



Histamine receptor H4 regulates mast cell degranulation and IgE induced FcεRI upregulation in murine bone marrow-derived mast cells



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ABSTRACT

There is increasing evidence that histamine regulates the immune system via histamine H4 receptors, therefore we sought to investigate the functions of the H4 receptor on mast cells. Mast cells were differentiated from murine bone marrow stem cells, and the expression of mast cell surface markers FcεRI and CD117 were measured using flow cytometry. Real-time qRT-PCR was used to determine the expression of mH4R; as a measure of antigen-dependent degranulation, β-hexosaminidase release assay was carried out using IgE sensitized mast cells. We determined that the expression kinetics of FcεRI and mH4R can be described with a function that has one maximum value in the time range of the culture's differentiation. Antigen-dependent degranulation of murine bone marrow-derived mast cells could be inhibited by a selective H4 antagonist/inverse agonist only when it was present during the IgE sensitization phase of degranulation. In addition, flow cytometric analysis revealed that the H4 antagonist/inverse agonist also inhibited IgE induced FcεRI upregulation. The inhibition percentage of H4 antagonist on IgE induced FcεRI upregulation was determined to be dependent upon the maturity of the mast cell cultures, and this time-dependency was consistent with the expression kinetics of both mH4R and FcεRI. These results imply that H4R has regulatory roles in FcεRI expression and FcεRI mediated functions in mast cells. In conclusion the present study shows that H4 receptors potentially play a role in IgE induced FcεRI upregulation and in the sensitization phase but not the effector phase of mast cell degranulation.

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1. Introduction

Histamine is an essential messenger molecule that plays important roles in different physiological systems and also in pathophysiological conditions such as immuno-inflammatory diseases. The diverse biological effects of histamine are mediated via four G protein-coupled receptors, each mediating distinct effects. H1 receptors trigger vasodilation and are of great importance in allergy; they also play a role in the regulation of sleep cycles. H2 receptors regulate gastric acid secretion, whereas H3 receptors are pre-synaptic modulators of histamine and other neurotransmitters in the central nervous system [1]. The recently identified fourth human histamine receptor, H4 is believed to be mainly expressed on hematopoietic cells, particularly mast cells, basophils and eosino-

Abbreviations: DNP-HSA, dinitrophenyl human serum albumin; FcεRI, high affinity IgE receptor; H4R, histamine receptor H4; mH4R, murine histamine receptor H4; mBMMC, murine bone marrow-derived mast cell; SCF, stem cell factor.

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phils [2–6]. H4 receptors in mouse, rat and guinea pig have also been cloned [7]. The histamine H4 receptor is a Gαi/o coupled receptor [8], and its physiological functions revealed so far are eosinophil [9], monocyte-derived dendritic cell [10] and mast cell chemotaxis [8], mast cell-dependent neutrophil migration [11], regulation of T cells and dendritic cells [12–13], and recently discovered functions in the central nervous system [14]. The signal transduction pathways of H4 receptors have been studied and it has been shown that activation of H4 receptor leads to calcium ion mobilization in mast cells [8] and inhibition of forskolin-stimulated cAMP increase [2,4–5]. Another study concluded that histamine induced mitogen-activated protein kinase phosphorylation via H4, which was inhibited by pertussis toxin [3]. Many other pharmacologic functions of H4, and its signaling pathways, especially in endogenous settings have not been studied yet.

Mast cells are bone marrow-derived cells that participate in innate immunity and reside in tissues in proximity to surfaces that interface the external environment. They play an important role in inflammation and hypersensitivity reaction type I. Mast cells bind the Fc region of Immunoglobulin E (IgE) through the high affinity IgE receptors (FcεRI) with 1:1 ratio [15]. Subsequently, IgE binds specific antigens, the FcεRI-IgE-antigen complex forms cross-links

and larger aggregates [15–17], resulting in the release of mediators stored in mast cell granules such as histamine and proteases, and de novo synthesis of mediators such as prostaglandins and leukotrienes [18]. A recently discovered function of IgE is the regulation of FcεRI expression on mast cells. Several studies revealed that the binding of IgE to FcεRI itself causes the high affinity IgE receptor expression to increase on mast cells and basophils as a positive feedback loop that leads to allergic cascades, but not all components of the IgE signaling pathway are upregulated equally [19–23]. The exact mechanism of this phenomenon is still under investigation.

In the present study, biological functions of H4 receptors on murine BMMC were examined. For this purpose mast cells were differentiated from murine bone marrow stem cells and the expression kinetics of FcεRI and mH4R were determined across the culture's differentiation using flow cytometry and quantitative polymerase chain reaction, respectively. The functional evaluation of mast cells was performed with degranulation assays, and using a selective H4 antagonist it was eventually concluded that H4 is potentially engaged in the regulation of the sensitization phase of mast cell degranulation and IgE induced FcεRI upregulation.

2. Materials and methods

2.1. Materials

DMEM, DPBS, HBSS, High Capacity cDNA Reverse Transcription Kit, Oligo TaqMan Universal PCR Master Mix, Taqman Gene Expression Assay Mm00467634_m1, Taqman Gene Expression Assay Mm01546394_s1 was purchased from Life Technologies (Carlsbad, CA). BSA, Penicillin–Streptomycin Solution Stabilized, mouse monoclonal anti-dinitrophenol antibody (IgE), calcium ionophore A23187, 4-nitrophenyl N-acetyl-β-D-glucosaminide, dinitrophenyl-albumin (DNP-HSA) was purchased from Sigma–Aldrich Co. (St. Louis, MO). Rat anti-mouse IgE FITC, rat anti-mouse CD117 PE, rat anti-mouse CD16/CD 32 (Mouse Fc Block), BD Pharmingen Stain Buffer was purchased from BD Biosciences (Erembodegem, Belgium). Recombinant mouse IL-3, recombinant mouse SCF was purchased from R&D Systems (Minneapolis, MN). Rat anti-mouse FcεRI FITC was purchased from Biolegend (San Diego, CA); FBS was purchased from Thermo Fisher Scientific (Waltham, MA); ACK Lysis Buffer was purchased from Quality Biological Inc. (Gaithersburg, MD); RNeasy Mini Kit was purchased from Qiagen (Venlo, Netherlands). UR-63325 (Palau Pharma S.A., Barcelona, Spain), a furo[3,2-d] pyrimidine derivative is a selective histamine receptor H4 antagonist/inverse agonist [24–26], and was synthesized as a reference compound in Sanofi (Budapest, Hungary) using the synthesis method described in the patent WO2009056551 (A1) – Furo[3,2-d] pyrimidine derivatives (Palau Pharma S.A., Carceller Gonzalez E, Medina Fuentes EM, Martina via J, Virgili Bernado M, 2009).

2.2. Bone marrow mast cell culture

The femurs of BALB/c male mice (8–12 weeks) were surgically removed and flushed with cold DMEM. Cells were collected, filtered through a 70 μm cell strainer and centrifuged (1200 rpm, 10 min). After treatment with ACK Lysis Buffer to lyse red blood cells, the cells were washed twice with DMEM and plated at 2×10^6 cell/ml in BMMC medium (DMEM with 2% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin) containing 50 ng/ml recombinant murine SCF and 10 ng/ml recombinant murine IL-3. Twice every week non-adherent cells were removed, centrifuged (1200 rpm, 10 min), fresh media added and transferred to a new dish for further culture. Cells were analyzed by flow cytometry for CD117 and FcεRI expression. Mast cells were considered

mature from 4 to 5 weeks, when more than 90% of cells were positive for both CD117 and FcεRI compared to isotype control, and were used in further experiments. Handling and animal experiments were conducted according to 86/60S/EEC 2010-63-EU directive. All experimental protocols were approved by Sanofi's Institutional Animal Care and Use Committee.

2.3. Real-time qRT-PCR analysis of mH4R mRNA

The expression of the mRNA encoding murine histamine receptor H4 was determined by real-time qRT-PCR. Total RNA was extracted from mBMCMs using RNeasy kit and reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit. PCR reactions were performed with cDNA using Taqman Universal PCR Master Mix, primers for murine histamine H4 receptor (Taqman Gene Expression Assay Mm00467634_m1) and primers for murine RPL37a (Taqman Gene Expression Assay Mm01546394_s1). The PCR reaction was as follows: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s; 60 °C for 1 min. The data was normalized to RPL37a data, and analyzed with 7500 System Sequence Detection Software v1.4 (Life Technologies, Carlsbad, CA).

2.4. Flow cytometric measurement of FcεRI expression

Cells (2×10^5 cell/tube) were incubated in stain buffer, isotype control antibody, or specific antibodies as specified (rat anti-mouse CD117-PE, BD; rat anti-mouse IgE-FITC; rat anti-mouse FcεRI-FITC) and Fc Block (rat anti-mouse CD16/CD32) for 20 min at 4 °C. For anti-mouse IgE-FITC staining cells were previously incubated with anti-DNP IgE in stain buffer for 20 min at 4 °C. Samples were then washed once by centrifugation (1300 rpm, 5 min) and resuspended in stain buffer. Flow cytometric analysis was conducted using FACSCanto II (BD Biosciences, Erembodegem, Belgium), with at least 10,000 events recorded during each measurement. Data collection and analysis were performed using FACSDiva (BD Biosciences, Erembodegem, Belgium) software.

2.5. β-Hexosaminidase release

Degranulation studies were performed by measuring β-hexosaminidase release, as described previously [8]. Briefly, mast cells previously sensitized with 1 μg/ml anti-DNP IgE overnight were challenged with dinitrophenyl-albumin for 10 min at 37 °C in HBSS medium (containing glucose and BSA). Non-sensitized mast cells were challenged for 10 min at 37 °C with calcium ionophore A23187. Reactions were stopped by centrifuging the cells (900 rpm, 5 min), and 50 μl of supernatant was mixed with 100 μl of β-hexosaminidase substrate (4-nitrophenyl N-acetyl-β-D-glucosaminide) solution adjusted to pH = 4.5 with citrate buffer, and incubated for 2 h at 37 °C. 50 μl of 0.4 M glycine solution (pH = 10.7) was added, and absorbance at 405 nm was measured. Total release of mast cell contents was achieved by adding 1% Triton X-100. Degranulation percentage was calculated using the formula: $(\beta\text{-Hex}(\text{supernatant}) - A) / (\beta\text{-Hex}(\text{total}) - A) \times 100$, where A is the β-hexosaminidase released from unstimulated cells.

2.6. Statistical analysis

Experimental data are presented as mean ± standard deviation from the number of independent samples. Statistical significance was determined using Student's *t*-test for two groups or ANOVA with Dunnett's test for multiple groups using Statistica v9 (StatSoft, Tulsa, OK). Normality of the data was inspected with the Shapiro–Wilk test. In all analyses $p < 0.05$ was considered significant. Log-dose vs. response curve analysis was performed using GraphPad Prism 5 v5.04 (GraphPad Software, La Jolla, CA).

3. Results

3.1. mH4R and FcεRI expression kinetics in mBMMC

Since the FACS analysis of H4 receptors has not yet been established thoroughly, and H4 antibodies validated for flow cytometry are not readily available commercially, the expression of mH4R mRNA as a function of time was studied in mBMMC, using real-time qRT-PCR. Quantifying relative mRNA expression from mBMMC samples showed that mH4R expression increased with the maturation of the cell cultures until a maximum level, then started to decline as the cultures became older. The mRNA expression measurements were repeated with three independent samples and although there was some variance between different cell batches, the trend of mH4R expression as a function of time was consistently found to reach a maximum between weeks 5 and 10. The mH4R mRNA expression levels were normalized to RPL37a expression, and relative expression data are presented in Fig. 1.

The expression of FcεRI on mBMMC cell surface was also examined during the differentiation of the cultures as a function of time using flow cytometry. FcεRI expression kinetics was detected to also reach a maximum when quantified as a function of time, similarly to mH4R expression kinetics. FcεRI levels were found to increase from the day of preparation (with a peak around week 3), however after the maximum expression FcεRI levels started to decline steadily (Fig. 2). The mH4R mRNA and FcεRI expression kinetics also correlate with the cell viability (data not shown) Fig. 3 and degranulation function (Fig. 4) time plots of the murine bone marrow-derived mast cells, as both cell growth rate and the degranulation percentage decrease steadily from week 5 to 6.

3.2. H4 antagonist/inverse agonist inhibits mBMMC degranulation only during sensitization phase

Degranulation assays with mBMMC cultures were conducted by measuring β-hexosaminidase release after IgE-dependent activation of the mast cells [8]. BMMCs were incubated overnight with 1 μg/ml anti-DNP IgE, and then the cells were challenged with 50 ng/ml DNP-HSA to trigger mast cell degranulation. The β-hexosaminidase release of mBMMCs was inhibited by UR-63325 (H4 antagonist/inverse agonist). UR-63325 was co-incubated with the mBMMC both during the overnight IgE sensitization and the

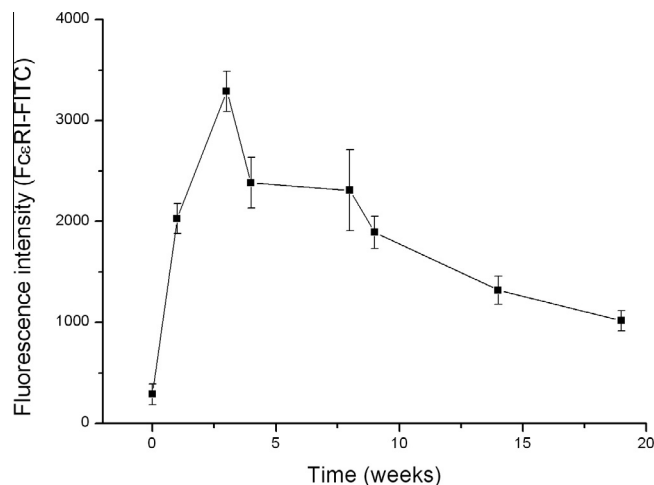


Fig. 2. FcεRI expression kinetics on mBMMC. FcεRI expression was quantified using flow cytometry. For these experiments only the FcεRI expression levels of double positive (CD117+, FcεRI+) mBMMC were considered. The data are presented in mean fluorescence intensity, one representative experiment of eight different batches of mBMMC cultures is shown. Data are mean ± SD, *n* = 3.

DNP-HSA challenge phases. This inhibition of β-hexosaminidase release by an H4 antagonist was found to be concentration-dependent as shown in Fig. 5. Maximum inhibition was achieved at 10^{-5} M UR-63325 concentration, since increasing UR-63325 concentration above 10^{-5} M did not result in increasing inhibition of degranulation (data not shown). In a subsequent set of experiments it was shown that UR-63325 could exert its inhibitory effect only when present during the IgE sensitization phase. Addition of UR-63325 (10^{-5} M) to mBMMC, only during the antigen challenge phase did not exhibit significant inhibitory effects on degranulation (Fig. 6). This implies that H4 receptor does not directly regulate mast cell degranulation, rather it plays a role in IgE-mediated sensitization processes. UR-63325 did not have any significant effect on the basal β-hexosaminidase release of the native mast cells (negative control), and in cytotoxicity experiments UR-63325 did not have any toxic effects on mBMMC (data not shown).

3.3. H4 antagonist/inverse agonist inhibits IgE induced FcεRI upregulation on mBMMC

During the sensitization of mast cells several signal transduction pathways are activated that result in IgE induced FcεRI upregulation. In this study it was shown that by antagonizing the mH4R receptor we were able to inhibit IgE induced FcεRI upregulation in mBMMC. Mast cells were incubated overnight with 1 μg/ml anti-DNP IgE in BMMC medium. After adding rat anti-mouse IgE-FITC, the expression of FcεRI was measured with flow cytometry. Approximately 80% of the mast cells (week 5 mBMMC) responded and their FcεRI expression increased due to IgE by 7.3-fold (Fig. 7) as also shown by previous studies [21]. In order to examine the role of H4 receptors in the regulation of FcεRI expression, UR-63325 was co-incubated with mast cells overnight along with IgE. The IgE induced FcεRI upregulation was inhibited with 3×10^{-5} M UR-63325 by 14.6% in week 5 mBMMC culture (Fig. 8A), 72.6% in week 9 mBMMC culture (Fig. 8B), and 27.9% in week 15 mBMMC culture (Fig. 8C). The FcεRI expression fold increase difference due to IgE in these experiments was 7.3, 7.3, and 5.7 in week 5, 9 and 15 mBMMC, respectively. In subsequent experiments, in order to attain a dose-response curve for this inhibition, mast cells were treated with 1 μg/ml anti-DNP IgE and with different concentrations of UR-63325, ranging from 3×10^{-5} M to 3×10^{-10} M. As well as in the case of degranulation, the inhibition of FcεRI

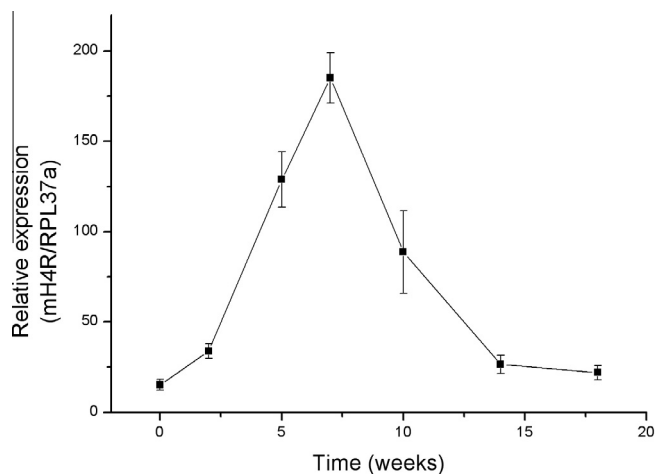


Fig. 1. Histamine receptor H4 expression kinetics on mBMMC. The mH4R mRNA expression of mBMMC was detected with real-time qRT-PCR. Total RNA from mBMMC was reverse transcribed and used as templates for PCR. RPL37a mRNA was amplified with specific primers as control in PCR reactions. Data are mean ± SD, *n* = 3.

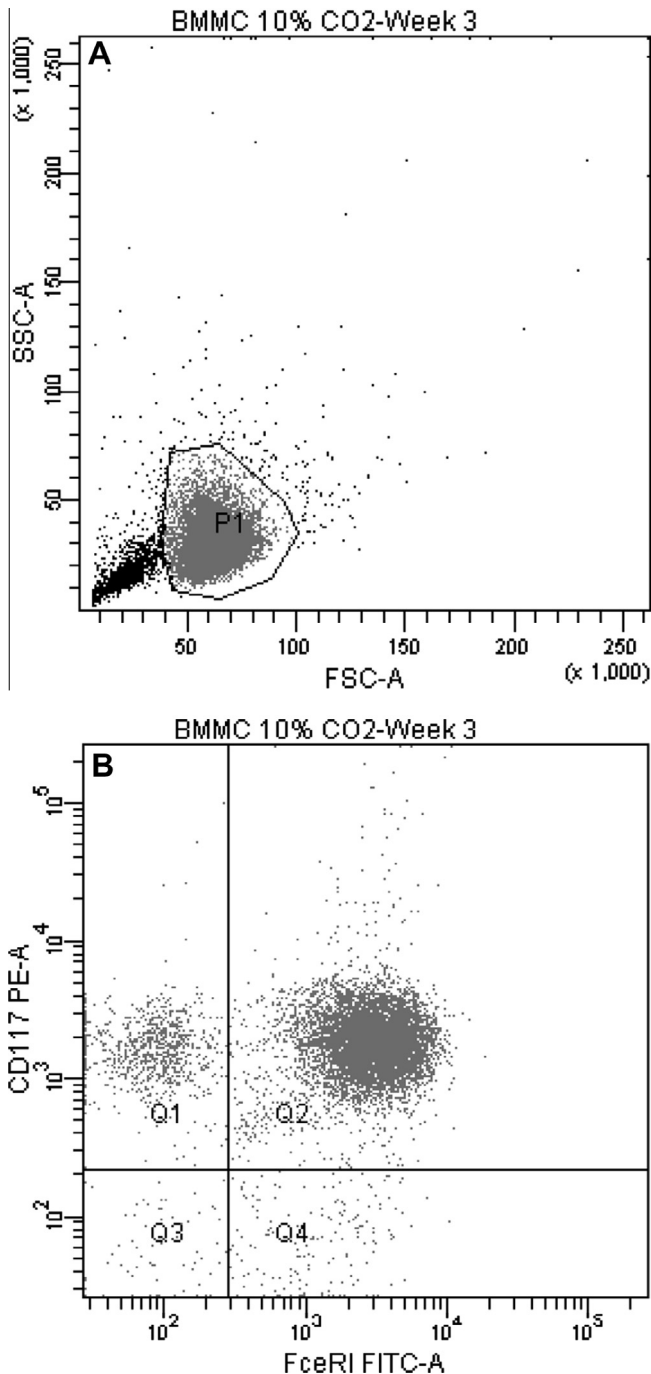


Fig. 3. Flow cytometric analysis of bone marrow-derived mast cells (3 weeks). The first FACS image shows the forward scatter (FSC) vs. side scatter (SSC) plot of each data point. The second image shows the fluorescence intensity values of FcεRI-FITC vs. CD117-PE of population P1 (gating out the cellular debris). The cells in the second quadrant (Q2) are considered double positive for FcεRI and CD117, thus mature mast cells. This procedure was used as a robust method of determining mast cell culture maturation, as described in the literature [8].

upregulation by an H4 antagonist was determined to be concentration-dependent (Fig. 9).

4. Discussion

Histamine is a ubiquitous biogenic amine secreted by mast cells as a potent pro-inflammatory mediator. Histamine mediates its diverse physiological functions via four distinct histamine receptors,

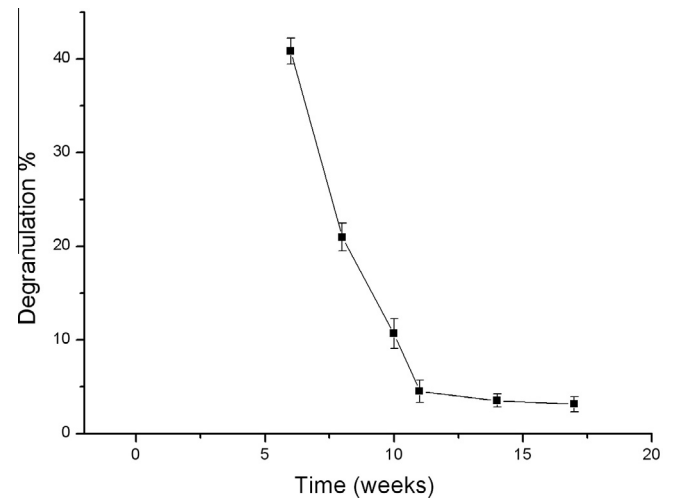


Fig. 4. Degranulation function of BMMC vs. time. The degranulation percentage of various BMMCs upon antigen challenge with 50 ng/ml dinitrophenyl-albumin. The first data point was recorded with 6 week old culture cells, since the cells can be considered mature BMMCs from this time point. Data are presented in mean $\pm$ SD, $n = 5$.

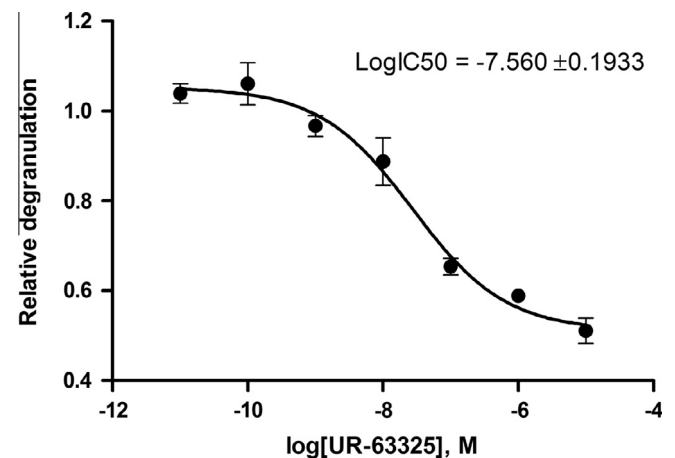


Fig. 5. UR-63325 (H4 antagonist) inhibits β -hexosaminidase release during mBMMC degranulation. mBMMCs (week 10) were incubated overnight with 1 μ g/ml anti-DNP IgE and UR-63325 with concentrations specified in the figure. The mast cells were then challenged with 50 ng/ml DNP-HSA. The data are presented in relative degranulation: degranulation % of inhibited mBMMC sample divided by degranulation % of sample not treated with UR-63325. Data are mean $\pm$ SD, $n = 5$ (ANOVA and Dunnett's test were performed). Data shown are representative of four repeated experiments.

of which histamine receptor H4 is the most recently discovered. Accumulating information in the literature suggests that H4 receptor expression is confined to cells of the hematopoietic lineage [2–5]. Others have previously demonstrated that the H4 receptor is expressed in both human and murine mast cells [2–4,8]. Hofstra et al. [8] have reported that histamine induces mast cell chemotaxis via the H4 receptor. This report along with other recent studies has suggested that the H4 receptor participates in the feed-back regulation of immunological responses. In the present study, we have explored further possible functions of the H4 receptor in mast cells. We first determined the expression kinetics of mH4R and FcεRI across the differentiation time span of murine bone marrow-derived mast cells (mBMMC). The expression level of H4 depended on many factors, one of which was the maturity of mBMMC cultures. We have shown using qRT-PCR and flow

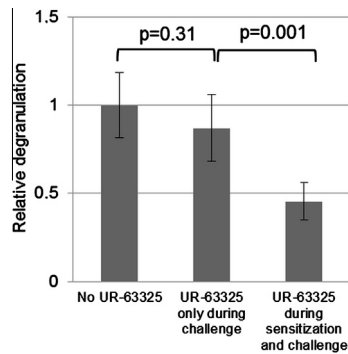


Fig. 6. H4 antagonist inhibits mast cell degranulation during sensitization phase, not antigen-challenge phase. All antigen challenges were performed using 50 ng/ml DNP-HSA. Data are in mean $\pm$ SD, $n = 5$ (p values were determined using Student's t -test). Data shown are representative of three repeated experiments.

cytometry, that the expression of both H4 receptors and Fc ϵ RI on mBMMC depends on the amount of time elapsed since the preparation of the cultures. The expression level of both mH4R and Fc ϵ RI receptors was found to reach a maximum, around week 3 and week 7, respectively, after which there was a decline in expression levels, which correlated with both the decline in cell viability and degranulation function of the mast cells. In order to characterize mBMMC with respect to their function, we determined mast cell degranulation by measuring released β -hexosaminidase.

The progress of an allergic response and development of inflammation involves two stages. During the allergen sensitization phase mast cells are sensitized with immunoglobulin E. In this

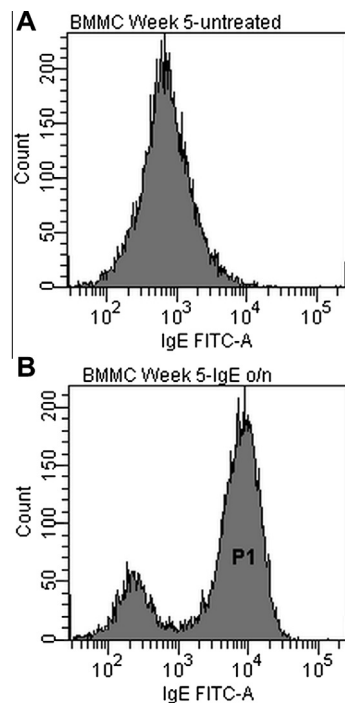


Fig. 7. IgE induced Fc ϵ RI upregulation in mBMMC. Murine mast cells (week 5) were incubated overnight (o/n) with 1 μ g/ml anti-DNP IgE. Subsequently both IgE treated and untreated mBMMC were labeled with IgE and rat anti-mouse IgE-FITC. The expression of Fc ϵ RI increases on treated cells due to IgE sensitization overnight (B), compared to that of cells not treated with IgE overnight before labeling (A). The fluorescence intensity of unstained cells does not differ for treated and untreated cells (data not shown). In inhibition experiments of Fc ϵ RI upregulation only the fluorescence intensity of the responsive population to IgE incubation (P1) was considered. Fluorescence intensity values of FITC (Fc ϵ RI FITC) are shown. The data is representative of three independent experiments.

phase the interaction of IgE with its receptor (Fc ϵ RI) upregulates Fc ϵ RI expression [19], additionally histamine may affect dendritic cell function [10,13] and T cell activation [12] via the H4 receptor. Subsequently, during the allergic reaction phase exposure of mast cells to antigens leads to mast cell degranulation and the release of histamine. Since the identification of the H4 receptor, many authors have suggested that histamine may be involved in the recruitment of mast cells [8] and eosinophils [9] to the site of inflammation, and plays a role in other components of the complicated process of allergic responses, but the role of H4 receptors in mast cell degranulation remains unclear.

The expression of H4 receptor in mast cells, eosinophils and dendritic cells has led to the identification of novel roles of the H4 receptor in immunological responses. Since a key function of mast cells is degranulation we sought to investigate the relationship between H4 receptors and mast cell degranulation. We report that the H4 receptor plays an indirect role in mBMMC degranulation. Here it was shown that a selective H4 receptor antagonist/inverse agonist inhibits mast cell degranulation only when it is present during the IgE-sensitization phase of mast cell activation. In a previous study conducted by Hofstra et al., histamine did not have any effects on degranulation and H4 receptor-deficient murine mast cells did not show any defects in degranulation [8]. In the study done by Hofstra et al. the effect of histamine in the antigen challenge phase was investigated: histamine was added to IgE-sensitized mast cells, and after antigen challenge no difference in calcium mobilization was detected compared to mast cells not pretreated with histamine. We have shown here that although histamine does not alter mast cell degranulation, H4 receptors modulate degranulation during the antibody sensitization phase (Fig. 5). Here we demonstrated that inhibition of the H4 receptor during the antigen challenge phase did not have any effects on mast cell degranulation, in accordance with Hofstra et al. [8], however an H4 antagonist/inverse agonist inhibited degranulation indirectly by affecting IgE-dependent mast cell sensitization processes (inhibition was concentration-dependent, with approximately 50% inhibition at maximal inhibitory concentration). This result implies that the regulatory functions of H4 on mast cell degranulation are to be sought in the early sensitization phase, rather than the actual degranulation phase. In our study we used the reference compound UR-63325 as a selective H4 antagonist/inverse agonist (IC_{50} -human = 24 nM, IC_{50} -murine = 38 nM, >200 fold selectivity vs. the other three histamine receptors [25]), because the efficacy has been proven both on human and mouse receptors and also *in vivo* in mouse pharmacology models and human clinical trials. Since in our experiments only the selective H4 antagonist/inverse agonist was added (without any histamine) to the cells during sensitization, and whether mast cells constitutively release histamine is not known, the effects of UR-63325 could be attributed to the high constitutive activity and/or functional selectivity of H4 receptors [27–29].

Since the presence of Fc ϵ RI on the mast cell surface and the consequent binding of IgE to Fc ϵ RI is essential to mast cell activation, we investigated the role of H4 receptors in IgE induced Fc ϵ RI upregulation, which occurs during mast cell sensitization [19]. Using an H4 receptor antagonist/inverse agonist we were able to significantly inhibit IgE induced Fc ϵ RI upregulation in mBMMC in a concentration-dependent manner. As the expression level of mH4R depends on the maturity of mBMMC cultures (Fig. 1), so does the magnitude of IgE induced Fc ϵ RI upregulation (Fig. 8). Relative inhibition of Fc ϵ RI upregulation by the H4 antagonist/inverse agonist could be measured, however this relative inhibition also changes as a function of mBMMC maturity. The time-dependence of Fc ϵ RI upregulation inhibition (with UR-63325) overlaps with the mH4R expression-kinetics based on our qRT-PCR results. This implies that not only does the expression level of H4 receptors

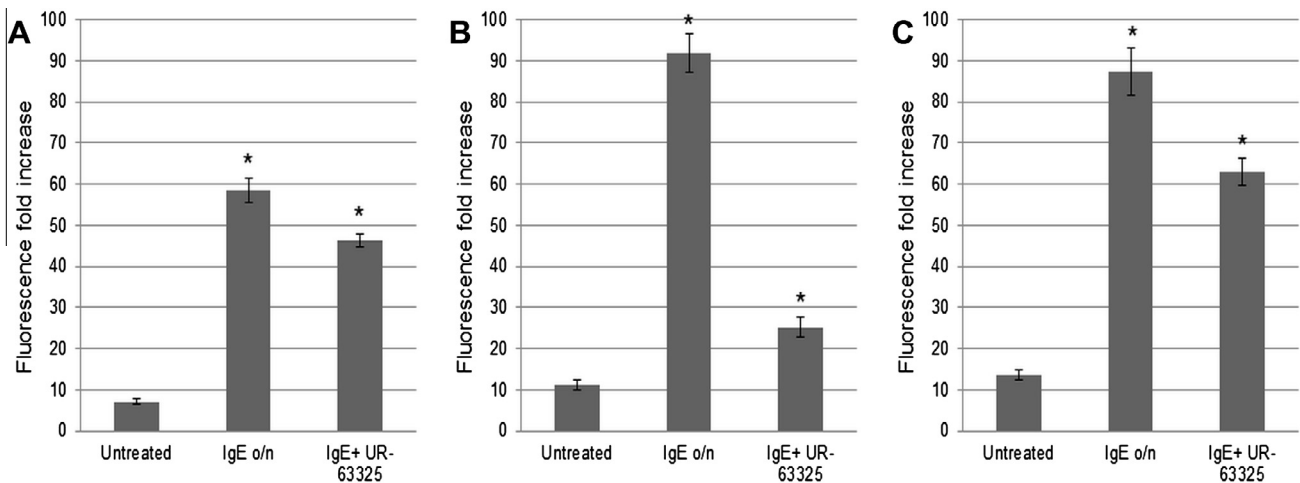


Fig. 8. UR-63325 inhibits IgE induced Fc ϵ RI upregulation in different mBMMC cultures. (A) Week 5, (B) week 9, and (C) week 15 mBMMCs were incubated with 1 μ g/ml anti-DNP IgE overnight (o/n), and co-incubated with or without 3×10^{-5} M UR-63325. Fc ϵ RI levels were measured by rat anti-mouse IgE-FITC labeling using flow cytometry. The mean fluorescence intensity values of unstained samples do not differ from one another significantly (data not shown). In the figure fluorescence intensity fold increase values are shown, which are calculated as the ratio of a sample's stained and unstained mean fluorescence intensity. The inhibition % of UR-63325 was determined using these fluorescence fold increase values. Data are mean $\pm$ SD, $n = 5$ (* $p < 0.001$, Student's t -test).

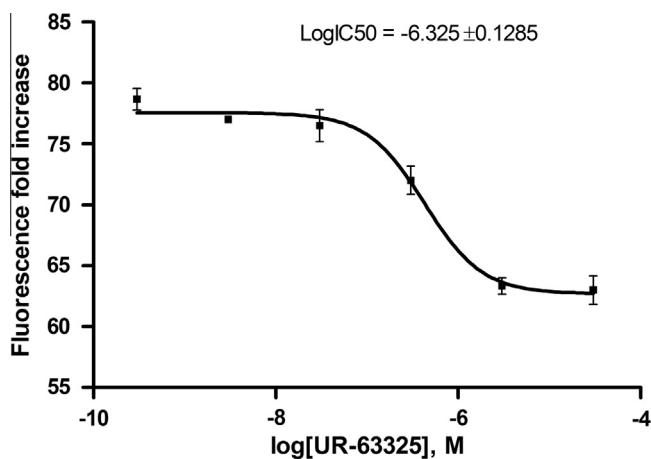


Fig. 9. UR-63325 inhibits IgE induced Fc ϵ RI upregulation in a concentration-dependent manner. mBMMCs (week 15) were incubated overnight with 1 μ g/ml anti-DNP and co-incubated with different concentrations of UR-63325, and analyzed with rat anti-mouse IgE-FITC using flow cytometry. Fluorescence intensity fold increase values are shown. Data are mean $\pm$ SD, $n = 5$ (ANOVA and Dunnett's test were performed with "Only IgE" as control group, all groups were $p < 0.05$). The figure is representative of three independent experiments.

change according to the maturation and aging of mast cells, but the sensitivity or involvement of the receptor in IgE induced Fc ϵ RI upregulation might vary with time as well. The inhibition of IgE induced Fc ϵ RI upregulation via H4 receptors might be associated with the inhibitory effects of an H4 antagonist/inverse agonist on mast cell degranulation. However, H4 might influence mast degranulation by means of various signal transduction pathways simultaneously. The difference in IC₅₀ values of UR-63325 during mBMMC degranulation, and IgE induced Fc ϵ RI upregulation (Figs. 5 and 9) could be attributed to the presence of multiple signaling pathways, or different constitutive histamine levels secreted by the cells as a result of different experimental settings.

The identification of the exact mechanism with which H4 contributes to IgE induced Fc ϵ RI upregulation is beyond the scope of this study. Our results are a descriptive exploration of H4 regulated mast cell processes, which have not been described in the literature previously, however the underlying mechanisms leading to

these results remains to be elucidated. By investigating the signal transduction pathways that govern the sensitization processes we can gain a deeper insight regarding the factors that regulate mast cells activation, and ultimately allergic reactions. A relevant study might be the investigation of IgE induced Fc ϵ RI upregulation in H4 receptor knockout murine mast cells, or performing calcium ion mobilization/cAMP functional assays. Another important aspect of this study would be the effect of a selective H4 agonist on IgE induced Fc ϵ RI upregulation, although in our experiments 4-methylhistamine, a selective H4 agonist did not induce mast cell degranulation (data not shown). It has been previously shown that the criterion for mast cell activation is the clustering of Fc ϵ RI-IgE dimers [30], and maximal histamine release is obtained when only 10% of the IgE receptors are aggregated [31]. Therefore further studies are needed to determine, whether the inhibitory effects of an H4 antagonist during the sensitization phase of mast cell degranulation is a consequence of the inhibition of IgE induced Fc ϵ RI upregulation.

Identifying the functions of H4 receptor enables a better understanding of immunological mechanisms. Several pharmacological functions of H4 receptors have been identified thus far, but to our knowledge their role in mast cells regarding IgE induced Fc ϵ RI upregulation has not been reported yet. Antagonists of the H4 receptor may be useful in the treatment of inflammatory diseases, such as asthma, allergic rhinitis or pruritus [32–34]. The data presented here imply that H4 regulates IgE dependent processes in mast cells, and may contribute to a better understanding in the pharmacological and clinical implications of H4 antagonists.

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