

Functional characterization of histamine H4 receptor on human mast cells



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ABSTRACT

Among the four different types of histamine receptors (H1–H4), H4R is predominantly expressed in immune cells and involved in immunomodulatory response. Here, in this study we determined the expression of H4R in human mast cells (HMC-1, LAD-2 and primary cord blood derived CD34+ human mast cells) and characterized its functional properties. Interestingly, we found that human mast cells responded to both histamine (natural ligand) and 4-methylhistamine (selective H4R agonist) for sustained intracellular calcium mobilization, degranulation and cytokine production. However, only histamine induced the release of cAMP, but 4-methylhistamine down regulates cAMP indicating that H4R mediates its effect through Gαi/o protein and H1R via Gαq protein. Furthermore, both histamine and 4-methylhistamine induced the production of cysteinyl leukotrienes and LTB4. Using human inflammation antibody array membrane, we found that H4R induced the expression of various inflammatory proteins, involving pro-inflammatory cytokines and chemokines and these are TGF-β1, TNF-α, TNF-β, PDGF-BB, TIMP-2, M-CSF, IP-10, IL-16, IL-6, IL-3, IL-10, MIP-1α, IL-1α, ICAM-1, Eotaxin-2, RANTES, IL-8, MCP-1, and IL-6sR. We also quantified the level of various inflammatory cytokines produced by human mast cells through H4R. It was observed that, the production level of Th2 cytokines IL-4 (401.34 pg/ml), IL-5 (64.21 pg/ml) and IL-13 (1044 pg/ml) and classical proinflammatory cytokines IL-6 (221.27 pg/ml) and IL-1β (34.24 pg/ml) and chemokines MCP-1 (106 pg/ml) and IL-8 (818.32 pg/ml). Furthermore, activation of H4R caused the phosphorylation of ERK and PI3 K in a time dependent manner. Taken together these data demonstrate that, the activation of H4R in human mast cells produced not only inflammatory mediators that are associated with allergic reactions but also other inflammatory conditions.

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1. Introduction

Histamine is an important mediator that orchestrates inflammatory and allergic responses (Shahid et al., 2009; Salcedo et al., 2013). The pleiotropic effects of histamine are mediated through at least pharmacologically distinct receptors namely H1R, H2R, H3R and H4R, which are all members of GPCR family (Thurmond et al., 2004). Accumulating evidence suggests that, histamine contributes

to the progression of allergic inflammatory responses by enhancing the secretion of pro-inflammatory cytokines like IL-1β, IL-6 as well as chemokines such as IL-8 and RANTES in inflammatory cells and local tissues as well (Umetsu et al., 2002; Dy and Schneider, 2004).

Previously it was thought that histamine modulates inflammatory and allergic responses mainly through its classical receptors H1R and H2R. However, after the discovery of the fourth receptor H4R, which is predominantly expressed on the cells of the immune system (Thurmond et al., 2008; Hodge et al., 2013) is found to be responsible for the selective recruitment and accumulation of inflammatory cells to the sites of allergic inflammation (Hofstra et al., 2003; Lim et al., 2005).

Activation of H4R triggers a series of signal transduction events such as, calcium mobilization, actin polymerization, cell shape change, up regulation of adhesion molecules leading to immune cell migration into sites of inflammation (Hofstra et al., 2003; Zampeli and Tiligada, 2009). Also, studies have shown that, asthmatic patients have increased number of degranulated mast cells in

Abbreviations: LAD-2, Laboratory of allergic diseases 2; HMC-1, Human mast cell line-1; MCP-1/CCl2, Monocyte chemo attractant protein-1; H4R, Histamine H4 receptor; MC, Mast cells; LTB4, Leukotriene B4; CyLTs, Cysteinyl leukotrienes; cAMP, Cyclic AMP; RANTES, Regulated on Activation T cell Expressed and Secreted; TIMP-2, Tissue Inhibitor of Metalloproteinases-2; 4-MH, 4-methylhistamine; Mep, Mepyramine maleate.

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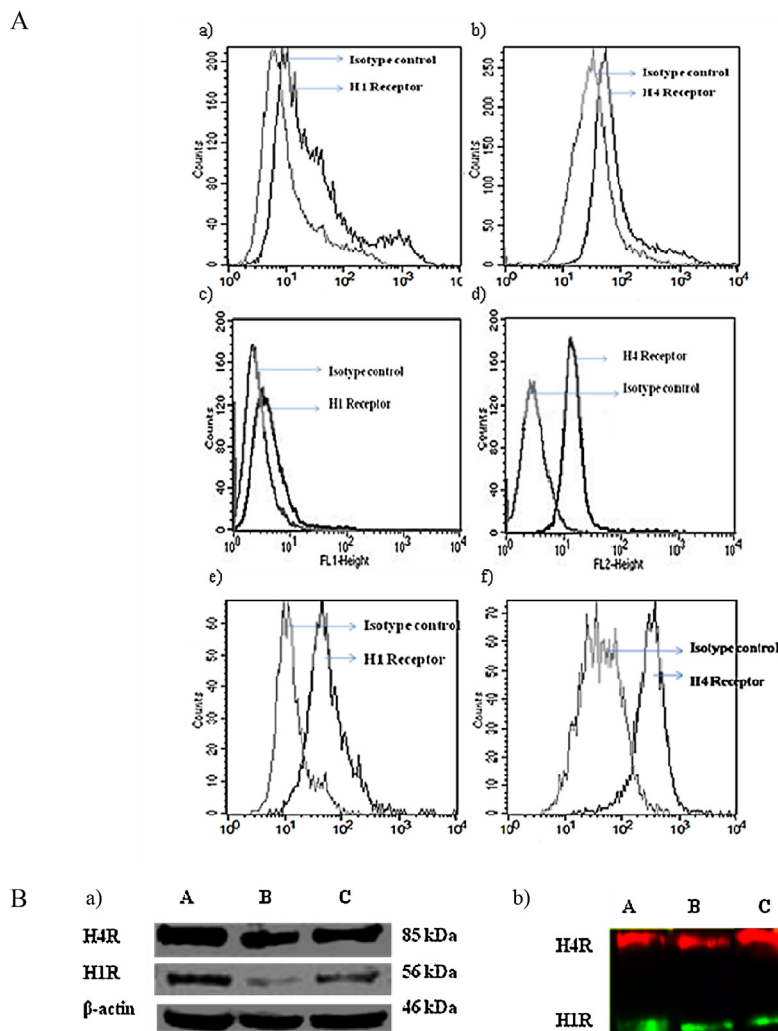


Fig. 1. A. Determination of histamine H1 and H4 receptors expression on HMC-1 cells, cord blood derived CD34+ human mast cells and THP-1 cells. Flow cytometry was performed using a fluorescent labelled H4R and H1R specific antibodies. (a and b) HMC-1 cells and (c and d) cord blood derived CD34+ human mast cells were expressed histamine H1R and H4R receptors. (e and f) THP-1 cell line that constitutively express histamine H1R and H4R was used as a positive control. Data shown are representative of three similar experiments. **Fig. 1B.** Expression analysis of histamine H1 and H4 receptors by western blotting. Representative results of immunoblotting for histamine H1, H4 receptors and β -actin are shown in (a) A) LAD-2, (B) HMC-1 and (C) cord blood derived CD34+ human mast cells. The proteins were visualized using ECL prime method and the image was captured by multi imaging system using Alpha view software, Cell biosciences. Multiplex western blot showing the expression of histamine H1 and H4 receptors on human immune cells. Cy3 and Cy5 linked fluorescent secondary antibodies were used to detect two different histamine H1 and H4 receptors expression on (b) (A) PBMC, (B) Granulocytes and (C) Jurkat cells. Data shown are representative of three similar experiments.

the airways as compared with normal subjects. It is well known that mast cells are the major producers of histamine (Zhang et al., 2007; Schwartz, 1994) and that mast cell activation amplifies the inflammatory response in the airways which in turn further affects lung physiology and function. Thus mast cells play a critical role in the pathogenesis of asthma (Reuter et al., 2010; Rivera, 2002). Though a great deal of research has been focused on mast cell activation via Fc ϵ RI, but very few reports highlight the involvement of H4R in cells associated with allergic asthma and the contribution of H4R mediated mast cell effects in allergic inflammation still remains little understood. Therefore, in order to better understand the functional aspects of H4R, we have utilized HMC-1, LAD-2 and cord blood derived CD34+ human mast cells to elucidate the histamine and 4-methylhistamine (selective H4R agonist) induced mediator release in human mast cells and the standard H4R antagonist JNJ777120 was employed to confirm the H4R mediated functional activation and also the downstream signal transduction pathways. Here, we demonstrate the novel finding that H4R activation leads to the production of not only Th2 cytokines (IL-5, IL-4 and IL-13) but also various other inflammatory proteins in human mast cells.

2. Materials and methods

2.1. Chemical and reagents

StemPro-34 medium, SCF, Penicillin–Streptomycin, L-glutamine, IMDM, rhIL-6 and CysLTs kit were purchased from Gibco invitrogen, USA. Recombinant human IL-3 was purchased from R & D Systems USA. Rabbit antihuman H4R antibody, mouse antihuman H1R antibody, Mouse IgG1, rabbit IgG, goat anti rabbit IgG-HRP, goat anti mouse IgG-HRP conjugates, goat anti rabbit-PE, goat anti mouse-FITC and 4- methylhistamine (H4R agonist) were purchased from Santacruz Biotechnology, USA, Human IgE from Abcam, Anti human IgE, JNJ 777120 (H4R antagonist), Histamine and Mepyramine maleate (H1R antagonist) from Sigma, USA, Human inflammation antibody array from Ray Biotech, Leukotriene B4 EIA Kit and Cyclic AMP Kit from Cayman Chemical Company, USA. ELISA Kits were purchased from ebioscience, USA and R & D Systems USA. ECL prime was purchased from GE Healthcare, USA. Phospho Akt antibody, Total Akt antibody, Phospho ERK1/2 (Thr202/Tyr204) antibody and total ERK antibody were

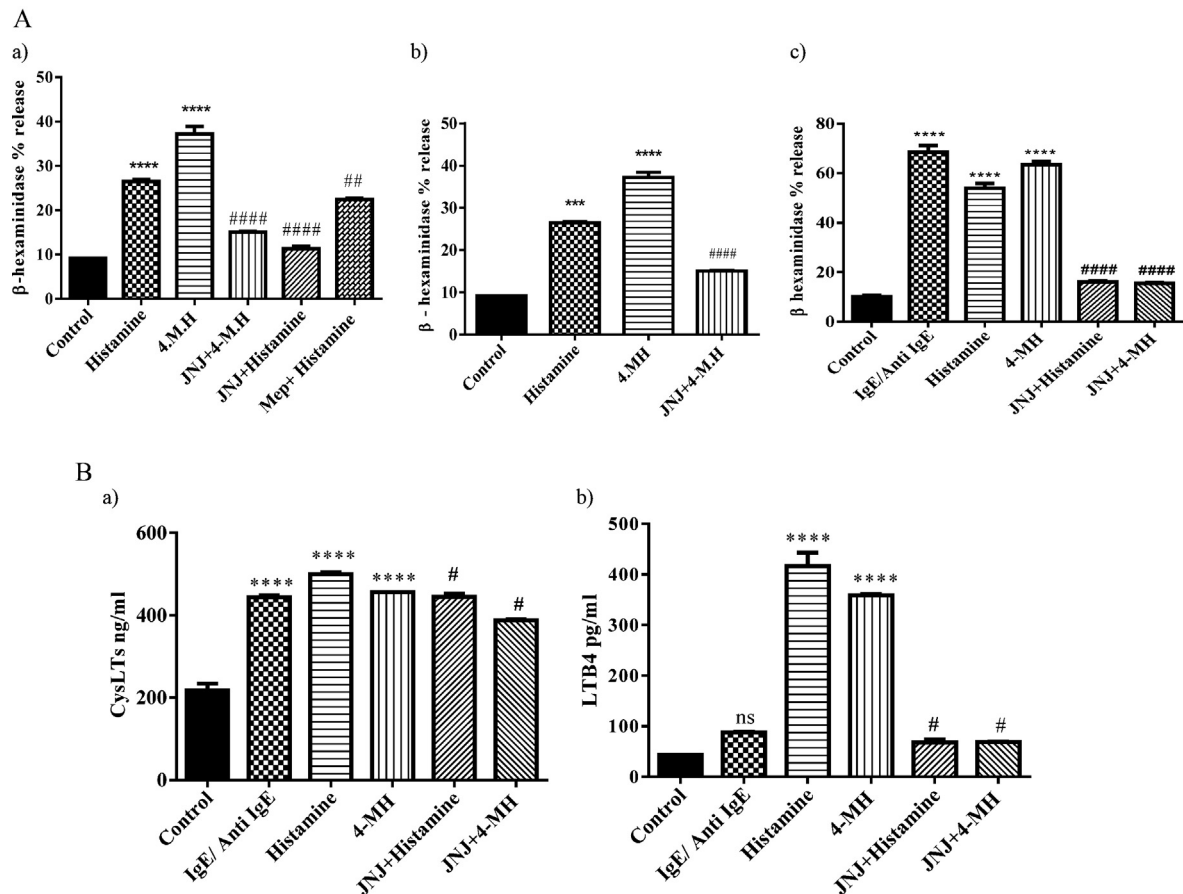


Fig. 2. A. Effects of histamine and 4-MH induced degranulation in human mast cells. Mast cells (a. HMC-1 cells, b. LAD-2 cells and c. cord blood derived CD34+ human mast cells) were sensitized with or without human IgE (1 μ g/ml) and washed before stimulation with antihuman IgE (1 μ g/ml), histamine (10 μ M), 4-MH (10 μ M) and pre-treated with 10 μ M of JNJ777120 and mepyramine (10 μ M) to study the inhibitory effect. Values are analysed using one-way ANOVA with Bonferroni's test. Data expressed as mean $\pm$ SD of three experiments done in triplicates. **** P < 0.0001, *** P < 0.0001, significantly different from control. #### P < 0.0001, significantly different from histamine and 4-MH. Fig. 2B. Measurement of LTB4 and CysLTs release upon activation with histamine and 4-MH in cord blood derived CD34+ human mast cells. Mast cells were stimulated for 30 min with histamine (10 μ M), 4-MH (10 μ M), IgE/Anti IgE (1 μ g/ml) and JNJ777120 (10 μ M) against 10 μ M of 4-MH and histamine. Quantification of (a) CysLTs and (b) LTB4 level was done using cell free supernatants by ELISA. Values are assayed using one-way ANOVA with Bonferroni's test. Data expressed as mean $\pm$ SD of three experiments done in triplicates. **** P < 0.0001, significantly different from control. # P < 0.01, significantly different from histamine and 4-MH.

purchased from Cell Signaling, Burlington, NC. SuperSignal West Pico chemiluminescent substrate was purchased from Thermo scientific USA.

2.2. Cell culture

LAD 2 cells (Kindly provided by Dr. Arnold Kirshenbaum, NIH, USA) were maintained in StemPro-34 medium supplemented with 100 ng/ml stem cell factor (SCF), glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 ng/ml) (Kirshenbaum et al., 2003). HMC-1 cells (Kindly provided by Dr. Joseph H Butterfield, Mayo Clinic, USA) were cultured in IMDM supplemented with 10% FBS, glutamine (2 mM), penicillin (100 IU/ml) streptomycin (100 ng/ml) and 5% CO₂ at 37 °C.

In vitro derived human mast cells were obtained as described in Radinger et al., 2010. Human cord blood was collected from a placenta during normal deliveries at SRM Medical college hospital in accordance with established institutional guidelines. Briefly, mononuclear cells were isolated by layering 3.8% sodium citrate-treated cord blood onto Lymphocyte Separating Medium (LSM). Mononuclear cells were washed and maintained in culture medium, consisting of 500 ml StemPro-34, 13 ml StemPro-34 nutrient Supplement, l-Glutamine (2 mM), Penicillin (100 U/ml)/Streptomycin (100 μ g/ml), rhSCF (100 ng/ml), rhIL-6 (50 ng/ml) and rhIL-3 (1 ng/ml). After 3 weeks, rhIL-3 is omitted.

Half of the medium was changed weekly, and the cells were cultured for at least 7 weeks. The purity of the mast cell at the time of experiment was 86–90% as determined using toluidine blue staining.

2.3. Flowcytometry

The expression of Histamine H1 and H4 receptor on the cell surface was evaluated by flow cytometry. Briefly, 1×10^6 cells were washed with SIR buffer containing 0.1% BSA. The cells were then incubated with rabbit IgG, mouse IgG1 (Isotype controls), rabbit anti-human H4R antibody, mouse anti-human H1R antibodies (1:50 dilutions) for 1 h at 4 °C. After washing, cells were incubated with PE labeled mouse anti-rabbit IgG and FITC-labeled goat anti-mouse IgG secondary antibodies. Cells were fixed in 1% paraformaldehyde and analyzed on a FACStarPLUS flow cytometer (BD Biosciences, CA).

2.4. Activation of signalling molecules

To determine the role H4 receptor in the downstream signal transduction, the mast cells (1×10^6) were stimulated with histamine (10 μ M) and 4-methylhistamine (10 μ M) for 0, 1, 3, 10, 30 min. Furthermore, to distinguish activation of histamine H4 and H1 receptors, the mast cells were pre-treated with the H4R

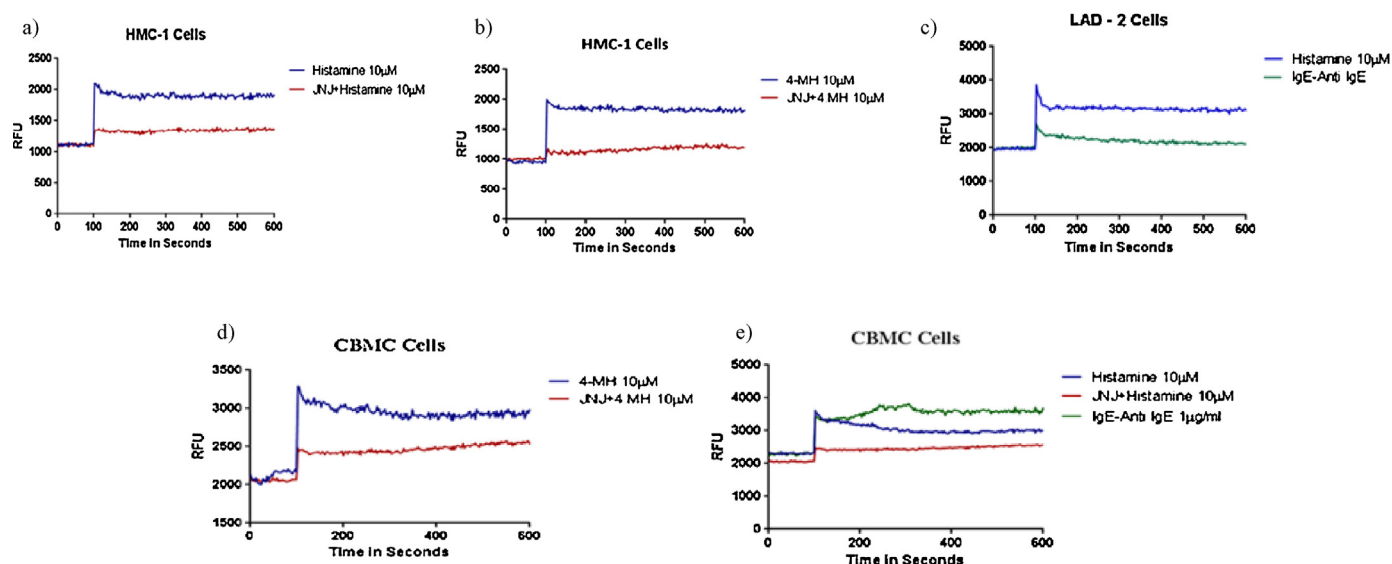


Fig. 3. Effects of histamine receptor agonist and antagonist on intracellular calcium release in human mast cells [(a and b) HMC-1 cells (c) LAD-2 cells and (d and e) cord blood derived CD34⁺ human mast cells]. The data showing increase of Ca²⁺ influx fluorescence from baseline to the maximum after stimulated with histamine (10 μ M) and 4-MH (10 μ M). JNJ777120 (10 μ M) showed inhibition Ca²⁺ release in response to histamine and 4-MH in (a, b) HMC-1 cells and (d, e) cord blood derived CD34⁺ human mast cells. (c) LAD-2 cells showed intracellular Ca²⁺ release with 10 μ M histamine and Anti IgE. Data shown are representative of three similar experiments.

antagonist JNJ777120 (10 μ M) and H1R antagonist mepyramine (10 μ M) followed by stimulated with 10 μ M of histamine and 4-methylhistamine. The cell lysates were separated on SDS-PAGE and blots were probed with anti-phospho-ERK1/2 and anti-phospho-Akt antibody followed by anti-rabbit- IgG-HRP (Santa Cruz). Respective blots were stripped and reprobed with anti-ERK or Anti-Akt antibody (Cell Signalling Technology) followed by anti-rabbit-IgG-HRP (Santa Cruz). The bands were visualized by SuperSignal West Pico chemiluminescent substrate.

2.5. Western blotting

The immune cells (1×10^6 cells/ml) were lysed in RIPA buffer. Proteins concentration was quantified by Bradford method and was separated by SDS-PAGE; Western blotting was performed with rabbit anti human H4R antibody, mouse antihuman H1R antibody and probed with goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP secondary antibodies (Santa Cruz, USA). Immunoreactive bands were visualized by ECL (GE Amersham, USA) and the image was captured using multi imaging system (Cell Biosciences, USA).

2.6. Degranulation assay

Mast cells (5×10^4 cells/well) were seeded into 96-well plates in a total volume of 50 μ l of SIR buffer containing 0.1% BSA and exposed to different concentrations of histamine, 4-methylhistamine, anti-human IgE for 30 min and for inhibition studies cells were pre-treated with 10 μ M of JNJ777120 and mepyramine. For total β -hexosaminidase release, control cells were lysed in 50 μ l of 0.1% Triton X-100. Aliquots (15 μ l) of supernatants or cell lysates were incubated with 15 μ l of 1 mM *p*-nitro phenyl-*N*-acetyl- β -d-glucosamine for 1 h at 37 $^{\circ}$ C. Reaction was stopped by adding 250 μ l of a 0.1 M Na₂CO₃/0.1 M NaHCO₃ buffer and absorbance was measured at 405 nm (Ali et al., 1944).

2.7. Cysteinyl leukotrienes and LTB₄ measurement

Mast cells (1×10^6 cells/well) were incubated with or without human IgE (1 μ g/ml) for overnight and washed twice in basal

medium. The cells were stimulated with histamine (10 μ M), 4-methylhistamine (10 μ M) or anti-human IgE (1 μ g/ml) for 30 min. For inhibition studies, cells were pre incubated with 10 μ M JNJ777120 (10 min) followed by stimulation was done with 4-methylhistamine and histamine. Cell free supernatants harvested for cysteinyl leukotrienes estimation using cysteinyl leukotrienes EIA kit and LTB₄ EIA kit (Invitrogen, Cayman chemicals) according to manufacturer's instructions.

2.8. Calcium measurements

Mast cells were plated (2×10^5 cells/well) on black flat-bottom 96-well plates and then they were labeled with FLUO-4 according to manufacturer's instructions. Intracellular Ca²⁺ release was measured in a multimode reader and fluorescence (excitation, 494 nm; emission, 516 nm) was measured per three seconds for 600 s and at a time of 100 s, histamine (10 μ M), 4-methylhistamine (10 μ M) and anti-human IgE (1 μ g/ml) were added to the respective wells

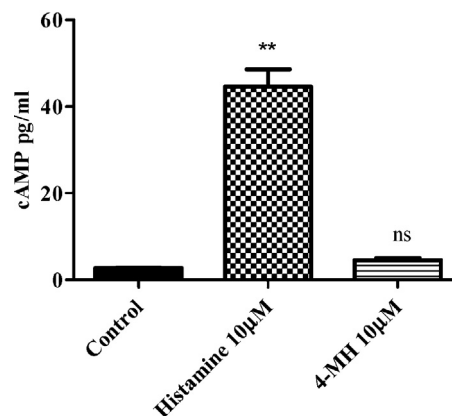


Fig. 4. Effects of histamine and 4-methylhistamine induced cAMP level in human mast cells. Mast cells were stimulated with 10 μ M histamine and 10 μ M of 4-MH. The cAMP level was quantified from the cell free supernatants by competitive ELISA. Data expressed as mean $\pm$ SD of three experiments done in triplicates. ** P < 0.001, significantly different from control. ns-refers to non significant.

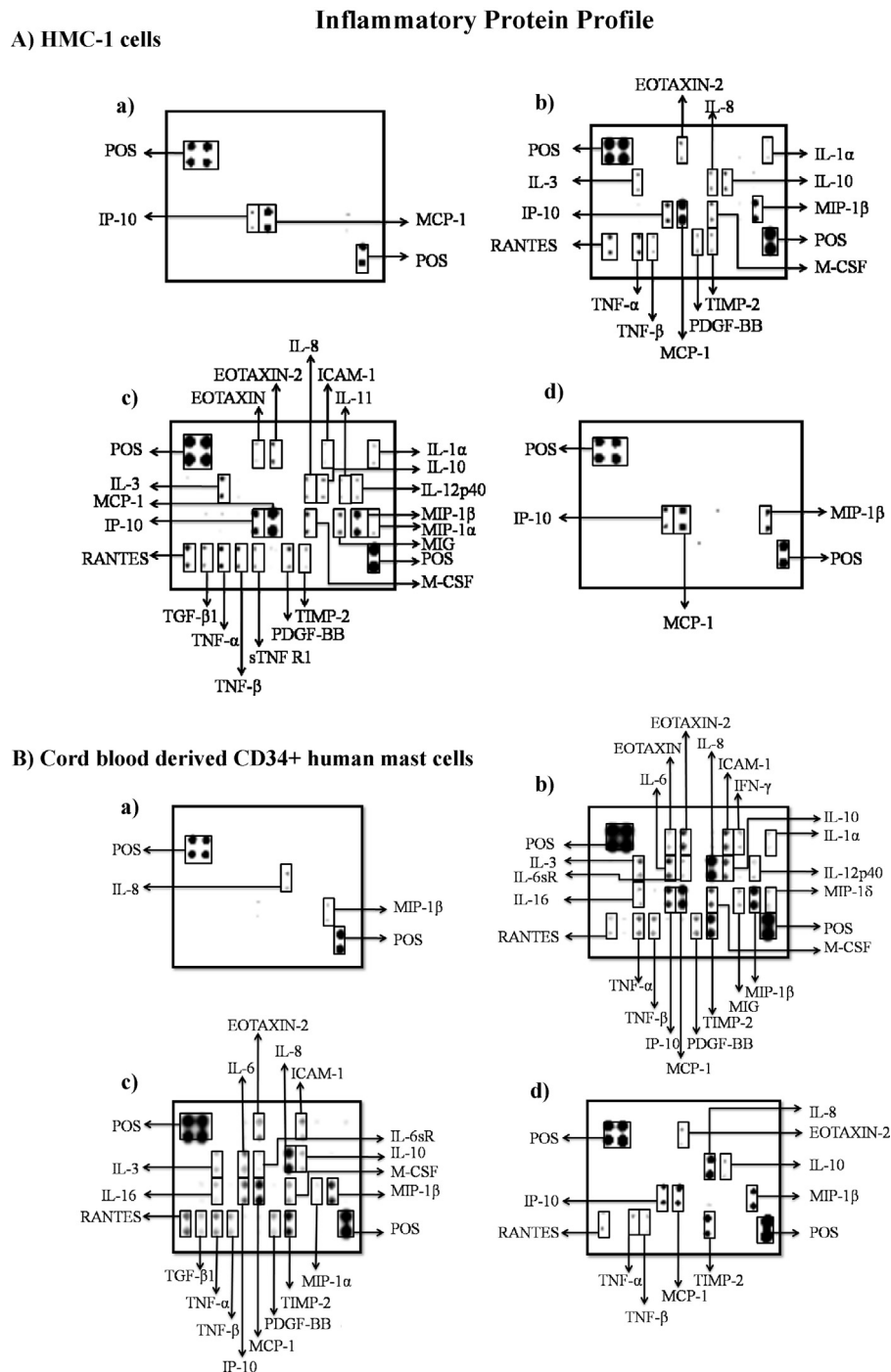


Fig. 5. Identification of H4R mediated inflammatory protein expression using human Inflammation antibody array technique in (5. A) HMC-1 cells and (5. B) Cord blood derived CD34+ human mast cells. Representative figure showing the expression inflammatory proteins [(a) control, (b) histamine, (c) 4-MH, (d) JNJ7777120 and 4-MH] after overnight stimulation with histamine (10 μ M) and 4-MH (10 μ M). Effect of H4R antagonist JNJ7777120 (10 μ M) against 4-MH (10 μ M) induced on inflammatory mediators release also shown.

and for inhibition studies, before stimulation cells were pre treated with 10 μ M of JNJ7777120 for 10 min.

2.9. cAMP assay

Mast cells (1×10^6 cells/well) were washed twice in basal medium and stimulated with histamine (10 μ M) and 4-methylhistamine (10 μ M) for 30 min. Cell free supernatants were harvested for cAMP analysis using cAMP EIA kit (Cayman chemicals) according to manufacturer's instructions.

2.10. Human inflammation antibody array

Mast cells (1×10^6 cells/well) were washed twice in basal medium, followed by stimulated with histamine (10 μ M), 4-methylhistamine (10 μ M) for overnight. For inhibition studies, cells were pre incubated with 10 μ M JNJ7777120 for 30 min, followed by stimulation was done with 4-methylhistamine for overnight. Cell free supernatants harvested for inflammatory protein analysis using inflammation cytokine array according to manufacturer's instructions. The image

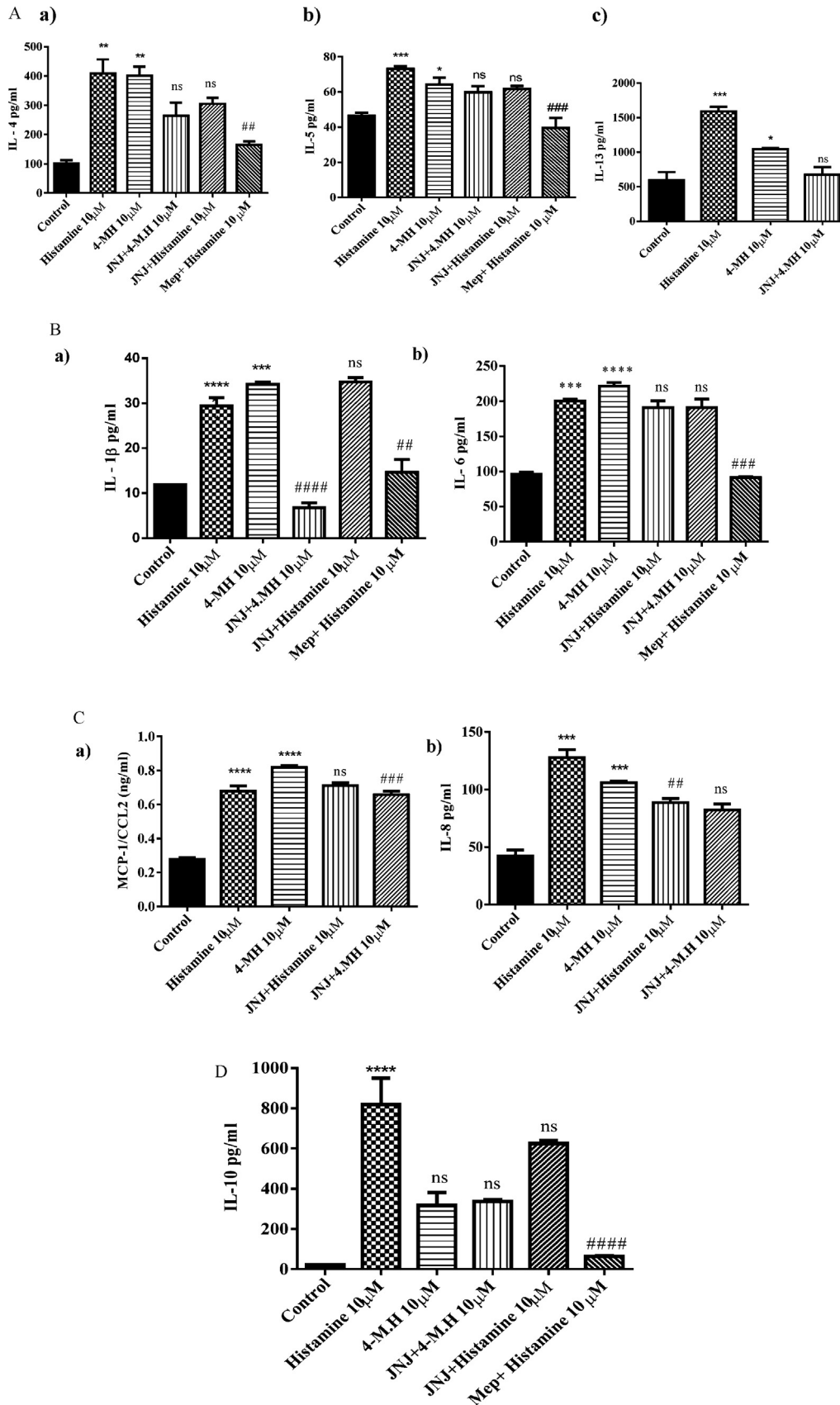


Fig. 6. A. Histamine and 4-MH induced Th2 cytokine release in cord blood derived CD34⁺ human mast cells and HMC-1 cells. Figure showing the level of (a) IL-4, (b) IL-5 and (c) IL-13 after 24 h of stimulation with histamine (10 μ M) and 4-MH (10 μ M). Pre incubation with the H1R antagonist mepyramine (10 μ M) and H4R antagonist JNJ7777120 (10 μ M) against histamine and 4-MH also shown. Values are analyzed using one-way ANOVA with Bonferroni's test. Data expressed as mean $\pm$ SD of three experiments done in triplicates. *** P < 0.0001, ** P < 0.001, significantly different from control. ## P < 0.001, significantly different from histamine and 4-MH. ns-refers to non significant. **Fig. 6B.** Determination of H4R mediated classical pro-inflammatory cytokines in human mast cells. Figure showing the level of (a) IL-1 β and (b) IL-6 after 24 h of

was captured by multi imaging system, Cell Biosciences, USA.

2.11. Measurement of cytokines and chemokines by ELISA

Mast cells (1×10^6 cells/ml per well in a basal medium) were stimulated with histamine (10 μ M), 4-methylhistamine (10 μ M) for 24 h. For inhibitor studies, cells were preincubated with 10 μ M of JNJ777120, mepyramine (30 min) and then stimulated with histamine (10 μ M) and 4-methylhistamine (10 μ M) for 24 h. Supernatants were collected and stored frozen at -20°C , until analysis. Inflammatory cytokines and chemokines such as IL-5, IL-4, IL-6, IL-8, IL-13, IL-10, IL-1 β and MCP-1/CCL2 levels were quantified from the same supernatant using sandwich ELISA as described in the manufacturer's protocols. The reaction was read at 450 nm in a micro plate reader (Biotek Synergy HT multimode reader, USA).

2.12. Statistical analysis

The difference in estimated parameters between the groups was analyzed using one-way ANOVA with Bonferroni's test. Data expressed as mean $\pm$ SD. All parameters were analyzed at 95% confidence intervals and P value of <0.01 was considered to be statistically significant. Statistical analysis of the data was performed using Graph Pad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

3. Results

3.1. Determination of Histamine H1 and H4 receptors expression in human immune cells

Among the four different types of histamine receptors (H1–H4), H4R is predominantly expressed in immune cells and is involved in immunomodulatory response. Here in this study, we determined the expression of H4R in human immune cells like monocytes, T-cells and mast cells such as HMC-1, LAD-2 and cord blood derived CD34+ human mast cells using flowcytometry and western blotting (Fig. 1A and B). Both H1R and H4R are present in all the cell types tested but compared to H1R, H4R expression was found to be high in human mast cells.

3.2. Functional activation of H4R in human mast cells

To further characterize the functional properties of H4R in human mast cells we used both the cell lines HMC-1, LAD-2 and primary cord blood derived CD34+ human mast cells. Upon activation, mast cells release their granular contents by the process of degranulation. In order to find out the extent of degranulation in mast cells by histamine and 4-methyl histamine, we assayed the release of β -hexaminidase. We found that in human mast cells, histamine induced 26.48%, 28.86%, 53.92% and 4-methylhistamine induced 36.76%, 37.21%, 63.44% of degranulation in HMC-1 cells, LAD-2 cells and cord blood derived CD34+ human mast cells respectively (Fig. 2A). Furthermore, we have used H4R antagonist JNJ777120 and H1R specific antagonist mepyramine to find out the histamine

and 4-methylhistamine induced mast cell degranulation. However, we found that JNJ777120 caused more inhibition than mepyramine (Fig. 2A, a). Histamine mediates its biological responses via the stimulation of GPCR that activate phospholipase C to cause intracellular Ca^{2+} mobilization (Leurs et al., 1995). Although, histamine binds to all of its four receptors (H1R–H4R) with varied affinity and distinct effects on different cell type, 4-methylhistamine binds only to H4R. Therefore, we studied the effects of 4-methylhistamine which is a selective and specific agonist for H4R and compared with histamine in human mast cells (Fig. 3). Activation by both 4-methylhistamine and histamine caused sustained intracellular calcium release in HMC-1, LAD-2 and cord blood derived CD34+ human mast cells and standard H4R antagonist JNJ777120 showed inhibition of H4R mediated intracellular calcium release. But cAMP level was increased when stimulated with histamine only. This is due to binding of histamine to H1R which couples to $\text{G}\alpha_q$ whereas, H4R activate $\text{G}\alpha_i/o$ which in turn inhibits cAMP production (Morse et al., 2001; Matsubara et al., 2005; De Esch et al., 2005) (Fig. 4).

3.3. Histamine H4 receptor mediated leukotriene generation in human mast cells

Upon activation, mast cells also has the ability to release a broad array of bioactive mediators, either preformed and stored in granules such as histamine and proteases, or newly synthesized mediators such as eicosanoids, cytokines, and chemokines (Gilfillan and Beaven, 2011). Therefore, we investigated histamine and 4-methylhistamine induced production of lipid derived mediators such as cysteinyl leukotrienes and LTB4 in HMC-1 cells and cord blood derived CD34+ human mast cells (Fig. 2B). Both histamine and 4-methylhistamine induced the release of cysteinyl leukotrienes and LTB4 release in cord blood derived CD34+ human mast cells. In addition, the leukotriene production was found to be (data not shown) insignificant which indicate that the immaturity of the HMC-1 cells.

3.4. Histamine H4 receptor mediated inflammatory protein release in human mast cells

The human inflammation antibody array technique was employed to profile Histamine H4 receptor mediated release of inflammatory proteins in HMC-1 cells and primary human cord blood derived CD34+ mast cells. Inflammation antibody array can detect 40 different inflammatory proteins from the same sample, in which we were able to detect fourteen inflammatory cytokines and chemokines in response to histamine (Eotaxin-2, IL-8, IL-3, IP-10, RANTES, TNF- α , TNF- β , MCP-1, PDGF-BB, TIMP-2, IL-1 α , IL-10, MIP-1 β and M-CSF) and twenty two in response to 4-methylhistamines (IL-8, Eotaxin, Eotaxin-2, ICAM-1, IL-11, IL-1 α , IL-10, IL-12p40, MIP-1 β , MIP-1 α , MIG, M-CSF, TIMP-2, PDGF-BB, STNF-R1, TNF- β , TNF- α , TGF- β 1, IL-3, MCP-1, IP-10 and RANTES) in HMC-1 cells (5A). Whereas, (5B) cord blood derived CD 34+ human mast cells released twenty three inflammatory proteins in response to histamine such as IL-3, IL-16, IL-6Sr, RANTES, IL-6, Eotaxin, Eotaxin-2, IL-8, ICAM-1, IFN- γ , IL-10, IL-1 α , IL-12p40, MIP-1 δ , M-CSF, MIP-1 β , MIG, TIMP-2, PDGF-BB, MCP-1, IP-10, TNF- β , TNF- α

stimulation with histamine (10 μ M) and H4R agonist 4-MH (10 μ M) and inhibitory effect by pre incubation with the H1R antagonist mepyramine (10 μ M) and H4R antagonist JNJ777120 (10 μ M) against histamine and 4-MH. Values are analyzed using one-way ANOVA with Bonferroni's test. Data expressed as mean $\pm$ SD of three experiments done in triplicates. *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.01$ significantly different from control. ### $P < 0.001$, significantly different from histamine and 4-MH. ns-refers to non significant. Fig. 6C. Measurement of H4R mediated chemokines release in human mast cells. Figure showing the level of (a) MCP-1 and (b) IL-8 after 24 h of stimulation with histamine (10 μ M) and H4R agonist 4-MH (10 μ M) and inhibitory effect by pre incubation with the (10 μ M) H4R antagonist JNJ777120 against 4-MH. Values are analyzed using one-way ANOVA with Bonferroni's test. Data expressed as mean $\pm$ SD of three experiments done in triplicates. *** $P < 0.0001$, **** $P < 0.0001$, significantly different from control. ### $P < 0.001$, significantly different from histamine and 4-MH. ns-refers to non significant. Fig. 6D. Measurement of H4R mediated release of anti-inflammatory cytokine (IL-10) in human mast cells. Figure showing the level of IL-10 after 24 h of stimulation with histamine (10 μ M) and 4-MH (10 μ M) and inhibitory effect by pre incubation with the H1R antagonist mepyramine (10 μ M) and H4R antagonist JNJ777120 (10 μ M) against 4-methylhistamine. Values are analyzed using one-way ANOVA with Bonferroni's test. Data expressed as mean $\pm$ SD of three experiments done in triplicates. ** $P < 0.001$, significantly different from control. ns-represents non significant.

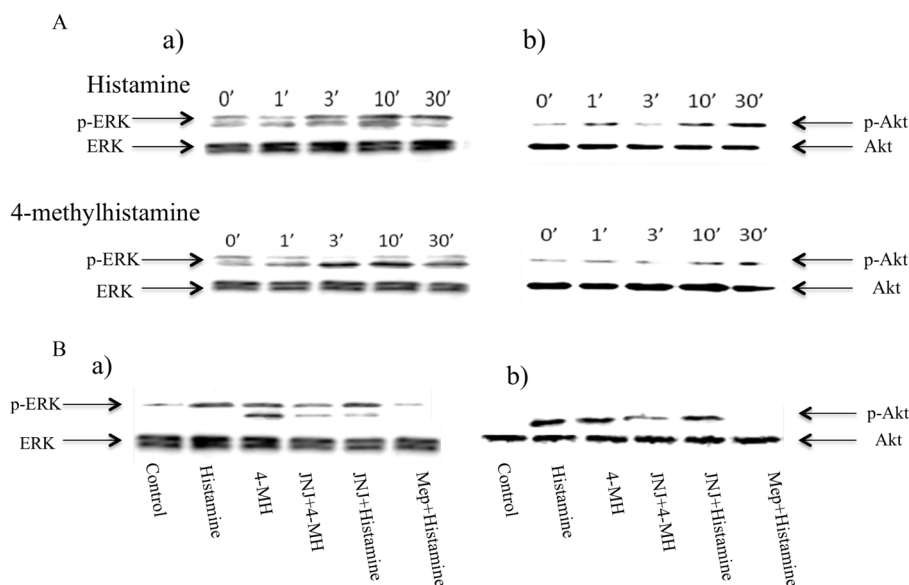


Fig. 7. A. Histamine and 4-methylhistamine activates sustained ERK and Akt phosphorylation in human mast cells. Mast cells (1×10^6) were stimulated with histamine ($10 \mu\text{M}$) and 4-methylhistamine ($10 \mu\text{M}$) for 0, 1, 3, 10, 30 min. Cell lysates were separated on SDS-PAGE and blots were probed with (a) anti-phospho-ERK1/2 and (b) anti-phospho-Akt antibody. Respective blots were stripped and reprobed with anti-ERK (a) or Anti-Akt antibody (b) followed by anti-rabbit-IgG-HRP. Immunoreactive bands were visualized by SuperSignal West Pico chemiluminescent substrate. Blots shown here is representative of three similar experiments. **Fig. 7B.** Effect of H1 and H4 antagonists on ERK and Akt phosphorylation in human mast cells. Mast cells (1×10^6) were pretreated with $10 \mu\text{M}$ of JNJ777120 and Mepyramine for 30 min followed by stimulated with $10 \mu\text{M}$ of histamine and 4-methylhistamine. Cell lysates were separated on SDS-PAGE and blots were probed with (a) anti-phospho-ERK1/2 and (b) anti-phospho-Akt antibody. Respective blots were stripped and reprobed with anti-ERK (a) or Anti-Akt antibody (b) followed by anti-rabbit-IgG-HRP. Immunoreactive bands were visualized by SuperSignal West Pico chemiluminescent substrate. Blots shown here is representative of three similar experiments.

and nineteen inflammatory proteins were expressed in response to 4-methylhistamine such as IL-3, RANTES, TGF- β 1, TNF- α , TNF- β , PDGF-BB, TIMP-2, M-CSF, MCP-1, IP-10, IL-16, IL-6, IL-8, IL-10, MIP-1 α , ICAM-1, Eotaxin-2, IL-1 α and IL-6sR as shown in Fig. 5.

3.5. Determination of histamine and 4-methylhistamine induced Th2/Th1 cytokines and chemokine release in human mast cells

Furthermore, we have quantified the level of inflammatory cytokines and chemokines produced by mast cells via H4R. Though Th2 response was dominant in allergic reactions, mast cells through H4R were able to produce not only Th2 cytokines - (Fig. 6A) IL-4 (401.34 pg/ml, IL-5 (64.21 pg/ml) and IL-13 (1044 pg/ml) but also other classical proinflammatory cytokines - (Fig. 6B) IL-1 β (34.24 pg/ml) and IL-6 (221.27 pg/ml), and immunoregulatory cytokines - (Fig. 6D) IL-10 (317.059 pg/ml) and also chemokines (Fig. 6C) such as IL-8 (106 pg/ml) and MCP-1(818.32 pg/ml). Though, both histamine and 4-methylhistamine induced the release of IL-6, IL-1 β , IL-4 and IL-5, only mepyramine inhibited the histamine induced cytokine release. Whereas, IL-1 β production by histamine and 4-methylhistamine was inhibited by mepyramine and JNJ777120 respectively.

3.6. Analysis of histamine and 4-methylhistamine induced activation of ERK and AKT in human mast cells

In order to find out the signalling pathway utilized by H1R and H4R downstream of Ca $^{2+}$, we have analyzed the activation of ERK and Akt in human mast cells. Also, to distinguish the H1R and H4R mediated effect on both ERK and Akt phosphorylation, mast cells were pre-treated with H1R antagonist mepyramine and H4R antagonist JNJ777120 and stimulated with histamine and 4-methylhistamine. As shown in Fig. 7A histamine and 4-methylhistamine induced the phosphorylation of ERK and Akt in a time dependent manner. However, the H4R antagonist JNJ777120 showed partial inhibition of both ERK and PI3 K activation whereas;

H1 antagonist mepyramine completely inhibited the PI3 K phosphorylation, but showed partial inhibition of ERK phosphorylation as shown in Fig. 7B.

4. Discussion

In the present study, we characterized the properties of H4R in human mast cell lines HMC-1, LAD-2 and primary cord blood derived CD34+ human mast cells. We found that both the mast cell types express cell surface histamine H1R and H4R receptors. Furthermore, they responded to both histamine and 4-methylhistamine for Ca $^{2+}$ mobilization, degranulation and cytokine production. Histamine activates natural killer cells (Damaj et al., 2009), eosinophils (Damaj et al., 2007; Reher et al., 2012) and monocytes (Gutzmer et al., 2005) to induce chemotaxis, degranulation and cytokine production (Zhang et al., 2007). The data presented here in demonstrates that histamine and 4-methylhistamine induce degranulation (>50% in cord blood derived CD34+ human mast cells) as that of IgE (Fc ϵ RI) receptors activation in human mast cells (Kinet, 1999). This shows that mast cells via H4R also trigger a powerful inflammatory cascade (Thurmond et al., 2008). Because histamine release in the lungs is a well recognized feature of allergic asthma and is associated with a cascade of events, including airway constriction, mucus secretion and selective recruitment of inflammatory cells to the lungs (Michel et al., 2004).

Previously it has been shown that histamine caused Ca $^{2+}$ mobilization in eosinophil (Reher et al., 2012), monocytes (Dijkstra et al., 2007), bone marrow derived mast cells (Hofstra et al., 2003) and dendritic cells (Gschwandtner et al., 2010). Here we demonstrate that both histamine and 4-methylhistamine induced a sustained intracellular calcium level in all mast cell types studied. However, only histamine but not 4-methylhistamine caused increase in cAMP level, which shows the differences in receptors subtypes (Seifert et al., 2013), that H1R couples to G α_q and H4R binds to G α_i/o

proteins and thereby inhibiting cAMP level (Morse et al., 2001; Matsubara et al., 2005; De Esch et al., 2005; Lippert et al., 2004).

Furthermore, it has also been shown that the cross-linking of high affinity FC receptors for IgE (FcεRI) by allergen caused cysLTs generation in human lung mast cells (MacGlashan et al., 1982; Venkatesha et al., 2005) and histamine induced H4R activation caused the release of LTB4 *in vivo* condition (Takeshita et al., 2003). We also showed that human mast cells were able to release cysteinyl leukotrienes and LTB4 via H4R. This clearly indicates that, H4R also triggers the release of lipid derived mediators as that of IgE induced activation. Here, the standard H4R antagonist JNJ7777120 was employed to confirm the H4R mediated functional activation in human mast cells.

Available data on activation of H4R in inflammatory cells indicated the release of different cytokines and chemokines, which are measured either individually or only few cytokines. For instance in dendritic cells H4R mediated the production of IL-1β (Simon et al., 2011), IL-31 from Th2 cells (Gutzmer et al., 2009), IL-17 from Th17 cells (Mommert et al., 2011), TNF-α from monocytes (Damaj et al., 2007) and secretion of IL-16 from CD8+ T cells (Gantner et al., 2002) and enhances LPS induced IL-6 production in mast cells (Desai and Thurmond, 2011) and also the upregulation of cell adhesion molecule CD11b in eosinophils (Buckland et al., 2003). Additional evidence provided the involvement of H4R in the down regulation of IL-12p40 production in monocyte derived dendritic cells (Gutzmer et al., 2005) and inhibition of TNF-α and IL-12 in slan-dendritic cells (Gschwandtner et al., 2010). An interesting finding of the present study was that, through H4R mast cells released a vast array of inflammatory cytokines and chemokines, that are closely associated not only in allergic asthma but also in other inflammatory conditions. These are mainly Th2 cytokines such as IL-4, IL-5, IL-13 and pro inflammatory cytokines like IL-6, IL-1β and immuno regulatory cytokine IL-10 and chemokines such as IL-8, MCP-1 which are chemo attractants of leukocytes. Thus, activation of mast cells via H4R can amplify inflammatory cascade through the release of various inflammatory mediators which in turn may activate other inflammatory cells into the site of inflammation. Previously, it was thought that the allergic reactions are mainly mediated through histamine H1 receptor and recent studies has been proven that not only H1R, H4R also involved in the development of various allergic inflammatory reactions (De Esch et al., 2005). In addition, we found that though both histamine and 4-methylhistamine induced the release of IL-6, IL-1β, IL-4 and IL-5, only mepyramine inhibited the histamine induced cytokine release. Whereas, IL-1β production by histamine and 4-methylhistamine was inhibited by mepyramine and JNJ7777120 respectively. Previous findings indicated that JNJ7777120 inhibited H4R mediated cytokines release (Desai and Thurmond, 2011; Beermann et al., 2012) in different cell types.

The most important observation in our study is using human inflammation array technique; we profiled the inflammatory proteins that are released upon activation by H4R. These are mainly TGF-β1, TNF-α, TNF-β, PDGF-BB, TIMP-2, M-CSF, IP-10, IL-16, IL-6, IL-3, IL-10, MIP-1α, IL-1α, ICAM-1, Eotaxin-2, RANTES, IL-8, MCP-1, and IL-6sR which are the powerful inflammatory mediators for inflammatory cells which are closely associated with allergy and asthma.

Furthermore, both histamine and 4-methylhistamine induced a sustained ERK and Akt phosphorylation in human mast cells and this effect was partially inhibited by JNJ7777120 and mepyramine completely inhibited Akt phosphorylation and partially inhibited ERK phosphorylation. This finding is in consistence with the previous reports (Desai and Thurmond, 2011) indicating that, the H4R activation required both ERK and PI3K phosphorylation for the production of IL-6 in bone marrow derived mast cells.

In conclusion, functional activation of H4R in mast cells leads to the production of various inflammatory mediators including, various cytokines, chemokines which are not only associated with allergic asthma, but also other inflammatory diseases. These findings make the H4R an extremely attractive target for the treatment of various inflammatory diseases. Further delineation of signalling pathways involved is under progress.

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