng/mL) as a thin layer covering the MethoCult well. At day 14, cells were retrieved from the MethoCult and maintained in IMDM containing only SCF (IMDM + SCF 10 ng/mL) at a density of 0.5 x 10^6 cells/mL for a 7 days resting phase. At day 21, medium was replaced with fresh IMDM containing SCF. A total of 34 cell cultures were performed for which, depending on the yield of each cell culture, a different numbers of experiments could be accomplished.

Flow cytometric analysis

Immunophenotyping

At day 21 of culture, viability of the cells was assessed using a 7-AAD viability staining solution. Cells were stained for surface makers with monoclonal anti-human CD45-peridinin chlorophyll (CD45-PerCP) (BioLegend, San Diego, USA), CD117-phycoerythrin (CD117-PE) (BD Biosciences) (n = 34), CD203c-allophycocyanin (CD203c-APC) (n = 31) (Pharmingen, BD Biosciences, Erembodegem, Belgium), FcεRI-phycoerythrin (FcεRI-PE) (eBioscience, San Diego, USA) (n = 25) and CD63-fluorescein isothiocyanate (CD63-FITC) (BD Biosciences) (n = 10).

Intracellular markers were determined as the number of cells positive for histamine (n = 21), tryptase (n = 9) and chymase (n = 9). Therefore, mast cells were fixed with 1 mL of 4% paraformaldehyde (PFA) solution for 30 minutes at room temperature, washed with and resuspended in PBS with 0.05% Triton-X-100 (PBS-TX, pH=7.4) (Sigma-Aldrich). Next, mast cells were stained with diamine oxidase-V500 (DAO-V500) (BD Biosciences) (19), monoclonal anti-human tryptase-Alexa Fluor 700 (anti-tryptase-AF700) (BioLegend) and monoclonal anti-human chymase-Alexa Fluor 700 (anti-chymase-AF700) (BioLegend), respectively, and incubated at 37°C for 30 minutes. Cells were washed and suspended in 300 µL PBS with 0.1% sodium azide and measured.

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). Since DAO is not an antibody, compensation with labelled DAO was executed manually by aligning the medians of positive as well as negative stained cell populations. Flow cytometric data were analyzed using Kaluza Analysis 1.3 software (Beckman Coulter, USA). A fluorescence minus one (FMO) sample was used to set a marker between positive and negative cells according to the 99th percentile. The percentages refer to the net number of cells positive for each parameter.

Functional analysis

At day 21 until day 35 function of the HuMC was evaluated in 10 experiments by passively sensitizing the cells with allogenic sera containing elevated titres of total IgE (400 - 2000 kU/L) 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 density of 0.5 x 10^6 cells/mL. Subsequently, 100 µL passively sensitized HuMC were incubated with 100 µL Tyrode's buffer as a negative control or 100 µL of 2 µg/mL monoclonal anti-IgE (BD Biosciences) as a positive control 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.

Histamine content was determined before and after activation as described above. A total of 7 experiments were conducted in duplicate. DAO staining with and without permeabilisation was used to set a marker between DAO positive and negative cells according to the 99^{th} percentile. Standardization was 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 mean of the duplicates in Molecules of Equivalent Specific Fluorochrome V500 (MESF-V500) for histamine content or Molecules of Equivalent Specific Fluorochrome APC (MESF-APC) for CD203c density. Histamine release was calculated by subtracting the intracellular content from activated cells from the content from non-activated cells.

Statistical analysis

Results are expressed as median and range (min-max). IBM SPSS Statistics version 23 software was used for data analysis. Non-parametric statistical analysis was performed where appropriate. A P-value of < 0.05 was considered significant.

Results

Mast cell characterization

Over all, CD45 positive cells showed cell viability of 95% (92 – 99) (figure 1B). As demonstrated in figure 1C, cells were subsequently characterized based upon expression of CD117 and CD203c. Two clear distinct cell populations could be identified: $CD117^{+}CD203c^{+hi}$ cells and $CD117^{-}CD203c^{+low}$ cells representing respectively 50% (21 - 97) and 50% (3 – 79) of the cells in culture. $CD117^{+}CD203c^{+hi}$ cells showed a basal expression of CD203c of 133 (86 – 432) MESF-APC/cell, whilst $CD117^{-}CD203c^{+low}$ cells showed a basal expression of CD203c of 70 (42-117) MESF-APC/cell (Wilcoxon: P = 0.02).

$CD117^{+}CD203c^{+hi}$ cells showed a significantly higher number of cells expressing the FcεRI as compared to $CD117^{-}CD203c^{+low}$ cells (figure 1D and 2A) (Wilcoxon: P < 0.0001), respectively 96% (87 – 99) and 86% (43 – 98) with a median difference of 11% (1 - 44). Besides, $CD117^{+}CD203c^{+hi}$ cells showed a significantly higher intracellular histamine content than $CD117^{-}CD203c^{+low}$ cells (figure 1E and 2B) (Wilcoxon: P < 0.0001), respectively 1388 MESF-V500/cell (187 – 10473) and 1011 MESF-V500/cell (198 – 8016) with a median difference of 414 MESF-V500/cell (11 – 2457). Both subpopulations showed an equal number of cells positive for tryptase (figure 1F and 2D), respectively 96% (66 – 100) and 96% (58 – 99) with a median difference of 2% (0 – 25). Lastly, $CD117^{-}CD203c^{+low}$ cells showed a slightly higher number of cells positive for chymase (Wilcoxon: P = 0.01) (figure 1G and 2C), respectively 97% (74 – 100) and 90% (61 – 99) with a median difference of 5% (0 – 25).

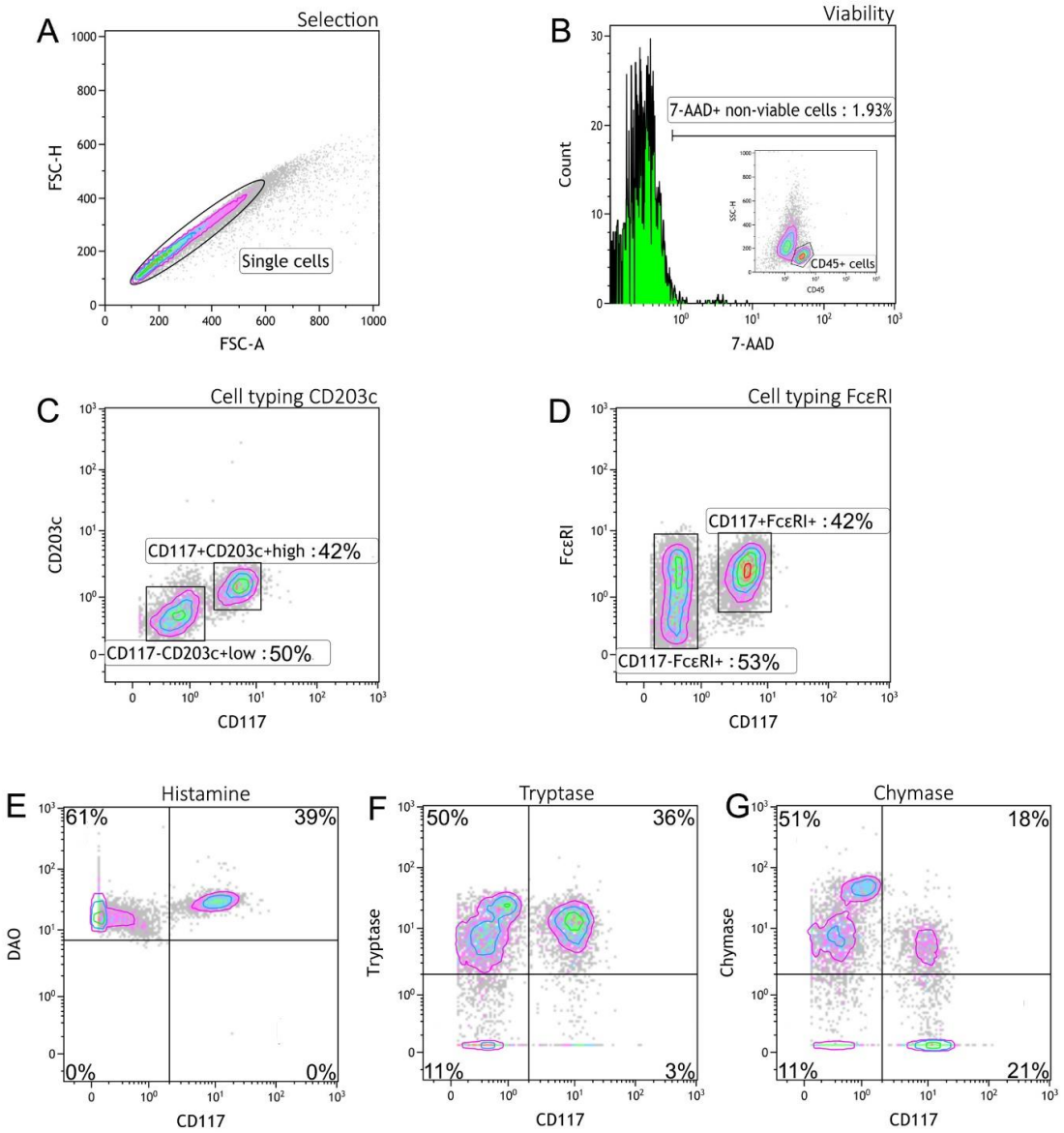

Figure 1: Phenotypic characterization of cultured cells.

Selection of single cells for analysis based on FSC-H (forward scatter-Height) and FSC-A (forward scatter-Area). All CD45 positive cells were evaluated for viability using 7-AAD staining. Viable $CD45^{+}$ cells were gated for further analysis **(A-B)**. Two distinct $CD45^{+}$ cell populations were identified: $CD117^{+}CD203c^{+hi}$ and $CD117^{-}CD203c^{+low}$ cells **(C)**. Both cell populations showed positivity for FcεRI **(D)**, histamine **(E)**, tryptase **(F)** and chymase **(G)**. The percentages refer to the number of cells positive for each parameter.

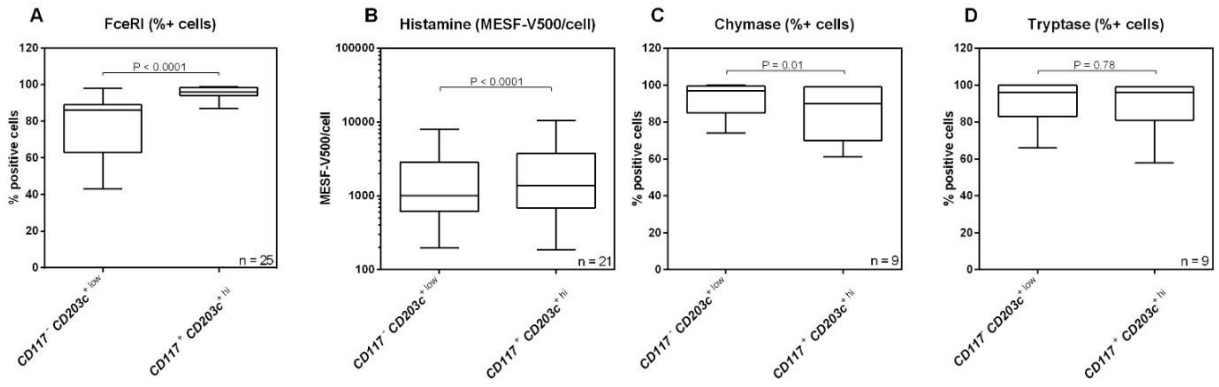

Figure 2. Cell characterization based on expression of CD117 and CD203c.

$CD117^{+}CD203c^{+hi}$ cells showed a significantly higher number of cells positive for FcεRI **(A)** on their cell membrane and a significantly higher histamine content **(B)** in comparison with $CD117^{-}CD203c^{+low}$ cells. $CD117^{+}CD203c^{+hi}$ cells showed a slightly lower number of cells positive for chymase **(C)** than $CD117^{-}CD203c^{+low}$ cells. The number of cells positive for tryptase **(D)** was equal in both subpopulations. The lines denote the medians. P values were calculated using Wilcoxon signed rank test. The N in the lower right corner represents the number of experiments.

Functional analysis

Expression of CD63 and upregulation of CD203c

Activation of passively IgE-sensitized cells with anti-IgE resulted in a significant upregulation in a net number of cells positive for $CD63^+$ of 21% (11 – 39) for $CD117^+CD203c^{+hi}$ cells (Wilcoxon: P = 0.005) and 27% (18 – 55) for $CD117^-CD203c^{+low}$ cells (Wilcoxon: P = 0.02) (n = 10). A discrete higher CD203c density up to 55 MESF-APC/cell (32 – 110) for $CD117^+CD203c^{+hi}$ cells (Wilcoxon: P = 0.005) and 62 MESF-APC/cell (19 – 187) for $CD117^-CD203c^{+low}$ cells (Wilcoxon: P = 0.02) could be observed.

Histamine content

Histamine content and release are illustrated in figure 3. Histamine content of non-stimulated cells was higher for $CD117^+CD203c^{+hi}$ cells as compared to $CD117^-CD203c^{+low}$ cells, respectively 994 (695 – 6815) MESF-V500/cell and 797 (629 – 4978) MESF-V500/cell (Wilcoxon: P = 0.02).

Stimulation of $CD117^+CD203c^{+hi}$ cells with anti-IgE resulted in a histamine release of 578 (366 – 1521) MESF-V500/cell (Wilcoxon: P = 0.04) in 5 cultures (figure 3C-D, left). In the remaining 2 cultures, no release of histamine was demonstrable.

Stimulation of $CD117^-CD203c^{+low}$ cells with anti-IgE resulted in a histamine release of 310 (217 – 366) MESF-V500/cell in 3 experiments (Wilcoxon: P = 0.11) (figure 3C-D, right). However, in the remaining 4 cultures no release of histamine could be observed.

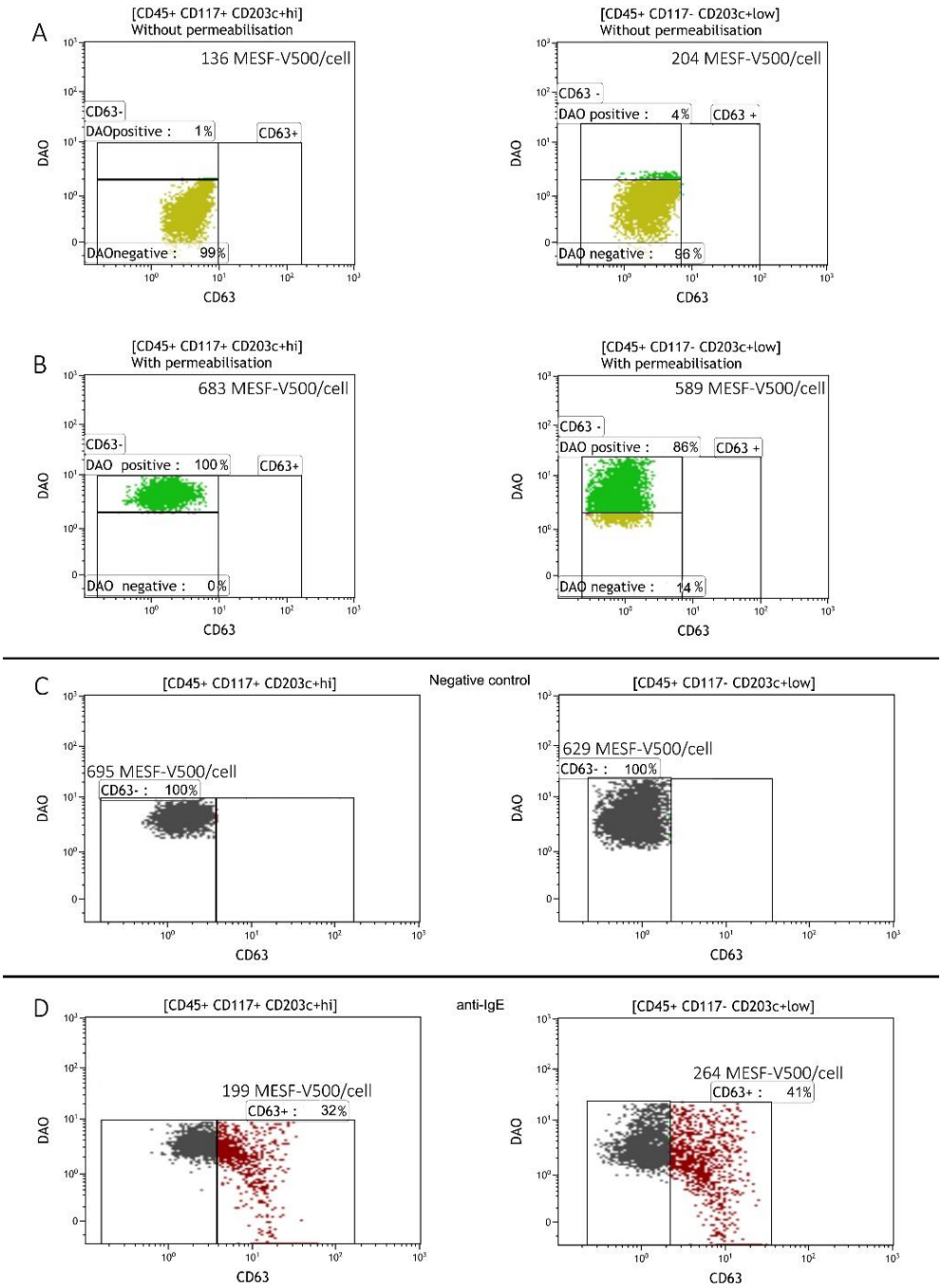

Figure 3: Histamine content and release after stimulation with anti-IgE.

Comparison of two subpopulations: $CD117^{+}CD203c^{+hi}$ cells **(left)** and $CD117^{-}CD203c^{+low}$ cells **(right)**. DAO specificity was ensured by staining without **(A)** and with permeabilisation **(B)**. Evaluation of cell function: Non-stimulated cells (negative control) showed neither $CD63^{+}$ upregulation, nor histamine release **(C)**. $CD117^{+}CD203c^{+hi}$ cells stimulated with anti-IgE showed a significant upregulation of cells positive for $CD63^{+}$ of 32%, with a histamine release of 496 MESF-V500/cell **(D, left)**. $CD117^{-}CD203c^{+low}$ cells stimulated with anti-IgE resulted in a significant upregulation of cells positive for $CD63^{+}$ of 41%, with histamine release of 365 MESF-V500/cell **(D, right)**. For more information, see results section.

Discussion

Studies on human mast cells have mainly been using cells cultured from different origins (12-14,17,18,21,22), as mast cells are tissue-resident cells, only present in small numbers. Here we confirm that human mast cells can be cultured from peripheral blood $CD34^+$ progenitor cells. However, these cultures yield two phenotypically distinct cell populations. Firstly, mature mast cells that express CD45, CD117 (c-kit), CD203c and FcεRI on their surface and contain histamine, tryptase and chymase (4,23), thereby resembling the connective tissue phenotype of mast cells (8,24). Secondly, a $CD117^-CD203c^{+low}$ subpopulation displaying characteristics of HuMC with a lower number of cells positive for FcεRI and a lower histamine content, but equal number of cells positive for tryptase and a slightly higher number of cells positive for mast cell specific chymase. We speculate these $CD117^-CD203c^{+low}$ cells to be immature mast cells with an internalized c-kit receptor upon binding of its ligand. This c-kit disappearance already has been described for HuMC as a mechanism by which the availability of the c-kit receptor is controlled (25-28). Functional analysis reveals that both, the $CD117^+CD203c^{+hi}$ and $CD117^-CD203c^{+low}$ subpopulation, display a significantly upregulated number of cells positive for CD63 and upregulated expression of CD203c after IgE-mediated cross-linking of their FcεRI, but in the vast majority of $CD117^-CD203c^{+low}$ cells this upregulation was not accompanied by a quantifiable histamine release. This dissociation between CD63 upregulation and histamine release already has been described for basophils (29-31), and could relate to stimulation conditions such as supra-optimal stimulation with anti-IgE.

To our knowledge this is the first description of flow cytometric quantification of histamine release by cultured HuMC. Our data reveal the existence of 2 phenotypically distinct cell populations that display a greatly similar response upon traditional activation through cross-linking of IgE. Furthermore, as this flow cytometric technique enables integration in a multi-parameter analysis with simultaneous quantification of various surface activation and degranulation markers, we suggest that it might capture data that cannot be obtained with traditional release tests. Especially on a single cell level or with some subpopulations, expanding experiments with intracellular signaling (32-35) or inhibitory receptors (36-39) might provide further insights into different pathophysiological mechanisms.

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Chapter 3: Influence of IL-6, IL-33 and TNF-α on human mast cell activation: lessons from single cell analysis by flow cytometry

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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.

	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.**

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 (24-27).

Non-IgE mediated activation with substance P

For non-IgE mediated activation with substance P, 10 new MCs cultures were generated from the peripheral blood of 10 healthy donors. These cultured human MCs, pre-incubated with IL-6 (n = 6), IL-33 (n = 10) and TNF-α (n = 6) 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.

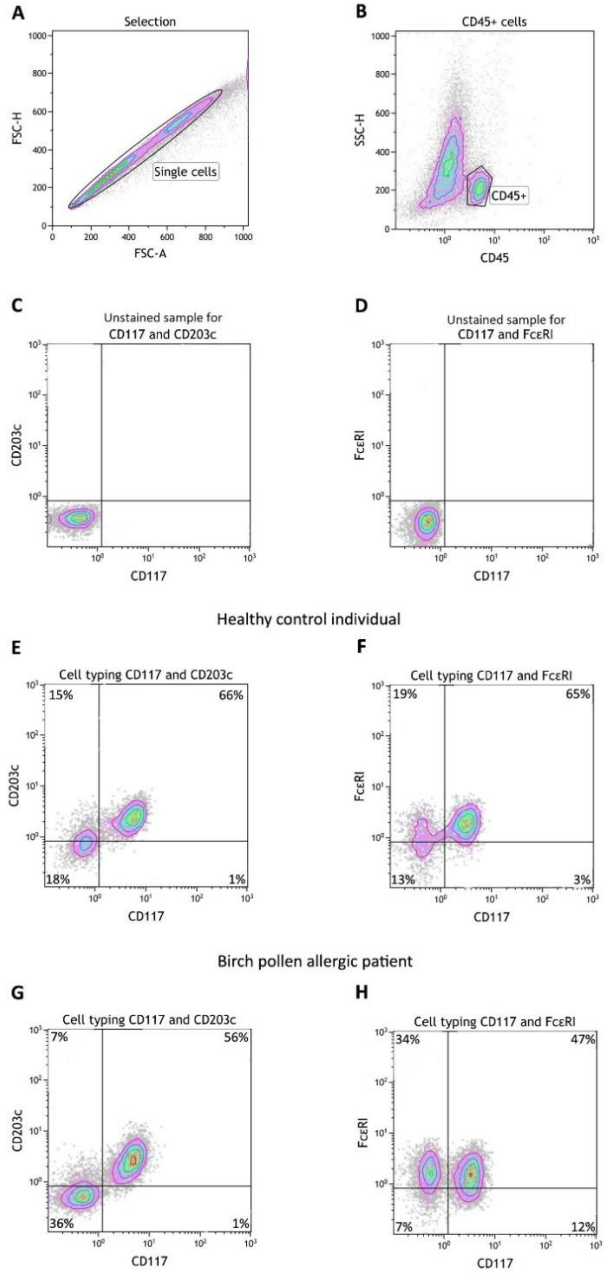

Figure 1. Phenotypical characteristics of mast cells cultured from peripheral blood of healthy control individuals or birch pollen allergic donors.

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.

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.

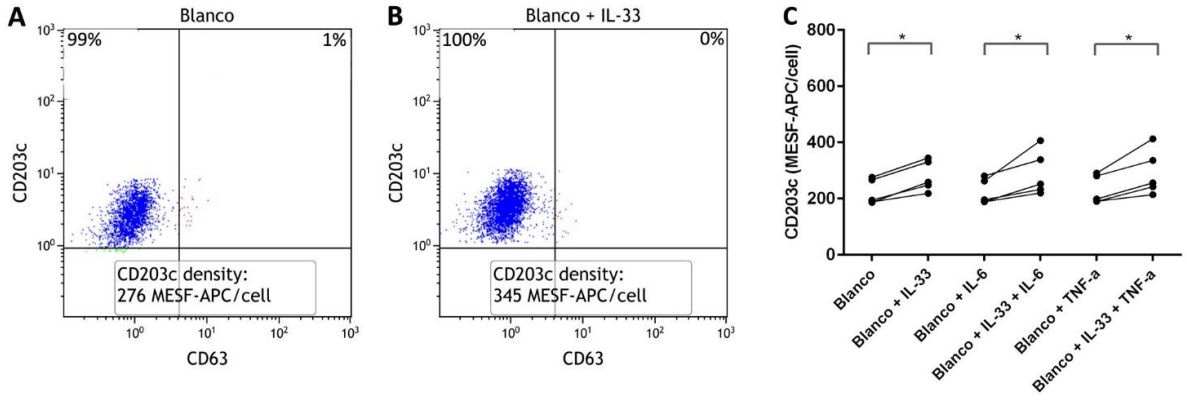

Figure 2. Effect of pre-incubation with pro-inflammatory cytokine IL-33 on *in vitro* cultured human mast cells. **(A)** MCs without pre-incubation with IL-33. **(B)** MCs pre-incubated with IL-33. **(C)** Comparison of pre-incubation with IL-33, IL-6 and/or TNF-α. Pre-incubation with IL-33 results in 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).

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.

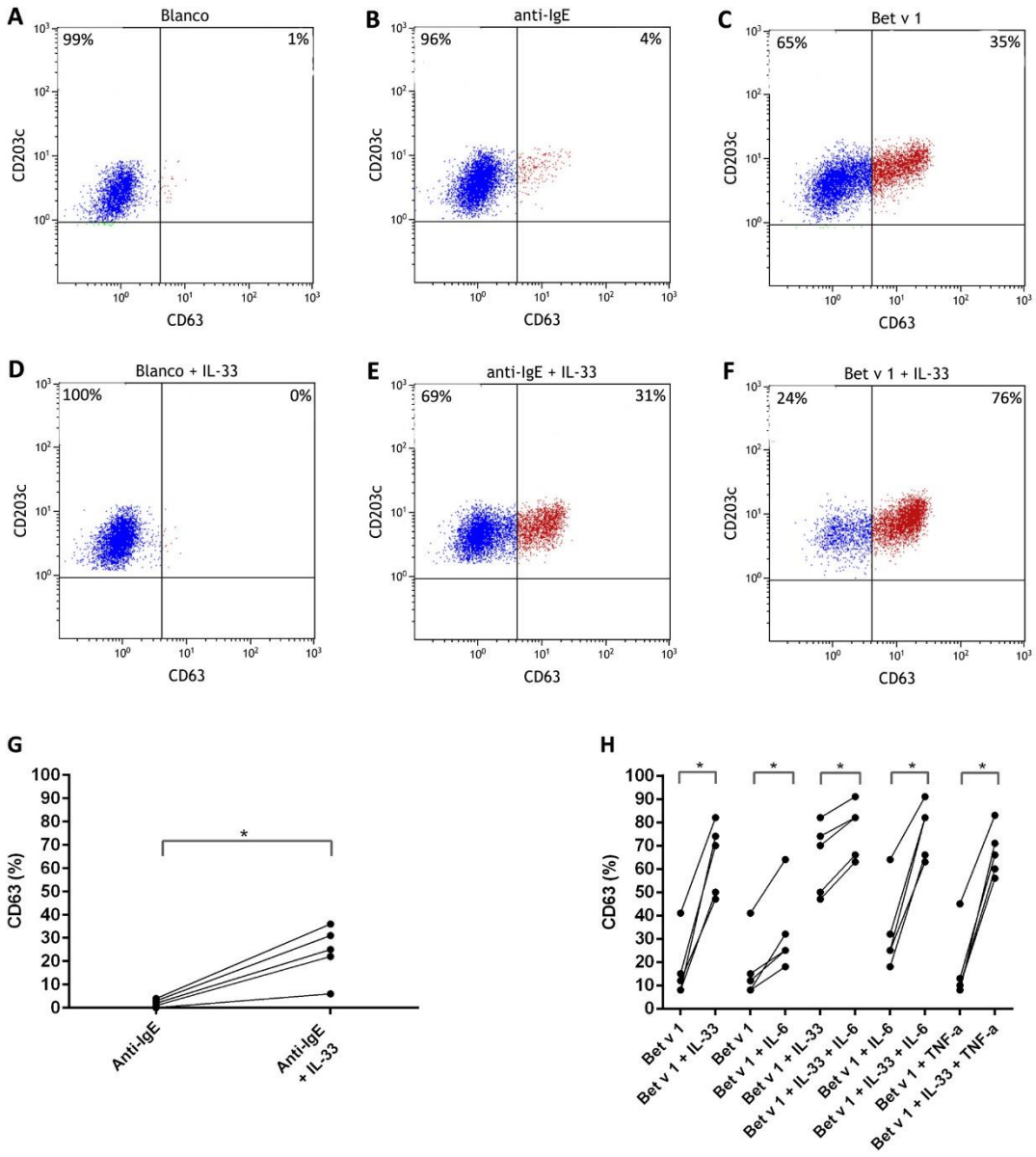

Figure 3. Effect of pre-incubation with pro-inflammatory cytokines on IgE/FcεRI-dependent activation of *in vitro* cultured human mast cells.

(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).

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).

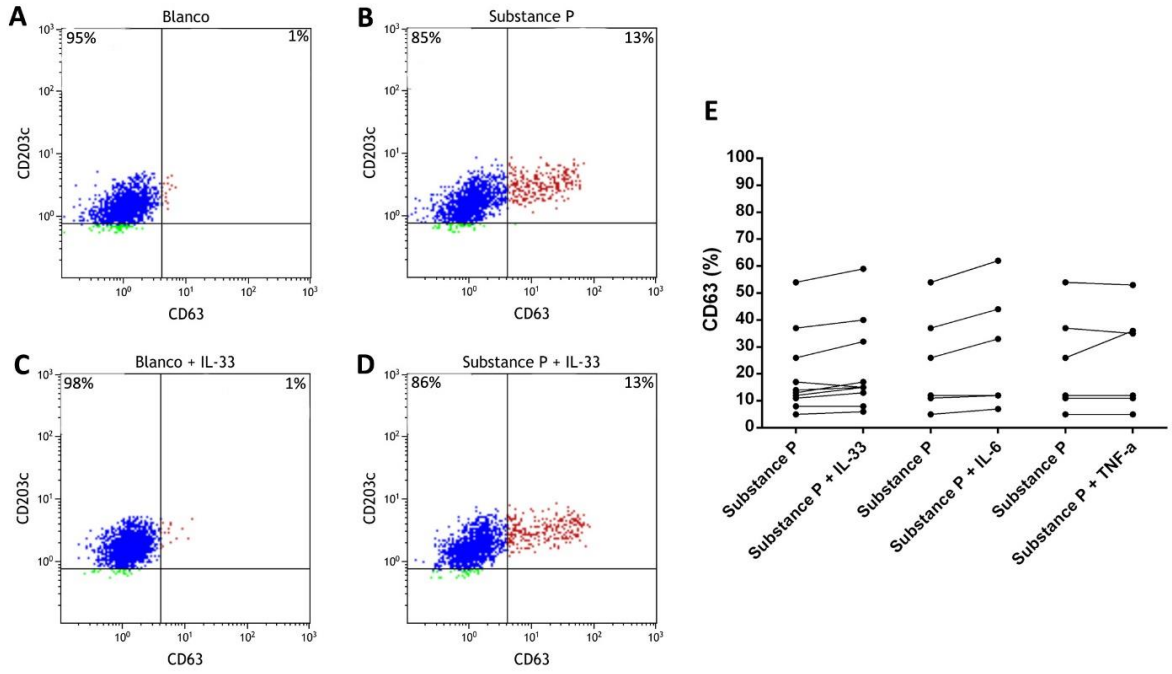

Figure 4. Effect of pre-incubation with pro-inflammatory cytokines on IgE-independent activation with substance P of *in vitro* cultured human mast cells.

(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 (n = 10), IL-6 (n = 6) or TNF-α (n = 6) 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.

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 IgE/DNP-human serum albumin (a commercially available antibody targeting dinitrophenyl (DNP) used for experimental induction of controlled allergic reactions). 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.

Acknowledgements

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Chapter 4: Flow cytometric analysis of drug-induced basophil histamine release

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Abstract

Background. Histamine and its release can be studied by multicolor flow cytometry on a single cell level by an enzyme affinity method (HistaFlow®). However, for the time-being, the clinical and scientific application of the HistaFlow® technique remains limited.

Objectives. This study aims at verifying the reliability of the HistaFlow® as an instrument to quantify IgE-mediated basophil responses to drugs, *i.e.* rocuronium, which are believed to be less potent basophil activators than large proteinaceous allergens.

Methods. Ten patients and three exposed control individuals were included in this study. Each subject underwent *in vitro* basophil activation tests (HistaFlow®) with 0.016 and 0.16 mmol/L rocuronium.

Results. Patients showed an activation of basophils ranging from 11 to 86% of CD63 positive basophils and a median histamine release per cell from 68 to 100% after stimulation with an optimal concentration of 0.16 mmol/L rocuronium. For the control individuals no activation was demonstrable.

Conclusions. This study confirms that the HistaFlow® technique is a reliable tool to study histamine release by individual cells in response to drugs. Although the HistaFlow® technique will probably not add to the diagnostic management of rocuronium allergy, our findings suggest that the technique could constitute an important asset for future studies on the pathomechanism(s) of immediate drug hypersensitivity reactions.

Introduction

Upon encountering allergens that cross-link FcεRI-bound specific IgE (sIgE), basophils release different mediators, such as histamine and leukotrienes. Traditionally, release of these mediators is quantified using assays measuring their extracellular content in the supernatant of all degranulated cells (1,2). Recently, we provided the proof-of-concept that histamine and its release can also be studied by multicolor flow cytometry on a single cell level by an enzyme affinity method (HistaFlow®) based on the affinity of the histaminase diamine oxidase (DAO) for its substrate histamine (3). However, for the time-being, the clinical and scientific application of the HistaFlow® technique remains limited.

This study aims at verifying the reliability of the HistaFlow® as an instrument to quantify IgE-mediated basophil responses to drugs, which are believed to be less potent basophil activators than large proteinaceous allergens. Rocuronium allergy, a rare but life threatening condition, was chosen as a model, mainly because IgE-mediated allergy to this neuromuscular blocking agent (NMBA) can robustly be established using a combination of skin testing, quantification of sIgE and traditional basophil activation tests (4).

Materials and methods

Patients and control individuals

Ten patients (5 female, median age 59 years (31-62) and 5 male, median age 51 years (38-63)) were selected and evaluated as detailed elsewhere (5). Briefly, patients had presented hypotension and/or bronchospasm within 5 min after injection of rocuronium and clinical suspicion of rocuronium hypersensitivity was documented by a positive skin test (ST) and basophil activation test (BAT). Three uneventfully rocuronium-exposed control individuals (1 female, age 57 years and 2 male, median age 39 years (12-66)) with a negative ST were also included. Participants gave a written informed consent as approved by the Ethical Committee of the University Hospital Antwerp (Belgium B300201316408).

In Vitro Activation of Basophils

Analysis of *in vitro* basophil activation was performed as described by Ebo *et al*. (3). Briefly, 200 µL endotoxin-free heparinized whole blood were challenged at 37°C for 20 min with 200 µL buffer as a negative control, 200 µL anti-IgE (Pharmingen, BD Bioscience, Erembodegem, Belgium) as a positive control, and 200 µL of an end concentration of 0.016 mmol/L and 0.16 mmol/L rocuronium bromide (Esmeron®, N.V. Organon, The Netherlands). Stimulation with an end concentration of 0.16 mmol/L has been proven to be the optimal concentration in previous research (5). Reactions were stopped by chilling on ice, adding 1 mL ice-cooled PBS with 10 mmol/L EDTA and spinning for 5 min (4°C, 200g). To select and quantify basophil activation, cells were stained with 20 µL of monoclonal anti-human IgE (clone GE-1, Sigma Aldrich GmbH, Steinheim, Germany) labeled with Alexa Fluor 405 (Molecular Probes, Invitrogen, Paisley, UK), 10 µL of monoclonal anti-human CD63-FITC (clone H5C6, BD Biosciences, Erembodegem, Belgium) and 10 µL CD203c-APC (clone NP4D6, Biolegend, San Diego, CA, USA) for 20 min on ice. Cells were lysed/fixed with 2 mL Phosflow Lyse/Fix buffer (BD Biosciences, Erembodegem, Belgium) for 20 min (37°C). Cells were washed with and resuspended in PBS with 0.1% Triton-X-100 (PBS-TX, pH=7.4). To stain intracellular histamine 10 µL PE-labeled DAO (BD Biosciences, Erembodegem, Belgium) was added and incubated at 37°C (45 min). Cells were washed and re-suspended 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 (violet - 405 nm, blue – 488 nm and red - 635 nm) to detect 8 colors including fluorochromes used as described by Ebo *et al*. (3). Correct compensation settings for these fluorochromes were performed using BD CompBeads (BD Biosciences, Erembodegem, Belgium). Fluorescence minus one (FMO) and DAO staining with and without permeabilization was used to set a marker between DAO positive and negative cells. Flow cytometric characterization of basophils relied upon a combination of side scatter (SCC), anti-IgE and CD203c. Standardization of intracellular histamine content was performed using standardized fluorospheres (SPHERO Ultra Rainbow Calibration particles, Spherotech, Lake Forest, IL) as described by the manufacturer. Results were expressed as % CD63 positive basophils and as the % median histamine release per basophil (%MHC). MHC percentage was calculated as the ratio of the difference between median fluorescence intensity (MFI)/cell in non-degranulating ($CD203c^{dim}$ and $CD63^{-}$) basophils minus the MFI/cell in degranulating ($CD203c^{hi+}$ and $CD63^{+}$) basophils, against the MFI per cell in non-degranulating ($CD203c^{dim}$ and $CD63^{-}$) basophils multiplied by 100 (%MHC = [($CD203c^{dim}CD63^{-}$ – $CD203c^{hi+}CD63^{+}$)/ $CD203c^{dim}CD63^{-}$] x 100).

Statistical analysis

Results were expressed as median and range. The Mann-Whitney *U*-test was used to state significant differences between the controls and patients. Differences were considered significant at a *P* value less than 0.05.

Results

When stimulated with rocuronium 0.16 mmol/L, basophils of the patients showed an up-regulation of CD63 ranging from 11 to 86% (Figure 1A) and median histamine release per cell (MHC) varying between 68 to 100% MHC (Figure 1B). In contrast, in rocuronium-exposed control individuals, no rocuronium-induced CD63 up-regulation nor histamine release was demonstrable (Figure 1). Figure 2 shows a representative HistaFlow® plot of a patient with an allergy to rocuronium (Figure 2).

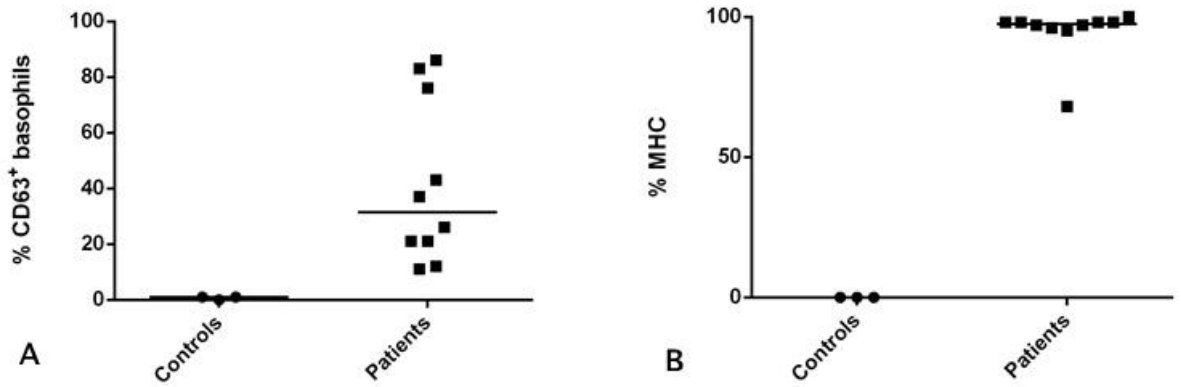

Figure 1: Basophil activation test and median histamine release per cell expressed as % MHC results after stimulation with 0.16 mmol/L rocuronium.

(A) Basophil activation test. Circles: Rocuronium-induced CD63 expression in control individuals with a negative ST after exposure to rocuronium. Squares: Patients allergic to rocuronium confirmed by a positive ST and BAT. The line represents the median.

(B) %MHC: Circles: Rocuronium-induced % median histamine release per cell (MHC) in control individuals with a negative ST after exposure to rocuronium. Squares: Responsive patients allergic to rocuronium confirmed by a positive ST and BAT. The line represents the median.

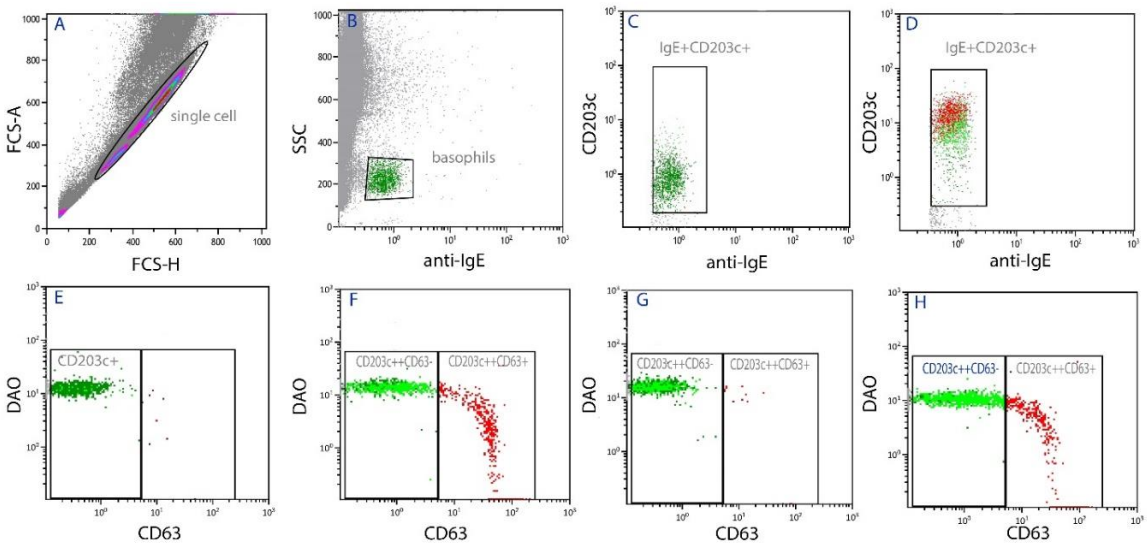

Figure 2: Representative sample of histamine release after IgE/FcεRI cross-linking and activation by rocuronium.

(A) Selection of unique cells based on forward scatter (FCS) area and height plot. **(B)** Basophils are gated out as IgE high positive cells and **(C)** $CD203c^{+}$ cells. **(D)** Activation with anti-IgE as a positive control led to an up regulation of CD203c. **(E)** Histamine (DAO) and CD63 expression upon stimulation with buffer as a negative control. **(F)** Activation of basophils with anti-IgE resulted in histamine release by 73% of basophils (DAO- cells). **(G)** Upon stimulation with 0.016 mmol/L rocuronium no histamine release was noticeable in this sample. **(H)** Stimulation with 0.16 mmol/L rocuronium led to a 52% release of the basophils' histamine content.

Discussion

Although for many the reference assay for effector cell activation remains the basophilic histamine release tests (6), these techniques have never entered mainstream diagnostic application and have now been largely supplanted by the basophil activation test (BAT). Traditional BAT relies upon flow cytometric quantification of alterations of specific activation or degranulation markers on the surface membrane of the cell (7). Moreover, it has been demonstrated that flow cytometry enables to combine analysis of surface markers with a simultaneous study of intracellular signaling molecules such as p38 MAPK (mitogen-activated protein kinase) (8) and STAT 5 (signal transducer and activator of transcription) (9) and, most interestingly, quantification of intracellular histamine and its release by *in vitro* activated basophils (3). However, for the time being, the technique of flow cytometric quantification of intracellular histamine content and histamine release, which is called HistaFlow®, is still in its infancy and literature is restricted to a proof-of-concept in birch pollen allergy (3) and a follow-up study in wasp venom immunotherapy (10). With respect to drugs, which are generally considered less potent basophil activators than larger proteinaceous allergens, the technique has only been applied in 3 cases who suffered from an immediate reaction to the opiate antitussive pholcodine (11) and one patient with a cephazolin allergy (12).

The current study confirms that the HistaFlow® technique also enables to demonstrate histamine release by small chemicals that generally elicit relative little basophil activation such as drugs. Since this semi-quantitative study does not aim at calculating the real intracellular histamine content, but rather at describing the mechanism of immediate drug hypersensitivity, the chosen wide spectrum calibration method seems sufficient. Although the HistaFlow® technique will probably not add to the diagnostic management of rocuronium allergy, our findings suggest that the technique could constitute an important research asset for future studies about immediate drug hypersensitivity reactions (IDHR) (13). First, HistaFlow® experiments closely mirror the *in vivo* pathway leading to symptoms of IDHR. Second, the technique captures data that are inaccessible for traditional mediator release assays requiring homogeneous cell populations and of which results merely represent an average of isolated cells analyzed. Third, as already exemplified, HistaFlow® allows an integrated analysis of mediator release, intracellular signaling and alterations of surface activation markers and inhibitory receptors such as CD300a (inhibitory receptor of 60 kDa). In fact, it is anticipated that by extending the experiments to intracellular signaling (8,14) and inhibitory receptors (15) the HistaFlow® technique might unveil novel insights into drug-specific basophil degranulation patterns. One particularly interesting area we believe to be perfectly manageable for experimental examination by the HistaFlow® technique relates to the pathomechanism(s) of IDHR. Because effector cell

degranulation does not per se require IgE/FcεRI cross-linking, the technique might also be valuable to study IgE-independent IDHR resulting from alternative means of cell activation (*e.g.* anaphylatoxins such as C5a and C3a, pathogen-associated molecular patterns (PAMPs) or direct mast cell activation). For example, the technique could contribute to resolve the recent controversy about the basophil activation potential of quinolones such as moxifloxacin (16).

In conclusion, this study confirms flow cytometry to constitute a reliable tool to study histamine release by individual cells in response to drugs in both an IgE-dependent and IgE-independent basophil activation and subsequent degranulation. Furthermore, it is anticipated that the technique might disclose fundamental insights into distinct drug-induced basophil degranulation patterns.

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Chapter 5: Drug-induced degranulation of in vitro cultured human mast cells: functional analysis at a single cell level

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Abstract

Background. It has been proposed that the G protein-coupled receptor MRGPRX2 might be involved in IgE-independent immediate hypersensitivity towards various drugs. However, data in human mast cells are restricted to experiments with a LAD2 cell line. Here we aim at further investigating this presumption by using flow cytometric analysis of drug-induced degranulation of mast cells cultured from peripheral blood progenitors.

Methods. MCs were cultured out of $CD34^+$ progenitors isolated from 50 mL whole blood from healthy volunteers. MCs were stimulated with substance P, the tetrahydroisoquinoline (THIQ)-motif containing drug moxifloxacin, the curarizing neuromuscular blocking agent (NMBA) rocuronium and the opiate morphine. Immunophenotyping of the cells comprised flow cytometric analysis of expression of CD117, CD203c and MRGPRX2. Degranulation of individual cells, *i.e.* appearance of the lysosome associated molecular protein 3 (LAMP3 or CD63), was analysed flow cytometrically.

Results. Mature MCs, defined as $CD117^+/CD203c^+$ cells, expressed 51% (15 – 83) MRGPRX2 on their cell membrane. Activation of the cells with substance P, moxifloxacin and morphine resulted in significant degranulation, *i.e.* surface appearance of CD63. Importantly, only the MCs expressing MRGPRX2 could be activated by these compounds. In contrast, stimulation with rocuronium did not result in degranulation.

Conclusions. We add evidence that our MCs culture generates functional mature MCs that behave as connective tissue mast cells, as they constitutively express the G-coupled receptor MRGPRX2 that is probably involved in degranulation of the cells in response to substance P, morphine and moxifloxacin.

Introduction

The activating and inhibitory mechanisms that finally culminate in drug-induced degranulation of mast cells (MCs) remain elusive but clearly extend beyond traditional IgE/FcεRI cross-linking. Recently, it has been shown that one of the pathways drugs use to trigger MCs independently from traditional IgE/FcεRI cross-linking probably relates to activation of the G-protein coupled receptor MRGPRX2 (1–5). This MRGPRX2-receptor has been reported to be highly expressed on MC_{TC}, while only little expression has been measured on MC_T (1,4,5). However, supportive data that immediate drug hypersensitivity reactions (IDHR) might relate to ligation of MRGPRX2 have mainly been gathered in mouse models or human LAD2 cells, a mast cell line obtained from a mastocytosis patient (1,3,6). As a matter of fact, these studies have suggested that basic peptides and several drugs such as fluoroquinolones, neuromuscular blocking agents or opiates can induce calcium mobilization and release of β-hexosaminidase by activation of LAD2 cells through the MRGPRX2-receptor (1,6). In contrast, to the best of our knowledge, for the time being no data on individual cultured or isolated MCs are available.

Along with others, we have recently shown that functionally active mast cells can effectively be cultured from $CD34^+$ progenitors obtained from peripheral blood (7–10). These peripheral blood cultured mast cells, designated as PBCMC, display a connective tissue phenotype (MC_{TC}) (11). Here, we aimed at assessing whether these PBCMC constitutively express MRGPRX2 and whether these cells could be activated through ligation of this receptor by substance P and drugs as recently suggested (1,6). For this purpose we took advantage of a flow cytometric technique enabling to study degranulation at the single cell level, and therefore reveal particularities that are inaccessible with traditional mediator release tests in which results merely reflect the sum of all stimulated cells.

Materials and methods

In vitro culture of human PBCMC

Human PBCMC were cultured as described elsewhere (11). Briefly, peripheral blood mononuclear cells were isolated from 50 mL whole blood and $CD34^+$ 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).

Antibodies

Cultured human MCs were stained for surface makers with monoclonal anti-human CD117-allophycocyanin (CD117-APC) (clone 104D2, BD Biosciences, Erembodegem, Belgium), CD203c-phycoerythrin cyanine 7 (CD203c-PECy7) (clone NP4D6, eBioscience, San Diego, USA), MRGX2-phycoerythrin (MRGX2-PE) (clone K125H4, BioLegend, San Diego, USA) and CD63-fluorescein isothiocyanate (CD63-FITC) (clone H5C6, BD Biosciences).

Activation of the cells

Cultured mature PBCMC, defined as cells expressing CD117 and CD203c, were incubated with pre-warmed (37°C) Tyrode's buffer (Sigma-Aldrich, St. Louis, USA) at a concentration of 0.5 x 10^6 cells/mL. Preliminary dose-response experiments were performed to determine the optimal concentrations for drug-induced activation of MCs. Next, 100 µL of the cells were stimulated with 100 µL Tyrode's buffer as a negative control, 100 µL of an end concentration of 0.074 mmol/L substance P (Sigma Aldrich) as a positive control (n = 10), 100 µL of an optimal end concentration of 0.57 mmol/L moxifloxacin hydrochloride (Sigma Aldrich) (n = 7), 100 µL of an optimal end concentration of 0.16 mmol/L rocuronium bromide (Esmeron®, N.V. Organon, The Netherlands) (n = 5) and 100 µL of an optimal end concentration of 0.5 mmol/L morphine hydrochloride (n = 5) during 3, 5 and 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 CD117-APC, CD203c-PECy7, MRGX2-PE and degranulation marker CD63-FITC for 20 minutes at 4°C. Next, cells were fixed with 1 mL Phosflow Lyse/Fix buffer (BD Biosciences, Erembodegem, Belgium) for 20 min. Finally, cells were washed and resuspended in PBS with 0.1% sodium azide and measured. Appearance of CD63 indicates anaphylactic degranulation of mast cells (12,13).

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). A fluorescence minus one (FMO) sample was used to set a marker between positive and negative cells according to the 99^{th} percentile. The percentages refer to the number of cells positive for each parameter. Based on the precision of our technique, the threshold for positivity was set at the appearance of 5% CD63 on the cell surface. At least 2000 viable cells were counted per sample. 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 MRGPRX2 density.

Statistical analysis

GraphPad Prism version 6 software was used for data analysis, Fisher's Exact Chi-Square test and Spearman's correlation test were performed. Results are expressed as median and range. A P-value of < 0.05 was considered significant.

Results

PBCMC were phenotypically and functionally evaluated on the single cell level. A representative sample for immunophenotyping is shown in figure 1. Mature human PBCMC can be defined as viable cells expressing both CD117 and CD203c (Figure 1A-D). Moreover, these non-stimulated mature PBCMC harbor 2 different subpopulations, *viz.* a population positive for the MRGPRX2-receptor and another population negative for the MRGPRX2-receptor on their cell membrane (Figure 1E-F). As illustrated in figure 2, mature PBCMC from each cell culture constitutively express the MRGPRX2-receptor on their cell membrane. However, this expression is highly variable from culture to culture with a median and range of 51% (15 – 83) number of cells positive for MRGPRX2 and a density of 410 MESF-PE/cell (292 – 482) for MRGPRX2 expression.

Preliminary dose-response experiments were performed to determine optimal concentrations for activation. A representative sample for functional analysis is shown in figure 3. There was no appearance of degranulation marker CD63 by resting cells (Figure 3A). PBCMC were stimulated with neuropeptide substance P (Figure 3B), THIQ-motif containing drug moxifloxacin (Figure 3C), neuromuscular blocking agent rocuronium (Figure 3D) and natural opiate morphine (Figure 3E). As demonstrated in figure 2 and 3, the number of MRGPRX2-positive cells remained unaltered throughout stimulation experiments. PBCMC could be activated with substance P (Figure 2A, 3B), moxifloxacin (Figure 2B, 3C) and morphine (Figure 2D, 3E), resulting in significant anaphylactic degranulation by appearance of CD63 in virtually all conditions (Chi-Square: $P < 0.01$). In fact, maximal activation of the cells was already attained after 3 minutes of incubation with the stimuli and remained on a plateau for 20 minutes of incubation. Moreover, the appearance of CD63 appeared to be limited to the MRGPRX2-positive cells (Figure 3) and a higher number of cells positive for MRGPRX2 correlated with a higher number of cells positive for CD63 (Figure 4). In contrast with the above, stimulation with rocuronium did not trigger degranulation of the cells (Figure 2C, 3D).

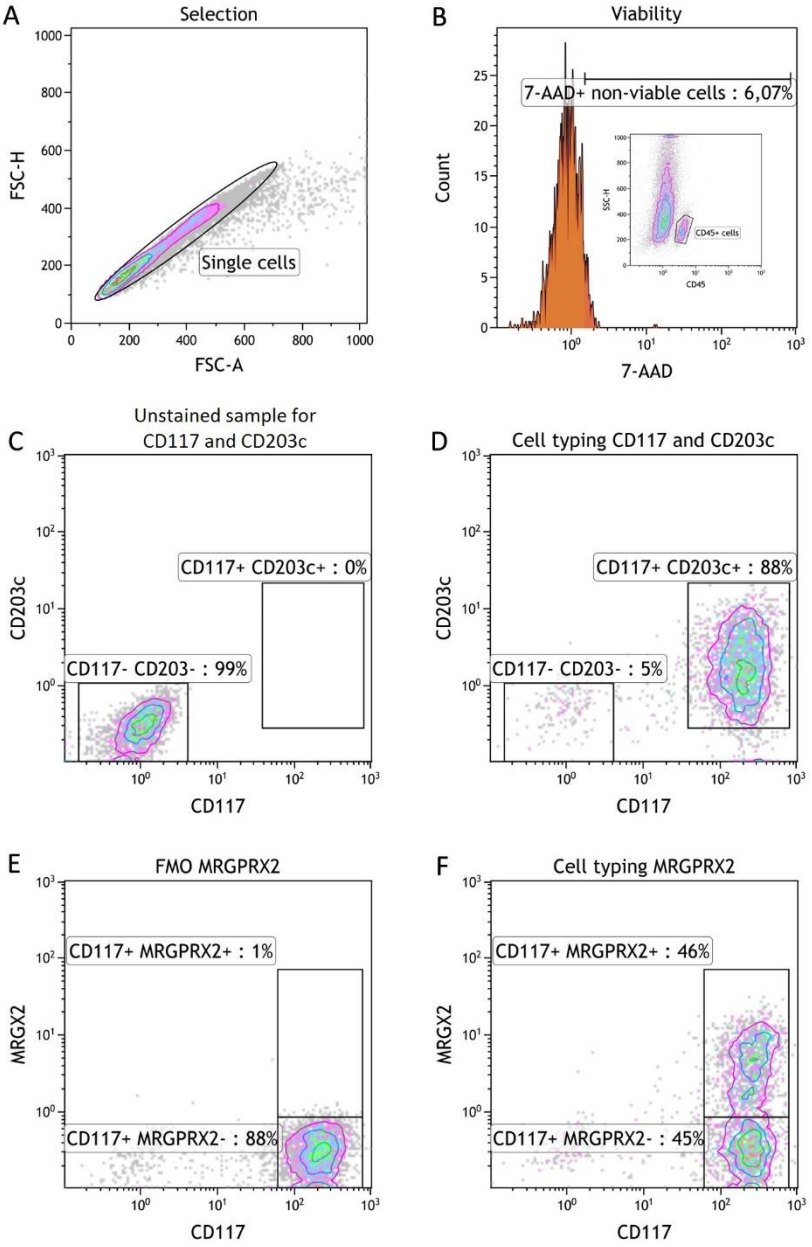

Figure 1. Immunophenotyping of cultured mast cells.

(A-B) Selection of single cells for analysis based on FSC-H (forward scatter-Height) and FSC-A (forward scatter-Area). All CD45 positive cells were evaluated for viability using 7-AAD staining. Viable $CD45^{+}$ cells were gated for further analysis. **(C)** Unstained sample for lineage markers CD117 and CD203c. **(D)** Mature mast cells are defined as cells expressing both CD117 and CD203c on their cell membrane. **(E)** Fluorescence minus one (FMO) sample for the G protein-coupled receptor, MRGPRX2. **(F)** Mature mast cells can be divided into cells positive for MRGPRX2 and cells negative for MRGPRX2 on their cell membrane. The percentages refer to the number of cells positive for each marker.

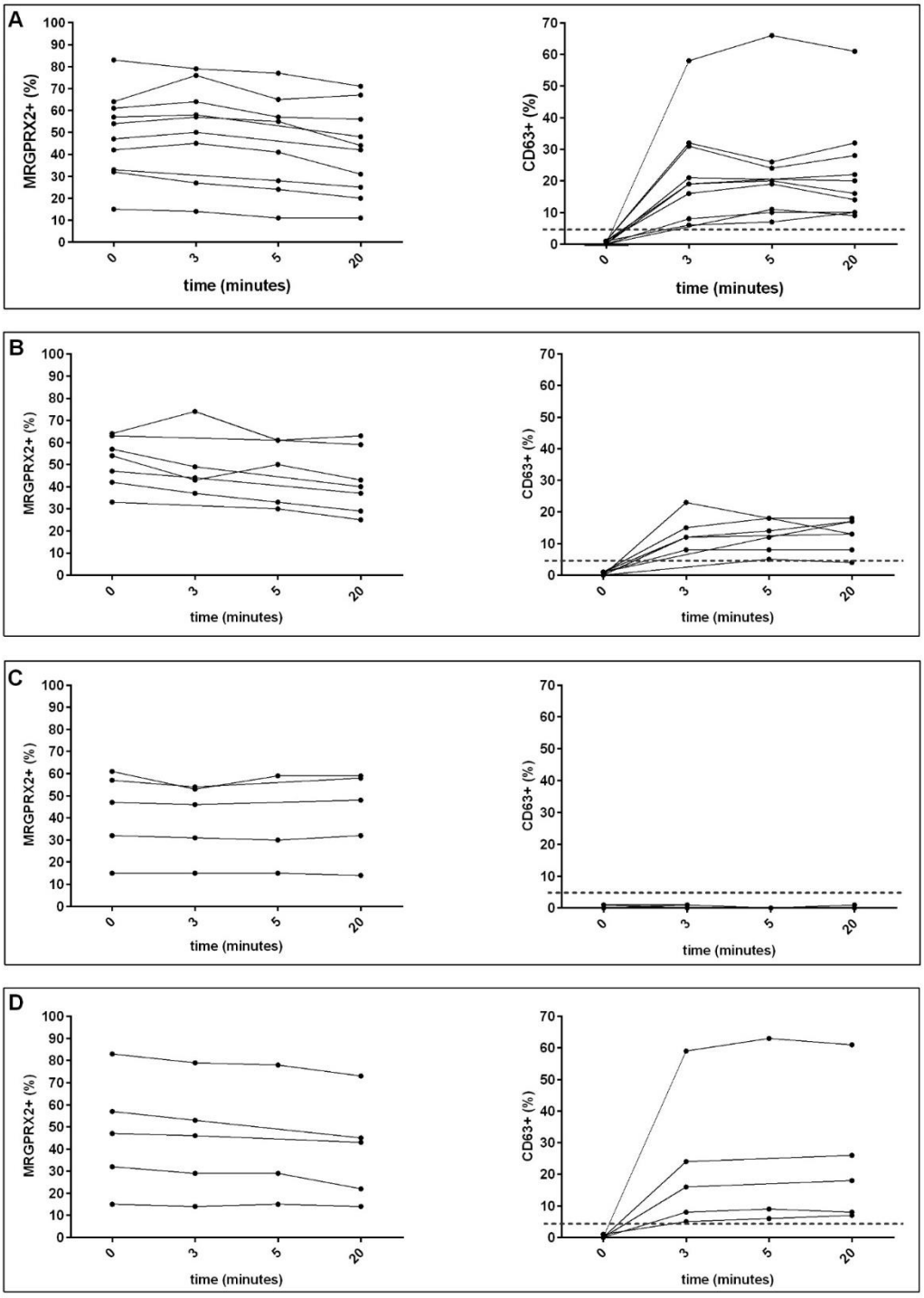

Figure 2. Time-curve of IgE-independent activation of *in vitro* cultured human mast cells by optimal concentrations of substance P (A), moxifloxacin (B), rocuronium (C) and morphine (D).

(A) Stimulation with substance P (n = 10). **(B)** Stimulation with moxifloxacin (n = 7). **(C)** Stimulation with rocuronium (n = 5). **(D)** Stimulation with morphine (n = 5). Total (basal) expression of MRGPRX2 on the cell membrane is demonstrated in the graphs on the left. Anaphylactic degranulation, *i.e.* appearance of CD63, is represented in the graphs on the right. The connecting lines represent values from the same human mast cell culture. The dotted line represents the threshold for degranulation.

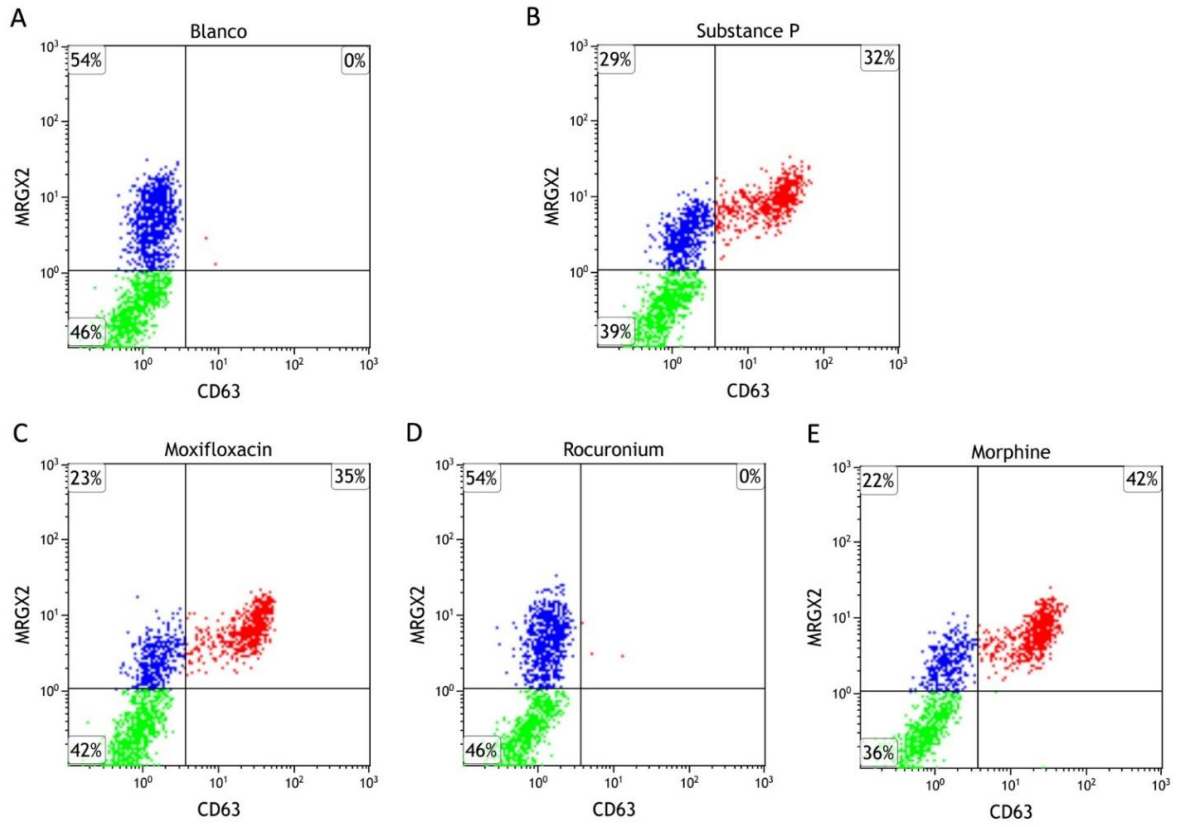

Figure 3. Illustration of IgE-independent activation of *in vitro* cultured human mast cells.

(A) Non-stimulated cells. **(B)** Mast cells stimulated with the positive control substance P. **(C)** Mast cells stimulated with the THIQ-motif containing drug moxifloxacin. **(D)** Mast cells stimulated with the neuromuscular blocking agent rocuronium. **(E)** Mast cells stimulated with the opiate morphine. The percentages refer to the net number of cells positive for each marker. Mast cells were stimulated with each stimulus during 3 minutes. Anaphylactic degranulation is indicated as the appearance of CD63. The percentage of MRGPRX2-positive cells remained similar during all stimulation conditions and seemingly the cells negative for MRGPRX2 did not up-regulate CD63 after activation.

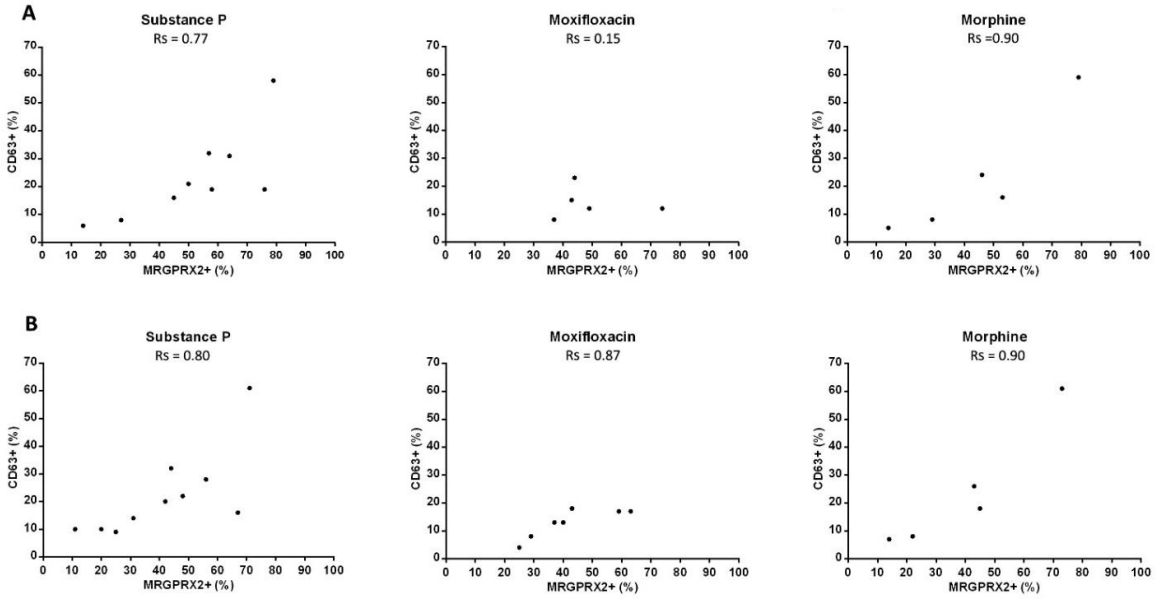

Figure 4. Correlation between the number of mast cells positive for MRGPRX2 and appearance of CD63 after stimulation with substance P, moxifloxacin and morphine.

(A) Incubation for 3 minutes. **(B)** Incubation for 20 minutes. Total number of MCs positive for MRGPRX2 on the cell membrane is represented in the x-axis. Anaphylactic degranulation, *i.e.* appearance of CD63, is represented in the y-axis. Spearman's rank correlation coefficients (Rs) were calculated using Spearman Correlation tests.

Discussion

The activating and inhibitory mechanisms that govern drug-induced activation and degranulation of human mast cells remain poorly understood, but definitely extend beyond classical IgE/FcεRI cross-linking. Recently, it has been demonstrated that an alternative explanation for immediate drug hypersensitivity implies activation of mast cells through engagement of the MRGPRX2-receptor (1,6). As already exemplified in the introduction the MRGPRX2-receptor, which belongs to the G protein-coupled receptor family, is constitutively expressed on connective tissue mast cells (MC_{TC}) but not on mucosal mast cells (MC_T). Here we demonstrate that mature PBCMC, cultured as described in the methodology section, can also constitutively express the MRGPRX2-receptor but that this expression is not homogeneous and is highly variable from culture to culture. In any way, the observation that our PBCMC express MRGPRX2 is in line with our former findings showing the cells to display a connective tissue phenotype (11).

In addition, our data add evidence on the single cell level to the recently published studies of McNeil *et al*. (1) and Lansu *et al*. (6) who have identified the MRGPRX2-receptor to be potentially involved in IgE-independent immediate hypersensitivity reactions towards various drugs containing a tetrahydroisoquinoline (THIQ)-motif, such as the fluoroquinolone moxifloxacin, and various opiates and (semi)synthetic opioids, such as morphine, by studying calcium mobilization and β-hexosaminidase release in the supernatants of murine-derived or LAD2-derived mast cells. In our study, we demonstrate that PBCMC can be activated in an MRGPRX2-dependent manner by stimulation with substance P, moxifloxacin and morphine resulting in rapid degranulation that starts within 3 minutes of incubation with the stimuli. These findings are not only consistent with Gaudenzio *et al*. (3) who demonstrated a fast release of individual granules after only 5 minutes of stimulation with substance P, but also with our own clinical observations of hypersensitivity in response to moxifloxacin in naïve patients, which were extremely rapid in onset (14). Furthermore, the genetic polymorphism of the human MRGPRX2-receptor might not only explain susceptibility for moxifloxacin hypersensitivity, but also the variations in clinical phenotype (15). Lastly, our data are in accordance with several research groups who have demonstrated activation of connective tissue mast cells by morphine (16–19) and who have proven that this activation implies ligation of the G-protein coupled receptor MRGPRX2 (6,20).

Alternatively, unlike McNeil (1), no activation of our PBCMC was demonstrable for rocuronium, an aminosteroid curarizing neuromuscular blocking agent that does not contain a THIQ-motif. The exact reason for this divergence remains elusive but could relate to fine functional differences between the human MRGPRX2 and its orthologue in mice (6,21). This hypothesis is further supported by Lansu *et al*. (6) who also failed to demonstrate activation of human LAD2 cells after stimulation with rocuronium. Anyhow, based upon these data caution has to be called upon a too generalized hypothesis that all curarizing neuromuscular blocking agents trigger human mast cell activation via MRGPRX2 (1,22).

Taken together, these experiments add further evidence that the applied culture conditions can generate mature human PBCMC that phenotypically and functionally resemble connective tissue mast cells (11,23,24). As a matter of fact, along with the expression of traditional surface markers such as CD117, CD203c and FcεRI they also express MRGPRX2, contain chymase, tryptase and histamine and are responsive to substance P, opiates and THIQ-motifs. Moreover, our discoveries might help to introduce a novel model to further unveil the activating mechanisms that govern mast cells activation and shift paradigms in immediate drug hypersensitivity reactions independent from IgE/FcεRI cross-linking.

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Chapter 6: Critical reflections and perspectives

The development of a human mast cell culture that generates functional mature and immature human connective tissue mast cells and the identification of priming cytokines creates multiple opportunities to close the fundamental gaps in our knowledge about the mechanisms governing both IgE- and non-IgE mediated activation of mast cells. Besides further unveiling the complex mechanisms that finally culminate in degranulation, it seems theoretically justified to anticipate this realisation to introduce a human model enabling to study the effect of various secretagogues and to identify the off-target potential of drugs and compounds to trigger mast cell-related adverse events. Moreover, closing the gap in our knowledge about the activating and inhibitory mechanisms of human mast cells, could shift our paradigms about the underlying mechanisms of these side effects and our therapeutic approach.

In this context, our human mast cell culture displays many advantages over existing methods described in literature. To start with, many current methods require at least 6 to 12 weeks to produce mature human mast cells and often use several pro-inflammatory cytokines, such as IL-6 or IL-9, to assure maturation of their cells. No need to stress that such culture conditions are extremely laborious, time consuming and costly. Furthermore, depending on the applied conditions these cultures generate variable phenotypes, which makes it challenging to compare data between investigations. Our cell culture already generates mature chymase and tryptase containing mast cells after 3 weeks of culture and only uses SCF and IL-3 to promote cell growth and differentiation, thus making our method less time-consuming and less influenced by pro-inflammatory cytokines during the maturation process.

Furthermore, the majority of studies uses buffy coats or stem cell concentrates discarded from the blood bank as a source for progenitor cells. Consequently, the outcome and quality of mast cells might be affected due to a few reasons: 1) buffy coats are only released from the blood bank after 24 hours, which could imply that the quality and number of progenitor cells might already be decreasing; 2) there is little information available on the donors, preventing them to include well stratified groups for their studies. To avoid these disadvantages, we demonstrate in this thesis that mature MCs can be cultured from very small volumes of peripheral blood, allowing to use fresh progenitor cells and to include well characterized patients and/or control individuals in our studies.

Last but not least, the majority of investigations assesses mast cell degranulation by use of traditional mediator release tests. These traditional assays measure the release of mediators (*e.g.* histamine, β-hexosaminidase, *etc.*) in the supernatant, thereby only measuring an average of all stimulated cells. Flow cytometry enables to evaluate mast cell activation on the single cell level, allowing to distinguish between subpopulations and to study phenotype, activation and degranulation patterns simultaneously. Thus, flow cytometry provides a valuable research tool to analyze various activating or inhibitory conditions.

Obviously our findings need to be confirmed and further exploration of human mast cell characterisation and behaviour is absolutely mandatory and could include various domains. Firstly, the overall number of cultures included for each study is limited and should ideally be enlarged to confirm our findings. Secondly, novel culture conditions could generate distinct human mast cell populations that respond differently on primers and triggers and might add to our insights in the heterogeneity of mast cells. Thirdly, it has been shown that effector cells such as basophils and mast cells can be conditioned and primed. In this thesis, it was demonstrated that short-term incubation with IL-33 and IL-6 exhibited a clear priming effect upon human mast cell activation. However, this priming effect seemed restricted to allergen-specific IgE/FcεRI-cross-linking degranulation of the cells that were passively sensitized with sera from birch pollen sensitized patients. In contrast, activation of MCs cultured from control individuals, passively sensitized with autologous sera and subsequently stimulated with anti-IgE failed to trigger mast cell activation and subsequent degranulation. We speculate that this failure to passively sensitise the cells probably results from the low total IgE titres in the sera from healthy control individuals, since these cells could be activated with allogenic sera containing higher total IgE concentrations. It is likely that different stimulation conditions, such as a more long-term incubation of the cells with cytokines or the use of other pro- or anti-inflammatory cytokines, might enable a more reactive phenotype and thus allow the cells to respond to lower total IgE values. Possible candidates for future studies are IL-4, which is known to increase the density of FcεRI on the cell membrane, or IL-6, which is known to increase the mediator content of mast cells. Future analyses should also include inhibitory receptors, such as CD300a, CD200R and CD32b, since their inhibitory mechanisms could influence the IgE-mediated activation.

With respect to immediate drug hypersensitivity reactions (IDHR), it was demonstrated that HistaFlow® enables to asses allergic IDHR after drug-induced basophil activation with rocuronium (an aminosteroid-derived curarizing neuromuscular blocking agent). Although this technique will probably not enter mainstream application in the diagnostic management of drug allergy due to the basophil activation test, it might constitute a novel asset for future fundamental studies on the pathophysiological mechanisms of IDHR to drugs and related compounds. Parallelly, it was demonstrated that our peripheral blood derived cultured mast cells also express MRGPRX2 and can degranulate in response to substance P, morphine and moxifloxacin (containing a THIQ-motif). According to the literature it is likely this activation to result from MRGPRX2 occupation, but results await verification and confirmation using a selective MRGPRX2-antagonist. Recently, Azimi et al. (2016) suggested the use of a tripeptide neurokinin-1 receptor antagonist (QWF) to block the binding of substance P to both the MRGPRX2-receptor and the neurokinin-1 receptor (1). In anyway, our findings suggest that our human mast cell model might contribute to the exploration of IDHR reactions that result from off-target occupation of the MRGPRX2-receptor.

In conclusion, we can state that our human mast cell culture provides a valuable research tool, allowing to culture human mast cells from small amounts of peripheral blood from individual patients. Moreover, mature connective tissue mast cells can be generated after only 3 weeks of culture and their phenotype, intracellular content and function can be evaluated flow cytometrically on the single cell level. Therefore, we anticipate that our technique might help to further clarify the underlying role of mast cells in the pathogenesis, clinical outcome and targeted treatment of several auto-inflammatory, auto-immune and allergic diseases.

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Chapter 7: Summary

The mechanisms that govern human mast cell activation finally culminating in degranulation remain poorly understood and only partially deciphered. Unfortunately, exploring the activating and inhibitory mechanisms of tissue-resident mast cells is difficult, mainly because of shortage of eligible mast cell populations. Therefore, for the time-being, most of our knowledge and insights of the mechanisms governing human mast cells has been gathered from specific neoplastic mast cell lines and in a lesser extent also mast cells cultured from peripheral blood. However, in most of these studies observations and findings merely reflect an average of all stimulated cells, as degranulation was generally quantified by using traditional mediator release assays. In contrast, data on single cells remain scarce, particularly from mast cells cultured out of peripheral blood.

For that reason, in this dissertation we first optimize a technique to culture sufficient numbers of well-differentiated and functional mast cells from $CD34^+$ progenitor cells from buffy coat. Moreover, we adapt the technique to culture the cells from small amounts of peripheral blood. In **chapter 2** flow cytometric analysis of these cells reveals that our culture conditions generate two phenotypically distinct subpopulations: *viz.* $CD117^-CD203^{+low}$ and $CD117^+CD203c^{+hi}$ mast cells. Although phenotypically different, both subpopulations express the high affinity IgE-receptor (FcεRI) and contain comparable amounts of chymase, tryptase and histamine. Taken together, these data indicate our culture to generate immature $CD117^-CD203^{+low}$ and mature $CD117^+CD203c^{+hi}$ connective tissue mast cells (MC_{TC}). Furthermore, we observe that both the immature and mature subpopulation can successfully be uploaded with IgE and subsequently activated with anti-IgE, demonstrating release of histamine at a single cell level.

Moreover, in **chapter 3** we provide the proof that these cells can also be activated by specific allergen that cross-links membrane-bound IgE by autologous passive sensitization experiments in birch pollen allergic patients. Besides, we demonstrate that allergen-specific degranulation can be significantly enhanced by short-term incubation with pro-inflammatory cytokines. In this regard, pre-incubation with IL-33 has a significant priming effect with an enhanced responsiveness of pre-incubated cells towards subsequent IgE/FcεRI cross-linking by both anti-IgE and birch pollen allergen. We also show a priming effect of IL-6 on allergen-specific stimulation of the cells, but the effect is less pronounced than for IL-33. Moreover, we demonstrate a synergistic enhancing of co-incubation with IL-33 and IL-6.

Almost simultaneously, we extend functional analysis of our cells with IgE-independent activation experiments that focus on the G protein-coupled receptor MRGPRX2. Our study demonstrates that mast cell stimulation with substance P results in degranulation of the cells by significant appearance of degranulation marker CD63 on the cell surface. Unlike IgE-dependent activation of mast cells, MRGPRX2-dependent stimulation with substance P cannot be primed by pro-inflammatory cytokine exposure with IL-33, IL-6 or TNF-α.

Next in this thesis, we demonstrate that our single cell analysis enables to further disentangle the underlying mechanisms of immediate drug hypersensitivity reactions (IDHR). Because validation of the Histaflow® is mainly based upon cell activation in response to IgE/FcεRI cross-linking by large proteinaceous allergens, we verified in **chapter 4** whether the reliability and validity of the technique was sufficient to analyse IgE/FcεRI-dependent basophil responses to drugs, which are believed to be less potent basophil activators than large proteinaceous allergens. Validation is achieved by using such a drug, namely the aminosteroid-derived curarizing neuromuscular blocking agent rocuronium. Parallelly, in **chapter 5** we investigate the suggested role of MRGPRX2 in non-allergic IDHR. Hence, our mature $CD117^{+}CD203c^{+hi}$ mast cell population can be divided into two subpopulations: *viz.* MRGPRX2-positive and MRGPRX2-negative mast cells. Only the MRGPRX2-positive mast cells degranulate in response to substance P, morphine and moxifloxacin, endorsing the hypothesis that the MRGPRX2-receptor is implicated in triggering degranulation by these compounds.

To conclude, we provide a valuable research tool to study the phenotype, intracellular content and function of individual human mast cells in several auto-inflammatory, auto-immune and allergic diseases.

Samenvatting

De mechanismen die bijdragen tot de activatie en degranulatie van humane mastcellen blijven tot op vandaag onvolledig ontrafeld. Helaas is het bestuderen van deze mechanismen voor weefsel mastcellen moeilijk door een tekort aan geschikte mastcelpopulaties. Daarom is onze kennis, tot op heden, voornamelijk gebaseerd op data afkomstig van neoplastische mastcellijnen en in mindere mate uit mastcelculturen afkomstig van het perifeer bloed. Deze studies maken echter gebruik van traditionele analysemethoden waarvan het resultaat slechts een gemiddelde waarde voor degranulatie van de gehele celpopulatie weergeeft en geen informatie verschaft op het niveau van de individuele cel.

Om die reden, hebben we in deze thesis eerst een celcultuur techniek geoptimaliseerd met als doel het verkrijgen van een voldoende aantal mature en functionele mastcellen gekweekt uit $CD34^+$ voorlopercellen geïsoleerd uit *buffy coat*. Bovendien hebben we deze techniek verder geoptimaliseerd om tevens mastcellen te kunnen kweken uit kleine hoeveelheden perifeer bloed. In **hoofdstuk 2** blijkt dat onze cultuur twee fenotypisch verschillende mastcel subpopulaties genereert: namelijk $CD117^-$ $CD203c^{+laag}$ en $CD117^+CD203c^{+hoog}$ mastcellen. Ondanks hun fenotypische verschillen, brengen beide subpopulaties de hoge affiniteit IgE-receptor (FcεRI) op hun celmembraan tot expressie en bevatten ze vergelijkbare hoeveelheden chymase, tryptase en histamine in hun granulen. Samengenomen, tonen onze resultaten aan dat onze cultuur immature $CD117^-CD203c^{+laag}$ en mature $CD117^+CD203c^{+hoog}$ bindweefselmastcellen (MC_{TC}) genereert. Daarenboven kunnen zowel de immature als mature mastcellen succesvol worden opgeladen met IgE en vervolgens geactiveerd worden met anti-IgE, waarbij er vrijstelling van histamine wordt waargenomen op het niveau van de individuele cel.

Verder leveren we in **hoofdstuk 3** het bewijs dat deze cellen, na passieve sensibilisatie met autoloog serum, eveneens geactiveerd kunnen worden door een specifiek allergeen dewelke membraangebonden IgE overbrugt. Bovendien tonen we in deze studie ook aan dat deze allergeen-specifieke activatie significant versterkt kan worden door korte incubatieperiodes met pro-inflammatoire cytokines. In dit opzicht heeft pre-incubatie met IL-33 een significant *priming* effect met een verbeterde responsiviteit van de geïncubeerde mastcellen naar opvolgende IgE-gemedieerde activatie door zowel anti-IgE als berkenpollenallergeen. Daarnaast heeft IL-6 eveneens een *priming* effect op allergeen-specifieke stimulatie van de cellen, maar dit effect is minder uitgesproken dan voor IL-33. Bovendien tonen we een synergistische verbetering van IgE-gemedieerde activatie aan wanneer mastcellen geïncubeerd worden met zowel IL-33 als IL-6.

Bijna gelijktijdig hebben we onze functionele analyses uitgebreid met IgE-onafhankelijke activatie experimenten die zich toespitsten op de G-proteïne gekoppelde receptor MRGPRX2. Onze studie toont aan dat stimulatie van mastcellen met substantie P leidt tot degranulatie van de cellen met een significante verschijning van degranulatiemerker CD63 op de celmembraan. In tegenstelling tot de IgE-afhankelijke activatie van de cellen, kan de MRGPRX2-afhankelijke stimulatie met substantie P niet versterkt worden door blootstelling aan de pro-inflammatoire cytokines IL-33, IL-6 of TNF-α.

In een volgend deel van de thesis tonen we aan dat onze analyse op het niveau van de individuele cel het mogelijk maakt om de onderliggende mechanismen van onmiddellijke overgevoeligheidsreacties ten opzichte van geneesmiddelen verder te ontrafelen. Omdat de validatie van de *HistaFlow®* techniek hoofdzakelijk gebaseerd is op IgE-gemedieerde activatie door grote eiwitallergenen, wordt in **hoofdstuk 4** gecontroleerd of de techniek eveneens betrouwbaar is om IgE-gemedieerde geneesmiddelenallergie door basofielen te meten, aangezien geneesmiddelen veel minder krachtige basofielactivatoren zijn dan grote eiwitallergenen. Ter validering van de techniek wordt een dergelijk geneesmiddel aangewend, het betref hier het aminosteroïde afgeleide curariserende neuromusculaire blokkerende middel rocuronium. Parallel onderzoeken we in **hoofdstuk 5** de rol van MRGPRX2 in niet-allergische onmiddellijke overgevoeligheidsreacties veroorzaakt door geneesmiddelen. Hieruit blijkt dat we onze mature $CD117^{+}CD203c^{+hoog}$ mastcelpopulatie kunnen onderverdelen in twee subpopulaties: namelijk de MRGPRX2-positieve en MRGPRX2-negatieve mastcellen. Enkel de MRGPRX2-positieve mastcellen degranuleren na stimulatie met substantie P, morfine en moxifloxacine, wat de hypothese ondersteunt dat de MRGPRX2-receptor een rol speelt in activatie door deze stoffen.

Om samen te vatten, kunnen we besluiten dat onze onderzoeksgroep er in is geslaagd om een waardevol onderzoeksinstrument te ontwikkelen, dat zowel het fenotype, de intracellulaire inhoud als de functie van individuele humane mastcellen kan beoordelen en zodoende kan helpen om de rol van mastcellen in verschillende auto-inflammatoire, auto-immune en allergische ziekten te bestuderen.

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Curriculum vitae

Curriculum vitae

Nathalie Cop werd geboren op 24 februari 1989 te Wilrijk.

Na het behalen van haar ASO diploma Latijn-Wetenschappen, aan het Sint-Ursula Instituut te Wilrijk in 2007; studeerde zij biomedische wetenschappen aan de Universiteit van Antwerpen.

Het diploma van Master in de moleculaire en cellulaire biomedische wetenschappen met als masterthesis 'Ontwikkeling van fysiologische analysemethoden in het zebravisembryo voor gebruik in een classificatiesysteem van teratogene stoffen' werd in 2013 met onderscheiding behaald. Gedurende haar opleiding behaalde zij eveneens het certificaat voor proefdierkunde (FELASA C).

Vervolgens startte zij als doctoraatsbursaal op het laboratorium Immunologie – Allergologie – Reumatologie in het kader van een GOA consortium (2013 – 2017) van de Faculteit Geneeskunde en Gezondheidswetenschappen aan de Universiteit van Antwerpen. Verder begeleidde zij studenten geneeskunde en biomedische wetenschappen tijdens hun onderzoekswerk in het kader van hun bachelorscriptie of masterthesis.

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