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Dampening of mast cell secondary responses to allergen involves specific signalling and epigenetic changes

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ABSTRACT

Allergic diseases are increasing worldwide. Allergen and IgE dependent mast cell (MC) activation is the major initiator of these clinical symptoms. During this study, the effect of multiple exposures to the same allergen, on MC degranulation was studied. First, MC recovery in terms of surface expression of high affinity receptor FcεRI, and granule content after a primary allergen challenge was confirmed. Overall, previous exposure of MCs to allergen challenge led to a significant reduction in pre-stored mediator release during the secondary challenge at various time points and with various doses of allergen *in vitro*. The dampened response was not due to any defects in very early steps in signalling involving FcεRI activation. Inhibition of dampening response during secondary challenge by various inhibitors like wortmannin, tranylcypromine and pargyline, indicated the involvement of PI3K signalling and chromatin modifications. Our study provides insight into new therapeutic avenues for treating allergic disorders targeting MCs.

1. Introduction

Increasing prevalence of allergies and related diseases is a matter of concern worldwide including developing countries [1]. According to WAO (World allergy organization), 30–40% of the world population is being affected by one or more allergic conditions. These allergic conditions include asthma, allergies to food, drugs and insects, life-threatening acute anaphylaxis, allergic rhinitis, conjunctivitis, urticaria, eczema and eosinophilic disorders, including eosinophilic esophagitis [2,3]. MCs are one of the main mediators of the allergic and inflammatory disorders.

MCs are multifunctional secretory cells of the immune system packed with secretory granules containing bioactive mediators such as TNF-α, histamine, serotonin and enzymes like tryptase and β-hexosaminidase. These mediators are released at the site of infection or inflammation and further play an important role in pathophysiology of allergic disorders such as asthma and anaphylaxis. Activation of MCs can be induced by triggers engaging various receptors on their surface such as high affinity FcεRI receptors. Cross-linking of FcεRI-bound IgE by multivalent allergen leads to aggregation of surface FcεRI, which further initiates a complex downstream signalling cascade resulting in pre-stored mediator release [4], production and secretion of lipid mediators and cytokines. Antigen induced activation through IgE and FcεRI receptors leads to T_H2- and IgE–dependent immune responses [5]. MCs have been shown to be associated with pathogenesis of allergic and inflammatory disorders such as IgE-mediated food allergy [6] asthma [7] arthritis, atopic dermatitis, psoriasis and multiple sclerosis [8] by inducing various mechanisms like tissue remodelling, local inflammation; and migration, accumulation and activation of innate immune cells [4].

The innate immune cells being the first to encounter intruders including pathogens and allergens [9] are the first to detect, to respond and also modulate or orchestrate the overall immune response to these intruders. Hence, any means to manipulate the responses of these innate immune cells can affect overall immune responses. Along with above characteristics MCs have the ability to survive for prolonged periods after activation compared with other innate immune cell types that may begin to die during the contraction phase of the innate response [10]. MCs can survive in the tissues and might proliferate in response to appropriate stimuli [11] and are able to replenish the granules and undergo repeated rounds of activation and recovery followed by mediator release and up regulation of cytokine mRNA

Abbreviations: MCs, Mast cells; BMMCs, Bone marrow derived mast cells; RBL-2H3, Rat basophilic leukemia; DNP-BSA, Dinitrophenyl-Bovine serum albumin; TNF-α, Tumor necrosis factor alpha; FcεRI, high-affinity IgE receptor; PI3K, phosphoinositide 3-kinase; WRT, Wortmannin; PAR, Pargyline; TCPA, Tranylcypromine; LSDI, Lysine-specific demethylase 1; CFSE, Carboxyfluorescein diacetate succinimidyl ester; SNAREs, Soluble NSF Attachment Protein REceptor; LPS, Lipopolysccharide; TCR, T cell receptor; PLC, Phospholypase C; Ag, Antigen

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[12,13]. Rapid desensitization [14,15] and endotoxin tolerance have been well studied in macrophages [16] and recently in MCs [17]. Both of these procedures result in hyporesponsiveness of BMMCs and PMCs involving different mechanisms like loss of surface IgE (internalization) or BCL3 involving pathway, respectively. Some of these altered responses have been described as trained immunity something akin to memory or tolerance in adaptive immune system [18].

The therapies available for allergic diseases may target MC membrane, intracellular and extracellular events [19]. Cell membrane targeting therapies involve use of chromones (disrupt calcium influx), omalizumab (anti-IgE) [20], β2 adrenergic receptor agonists (Increase cytosolic cAMP levels) and CCR3 antagonists (inhibit degranulation). Intracellular and extracellular therapies target signalling pathway such as MAP or Syk kinase inhibitors, and antagonists and inhibitors of certain receptors, respectively [19]. Cell membrane targets are normally used as MC stabilizers with potential therapeutic applications. Except few, most of them target MCs either indirectly or weakly. And single therapy is not sufficient to treat allergies; targeting mast cells directly at molecular level may provide add-on therapy.

In this study, we targeted MCs directly to limit the pathogenesis of allergies; we investigated modulations of MC responses after antigen challenge. Here, we describe the post antigen challenge effects on MC responses, induced tolerance and its mechanism in MCs. Experiments were carried out using Rat Basophilic Leukaemia (RBL-2H3) cell line and further confirmed in primary murine MCs generated from precursors isolated from bone marrow (BMMCs) *in vitro*. To mimic allergic condition *in vitro*, cells were sensitized with antigen specific IgE and challenged with antigen (DNP-BSA) twice. Antigen induced degranulation (release of early phase mediator such as β-hexosaminidase and histamine) of MCs was investigated and found to be reduced/dampened after second exposure with same antigen in various conditions. We further investigated the underlying mechanisms in modulation of MC responses/antigen induced tolerance. This study may provide an insight into new therapeutic avenues for treating allergic disorders targeting MCs.

2. Material and methods

2.1. Animals

Inbred C57BL/6 mice (8–12 weeks) were procured from the National Institute of Nutrition, Hyderabad, India. They were maintained under pathogen free conditions at 25 °C and 50% relative humidity and a 12 h light and dark cycle in the central laboratory animal resources at Jawaharlal Nehru University (JNU), New Delhi, India. Both water and food were provided *ad labitum*. All experimental protocols were approved by the JNU Institutional Animal Ethical Committee (IAEC code 13/2013 dated 18 December 2014).

2.2. Cell cultures

Rat basophilic leukemia mast cells (RBL-2H3) were purchased from ATCC, USA and maintained in RBL complete medium containing equal parts of Minimum Essential Medium Eagle with Earle's salts and Iscove's Modified Dulbecco's Medium both from Gibco, Life technologies, Grand Island, NY, USA supplemented with 17% heat- inactivated Fetal Bovine Serum (FBS) (Gibco, life technologies, Grand Island, NY, USA), 25 mM HEPES (N-[2-100 hydroxyethyl]piperazine-N0-[2-ethanesulfonic acid]) (Sigma, MO, USA), and 120 μg/ml gentamicin sulphate [21]. Cells were maintained as sub-confluent monolayers at 37 °C in a humidified atmosphere containing 5% CO_2 and passaged with trypsin (Sigma, MO, USA). TIB-142 cell line (IGEL a2 [15.3], ATCC, USA) was used as source of anti-DNP IgE. This cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat- inactivated FBS, 1 mM Sodium pyruvate (Gibco), HEPES and antibiotics between 0.5×10^6 and 2×10^6 cells/ml density in 5% CO_2 incubator at 37 °C. Cell free supernatants were collected after each passage; filter sterilized and stored at −80 °C. These supernatant from TIB-142 cells were used to sensitize MCs *in vitro* at optimum dilution [21,22].

2.3. Generation of murine bone marrow derived mast cells

BMMCs were generated by isolating bone marrow cells from Femur and tibias of 8–12 weeks old C57BL/6 mouse by repeated flushing with RPMI medium as described earlier [23]. Briefly, cell culture was maintained in RPMI-1640 medium supplemented with 17% FBS (Gibco), 4 mM L-glutamine (Sigma), 5×10^5 M β-mercaptoethanol, 1 mM sodium pyruvate (Sigma), 0.1 mM nonessential amino acids (Sigma), antibiotics (Penicillin & Streptomycin, Himedia) and murine IL-3 (20 ng/ml; Peprotech) and murine stem cell factor (SCF) (20 ng/ml; Peprotech) at 10^6 cells/ml density at 37 °C and 5% CO_2. Cells were passaged by discarding adherent cells and collecting non-adherent cells by centrifugation at $239 \times g$ for 10 min at room temperature, then seeding 1 million cells per ml in new flask containing fresh culture media twice a week. At different time points, the cells were sensitized by IgE, and stained with PE (Phycoerythrin) conjugated anti mouse IgE antibodies (Biolegend) (for indirect detection of FcεRI, by detecting IgE bound to FcεRI), and also with APC (Allophycocyanin) conjugated anti mouse CD117 (c-kit) antibodies (Biolegend) to detect mature MCs. After 28 days BMMCs were confirmed by flow cytometry showing more than 95% c-kit and FcεRI (by indirect IgE staining) [24] (Supplementary Fig. S1).

2.4. Stimulation of MC degranulation during primary and secondary allergen challenge

BMMCs (0.5×10^6) and RBL-2H3 cells (0.3×10^6) were seeded in 24 well plates and allowed to settle for 3–4 hr. Then cells were sensitized with anti-DNP-BSA IgE (TIB 142 supernatant, 1:100 dilution) at 37 °C for 16–18 h [21,22]. After incubation, cells were washed twice with pre-warmed RPMI (without phenol red; Sigma, used as stimulation buffer in all experiments in this study) to remove excess or unbound IgE. Degranulation from MCs was induced by receptor cross-linking using DNP-BSA (100 ng/ml, Sigma, MO, USA) for 45 min unless mentioned otherwise at 37 °C and supernatants were collected after mentioned time intervals. Mock stimulation was done by using RPMI media only in IgE-sensitized cells. At the end of the experiment, cells were lysed with lysis buffer containing 0.2% Triton-X 100 (Sigma-Aldrich) to measure total and cell-associated β-hexosaminidase.

For secondary challenges, cells were activated as described above. Briefly, after primary challenge, cells were washed with ice cold RPMI and incubated in fresh culture medium for 6 hr at 37 °C. After incubation, cells were harvested by trypsinization and seeded for secondary challenge keeping the cell density same as primary challenge (0.3×10^6 RBL-2H3 and 0.5×10^6 BMMC). Then sensitized with IgE for 16–18 hr and stimulated with 100 ng DNP-BSA for 45 min. The time gap between the two challenges was 24 hr in both cell types.

For time kinetics, MCs were activated for various time points ranging from 0 min to 75 min. 100 ng DNP-BSA (diluted in RPMI without phenol red) was added and incubated for 15 min supernatant was collected and replaced with fresh DNP-BSA (100 ng). This step was repeated several times at 15 min time interval. For time kinetics during secondary challenge, stimulation with 100 ng DNP-BSA for 45 min was taken as primary challenge and secondary challenge was performed similarly as described above.

For dose response, various doses of antigen (DNP-BSA, 10 ng, 100 ng, 500 ng and 1000 ng/ml) were used to stimulate cells for primary challenge. During secondary challenge, cells were treated with respective doses of DNP-BSA used in primary challenge. In another experiment, first time challenged cells with different doses of antigen; cells were stimulated with all the doses during secondary challenge,

when they were activated with each dose separately for first time.

For inhibition experiments, Phosphoinositol-3 Kinase (PI3K) inhibitor wortmannin (WRT, 100 nM, Sigma Aldrich, USA) was added 30 min before activation whereas Lysine-specific demethylase 1 (LSDI) inhibitors (both from Cayman Chemicals, USA), Pargyline (Par, 1000 μM) and Tranylcypromine (TCPA, 250 μM) were added 1hr before each activation.

In all the experiments, before each challenge cells were counted and confirmed to be more than 95 per cent viable by trypan blue staining.

2.5. β-Hexosaminidase release assay

To determine β-hexosaminidase activity samples were assayed for enzyme colorimetric assay as described earlier [22]. Briefly, 20 μl of the supernatants and cell lysates were incubated with 50 μl of the substrate solution (27.3 mg/ml of *p*-nitrophenyl *N*-acetyl-*β*-D-glucosaminide (Sigma Aldrich, MO, USA) in 0.1 M citrate buffer (pH 4.5) for 1 hr 15 min at 37 °C. The reaction was terminated by the adding 100 μl of the stop solution (0.2 M NaOH, 0.2 M NaCl and 0.2 M glycine). Absorbance was taken at 405 nm in multi-mode micro plate reader (Spectramax M2e, Molecular Devices), and the extent of degranulation/exocytosis was expressed as a percent of the total β-hexosaminidase activity released in supernatants. Residual β-hexosaminidase present in lysates was calculated as cell associated.

2.6. Histamine release assay

To analyse histamine released from cells, samples were assayed using fluorometric assay as explained elsewhere [25]. 20 μl of 1 M NaOH and 10 μl of 1% (w/v) substrate *o*-phthaldialdehyde (OPA) (Sigma, dissolved in methanol) were added to 100 μl of cell supernatants and cell lysates. Mixed samples were incubated for 4 min at room temperature in dark. 10 μl of 3 M HCl was added to terminate the reaction. Protein precipitates were removed by centrifuging samples at 10,621*g* for 3 min, supernatant were collected and added to 96 well plates. Plates were read at 360 nm excitation and 450 nm emission using microplate reader (Thermo Scientific Varikoskan Flash) to determine the fluorescent histamine-OPA products. Released histamine was calculated as percent of total present in the cells.

2.7. Flow cytometric analysis of surface receptors on MCs

0.3×10^6 RBL-2H3 cells and 0.5×10^6 BMMCs were sensitized with DNP-BSA specific IgE (TIB 142 sup) for 16–18 hr and treated with DNP-BSA (100 ng/ml) for 45 min to induce activation by FcεRI cross linking during primary and secondary challenge. FcεRI expression on MC surface was studied indirectly by staining for bound IgE. IgE sensitized cells were taken as control. Activation was stopped by washing cells with ice cold PBS. Cells were harvested and incubated with anti-mouse CD16/32 Fc Block (1 μg/ml) for 20 min at 4 °C prior to staining. Cells were then incubated with anti-mouse IgE-PE antibody (Biolegend, San Diego, CA, USA) and their isotype for 20 min on ice in dark followed by washing with PBS and fixation with 2% PFA. To directly detect total surface FcεRI, resting, IgE-sensitized and activated cells were incubated with purified mouse anti-rat FcεRI (BD Pharmingen) or its isotype control, followed by biotinylated goat anti-mouse IgG (Biolegend) and finally with streptavidin-FITC (eBioscience). Samples were acquired within 24 hr of staining on BD FACS Calibur (BD Biosciences) flow cytometer. Data was analysed and collected from at least 10,000 total events per samples using Cell Quest Pro software.

2.8. Transmission electron microscopy (TEM)

15–20 million BMMC and RBL-2H3 cells were sensitized overnight with TIB-142 supernatant and activated with 100 ng DNP-BSA for 45 min during primary and secondary challenge. IgE sensitized cells were taken as control. Cells were trypsinized and collected after 45 min and 6 hr of antigen challenge and washed with ice cold PBS and then fixed in 2.5% Glutaraldehyde and 2% paraformaldehyde (Fisher Scientific) made in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4 °C. Cells were then washed with PBS and pellet was collected. The samples were then processed for Transmission Electron Microscope (JEOL 2100F) in Advanced Instrument Facility (AIRF, Jawaharlal Nehru University).

2.9. Statistics

Results were obtained from at least three independent experiments. Statistical analysis was done using Student's *t*-test with Microsoft 2010 and ANOVA (one way/two way) was carried out using Graph Pad Prizm. Data represented as mean ± SEM. Statistically significant difference between control and treated samples was considered when a p value of less than 0.05 was obtained.

3. Results

3.1. Recovery of MC granule content and FcεRI receptors after primary challenge with antigen

MCs possess numerous granules that appear electron dense due to presence of matrix made of heparin-protein complex, and degranulation upon activation with antigen leads to release of pre-formed mediators within minutes [22]. To assess the morphological changes in MCs during and after antigen induced activation which causes degranulation *in vitro,* cells were analysed by TEM. BMMCs were confirmed by double staining for c-KIT and FcεRI after 28 days (Supplementary Fig. S1). TEM images of IgE sensitized cells taken as control (mentioned as resting) showed intact granules (Fig. 1A, D). Whereas, allergen challenged cells showed empty granules whose content had been released (Fig. 1B, E). This was an indication of degranulation after antigen challenge that showed up as loss of electron density. After 6 hr of antigen challenge, recovery of the granules can be seen with partially filled showing beginning of replenishment of the granules as electron dense regions begin to emerge again (Fig. 1C, F). Cells start to recover within hours after activation. Both RBL and BMMC showed similar results. After activation, MCs retain their proliferative capacity and do not differ from resting cells (Supplementary Fig. S2) as shown by CFSE based proliferation assay and live cell recovery. These results indicate recovery of pre-stored granule contents, as well as proliferative capacity of MCs after a primary challenge with allergen.

Two events that follow MC activation by allergen challenge/FcεRI cross-linking are early signalling events which cause internalization of FcεRI followed by massive release of granular contents including β-hexosaminidase [23,26]. In order to check if MCs can recover such primary challenge, we checked the level of FcεRI on surface as well as the cell-associated β-hexosaminidase enzyme as granule cargo. Secretion of β-hexosaminidase through granule exocytosis from MCs is measured routinely 45 min after challenge to know the extent of degranulation in response to allergen challenge [27,28]. To validate the recovery of MCs from activation due to allergen challenge, cell associated as well as total granular content (Supplementary Fig. S3) was measured as β-hexosaminidase activity, 45 min and 24 hr after antigen challenge. Residual content, which remains inside the cell after degranulation, was taken as cell-associated, whereas total granule content was calculated by addition of released and residual content. Total granule content after 24 hr of antigen challenge appeared equal to that of control showing complete replenishment of granules (Fig. 1G, H). Activation for 45 min led to degranulation of approximately 45% of the total granular content as only 55.9 ± 7.1% β-hexosaminidase compared to control remained inside the cells, which further was increased after 24 hr, indicating the complete recovery and replenishment of granules from antigen challenge.

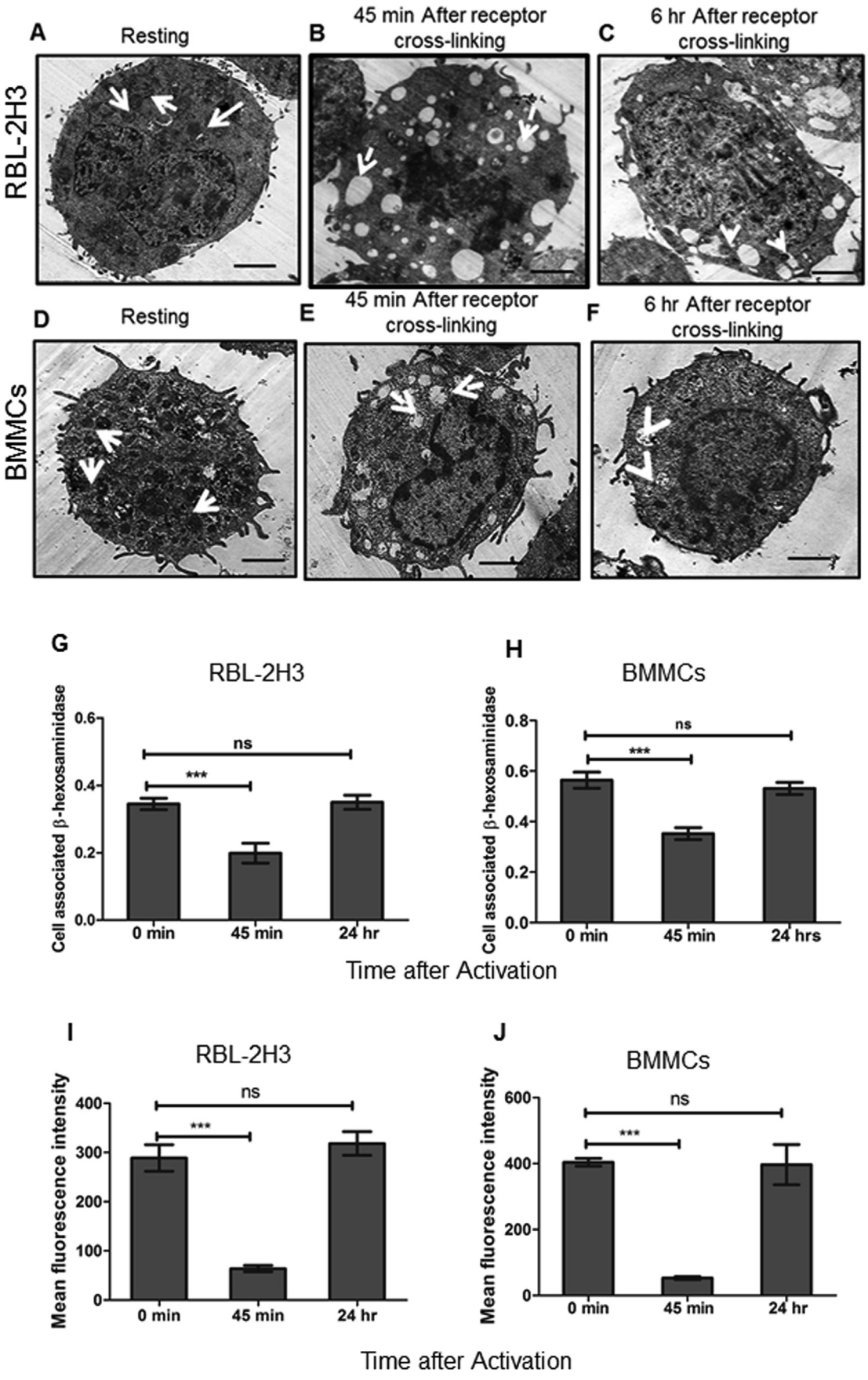

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Fig. 1. Mast cells degranulation and recovery of granule content and cell surface expression of FcεRI after primary antigen challenge. 15–20 million BMMC (A–C) and RBL (D–F) cells were sensitized with anti-DNP-BSA IgE (TIB 142 sup) for 16–18 hr and then activated with or without 100 ng/ml DNP-BSA for 45 min followed by washing, harvesting and fixing cells with 2.5% glutaraldehyde and processed for Electron Microscopy. Representative TEM images are showing resting mast cell (A & D), Activated degranulating cell (B & E) and cells recovering granule content after 6 h of activation (C & F). For recovery, cells were incubated at 37 °C in fresh culture medium for indicated time. Each image is representative of minimum twelve images. Magnification in all the panels is 2000× and Scale bar is 500 nm. Solid arrows indicate intact, Dashed; empty, and Arrow heads; partially filled granules in resting and activated mast cells. Supernatants and lysates were collected after indicated time points and assayed for β-hexosaminidase activity. β-hexosaminidase present in cell lysates was taken as cell associated granular content in RBL (G) & BMMC (H). Data was calculated and plotted as absorbance (OD at 405). Staining with PE-anti-IgE antibody and its isotype control for FcεRI expression was also performed at indicated time points as explained in material and methods. Shown is the graphical representation of histograms (showing in Supplementary Fig. S5) mean fluorescence intensity ± SEM (I & J) (one way ANOVA ***$p \leq 0.001$, ns: Not significant, n = 4).

Further, surface expression of total and IgE-bound FcεRI receptor was analyzed by FACS to check whether expression of FcεRI on surface can also be recovered after activation of MCs. These receptors get internalized upon binding of antigen to already bound IgE [29]. Approximately 80% and 65% reduction in surface bound IgE-FcεRI was observed after 45 min of activation indicating internalization as compared to control (IgE sensitized cells and resting) by indirect and direct staining, respectively. After initial challenge, cells were harvested and seeded again in fresh medium and sensitized with IgE overnight and then analyzed by FACS for receptor staining. More than 75.47 ± 3.5% and 85 ± 1.2% receptors were internalized at the end of incubation with antigen in case of RBL and BMMC (Fig. 1I, J) respectively (as indicated by Mean Fluorescence Intensity (MFI)). There was no significant difference in surface expression of FcεRI, or the amount of IgE bound to these receptors, after recovery in comparison to control, indicating that FcεRI receptors are completely re-expressed on cell surface after primary challenge, and equally able to bind IgE, as shown in Supplementary Figs. S4 and S5. Taking together above results showed that MCs were able to replenish their granule content and surface expression of total as well as IgE-bound FcεRI after primary allergen challenge.

3.2. MCs showed a decreased release of pre-stored mediators during secondary challenge with allergen

We observed complete recovery of MCs after primary allergen challenge in the form of replenishment of granule contents and re-emergence of surface receptors as well as no change in proliferation capacity of the cells. Next, we explored if MCs can be re-activated after initial challenge and what are the effects of first challenge on secondary MC responses. For this, after 24 hr of primary challenge cells were re-challenged and β-hexosaminidase released was compared (Fig. 2A). There was a 35.47% (From 47.56% to 30.69%) inhibition (Fig. 2B) and a 61.18% (from 33.03% to 12.82%) inhibition (Fig. 2D) in secretion during secondary challenge in comparison to secretion during primary challenge in case of RBL-2H3 and BMMCs, respectively. 34.88% inhibition was observed in histamine release after secondary challenge (Fig. 2F). To find out whether this reduction in degranulation was due to decrease in secretory granules inside the cells, total granule content was analyzed after each challenge as taken by addition of cell associated and released β-hexosaminidase (Fig. 2C & E). Total granule content was same during primary and secondary challenge, indicating the recovery or biogenesis of granules is normal after degranulation during primary challenge, and decrease in degranulation is not associated to change in total granule content.

We also investigated if the extended resting period could influence the MC responses observed for secondary antigen challenge. Cells were incubated in fresh medium for 24 hr rather than 6 hr (Fig. 3A), harvested and seeded for secondary challenge. The time difference between both challenges was 48 hr in this case. Percent inhibition observed was 32.2% in β-hexosaminidase release (Fig. 3B) while histamine release was reduced by 32.65% (Fig. 3C). The reduction in degranulation was comparable to previous results observed with 6 hrs of rest. Therefore, further experiments were carried out with a 6 hr rest, or a total of 24 h gap between the two allergen challenges.

Further, time kinetics (Fig. 4A) revealed that cells released maximum β-hexosaminidase within initial 15 min (30.09%, primary challenge and 18.22%, secondary challenge; $p \leq 0.001$) (Fig. 4B). Significant decrease (~38% reduction) on comparison of 15 min data (31.02–19.24%), ~74.97% reduction in 30 min (from 9.95% to 2.49%) and ~90% reduction in 45 min (from 5.8% to 0.54%) was observed during secondary challenge. Significantly higher extent of inhibition is seen at later time points in comparison to earlier time points. Thus, MCs respond to second exposure of same antigen with reduced mediator release, and continuous antigen challenge at optimum dose leads to complete inhibition after 45 min of induction.

3.3. Effect of doses of antigen used during primary challenge on pre-stored mediator release from MCs during secondary challenge

To explore MCs' responses to different doses of antigen, RBL cells were stimulated with various doses of DNP-BSA as described in material and methods. There was significant reduction in percent release of β-hexosaminidase during secondary challenge to their respective primary dose (10 ng/ml dose; from 19.76% to 14.88%, 100 ng/ml dose; 47.32% to 33.35%, 500 ng/ml dose; 38.6% to 25.88%, observed percent inhibition was 24.69%, 29.29% and 32.95%, respectively). An optimal degranulation was observed at 100 ng/ml dose (47.32% release), and 500 ng/ml dose (38.6% release) found to be sub-optimal (Fig. 4C). Among two extreme concentrations of antigen, 10 ng/ml (low concentration) could induce MC degranulation upto 20 percent but 1000 ng/ml (high concentration) was able to induce minimal mediator release (5.33%). No significant reduction was observed in control and 1000 ng/ml dose.

RBL cells activated during primary challenge with one individual dose were again activated with all four doses for the second time and assayed for β-hexosaminidase. Release of β-hexosaminidase was reduced after second exposure of the same antigen. Overall, highest reduction was found in case of 500 ng dose which was comparable to 100 ng dose, when compared to control (Fig. 4D). Primary treatment with 10 ng dose did not cause much inhibition except slight decrease at 100 ng dose after secondary challenge. Whereas, 1000 ng dose which failed to induce enough degranulation for the first time, resulted in significant more inhibition than 10 ng dose in mediator release (Fig. 4E). These results suggest that maximum dampening of MC secondary responses is seen with optimum and sub-optimum doses of allergen during primary challenge, and not with extremely low or high dose of allergen.

3.4. Primary allergen challenge to MCs does not affect early signalling involving FcεRI internalization during secondary challenge

To study if the initial signalling represented by FcεRI internalization is affected by primary challenge, influence of primary challenge on the amount of total FcεRI and IgE-FcεRI on the MC surface was assessed by using anti-FcεRI and anti-IgE antibodies, respectively. Cells were sensitized with IgE and activated two times with same antigen as described in material and methods. Cells were analysed for internalization of IgE-bound FcεRI receptors after antigen challenge by flow cytometry by (Figs. 5 and 6). We have already seen that FcεRI receptors are recycled

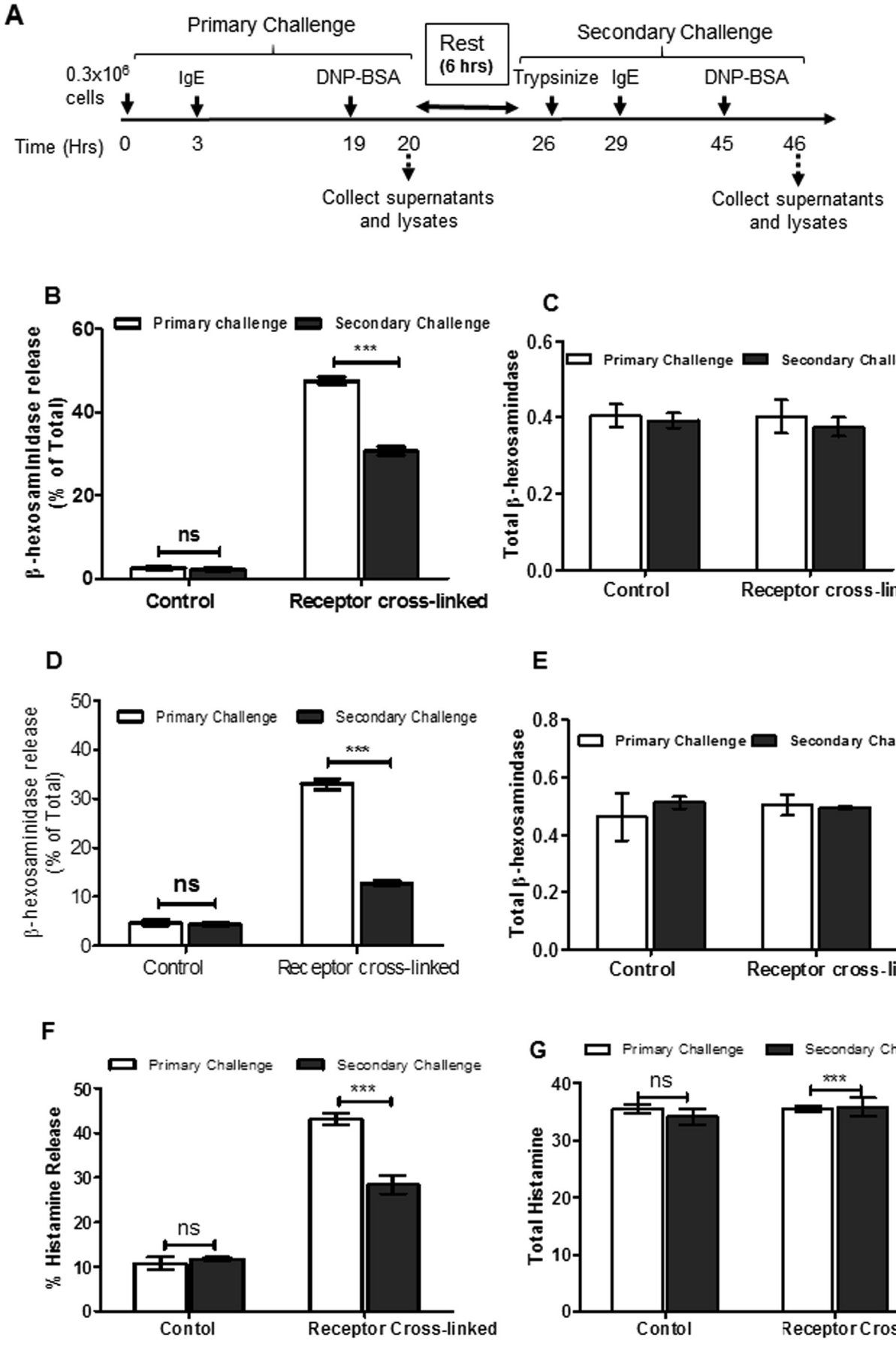

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Fig. 2. Mast cell mediator release in response to secondary allergen challenge. Time line of the experiment is shown (A). 0.3×10^6 Cells were seeded in 24 well plate into two sets and sensitized with anti-DNP-BSA IgE antibody (TIB 142 sup) after 3 hr. After incubation of 18 h, cells were washed and challenged with DNP-BSA (100 ng/ml) for 45 min. IgE sensitized cells were taken as control. Supernatants were collected after 45 min of stimulation. One set was lysed immediately after activation and cells in second set were given 6 hr of rest in fresh medium then harvested by trypsin (RBL-2H3) and seeded for secondary challenge maintaining the cell counts similar to primary challenge. 24 hr after the initial activation, RBL cells (B and C) and BMMCs (D and E) were sensitized with DNP-specific antibodies and activated again similarly as above, percent degranulation (B and D) and total β-hexosaminidase (C and E) as well as percent histamine release and total histamine (F and G) were analyzed after each challenge. Each bar graph represents mean ± SEM of independent experiments (n = 7 for RBL and n = 4 for BMMC) in duplicate, and asterisks indicate statistically significant differences in the percentage of β-hexosaminidase release between control and experimental samples (two way ANOVA ***$p \leq 0.001$).

or re-expressed on the surface during recovery from first encounter of antigen and cells get re-activated with reduction in mediator release (Fig. 1G, H and Fig. 2B, D and F). Approximately 30 percent (compared to resting cells) while more than 55 percent (compared to IgE sensitized cells) receptor internalization, was observed during each challenge when analysed by using anti-FcεRI antibodies (Fig. 5A and B). The visible difference between MFIs of resting and IgE-sensitized cells in Fig. 5 can be due to presence of IgE which regulates stabilization and up-regulation of FcεRI on the surface [30]. More than 70 ± 4.0 percent IgE got internalized in case of RBL-2H3 cells (Fig. 6B and C) whereas in

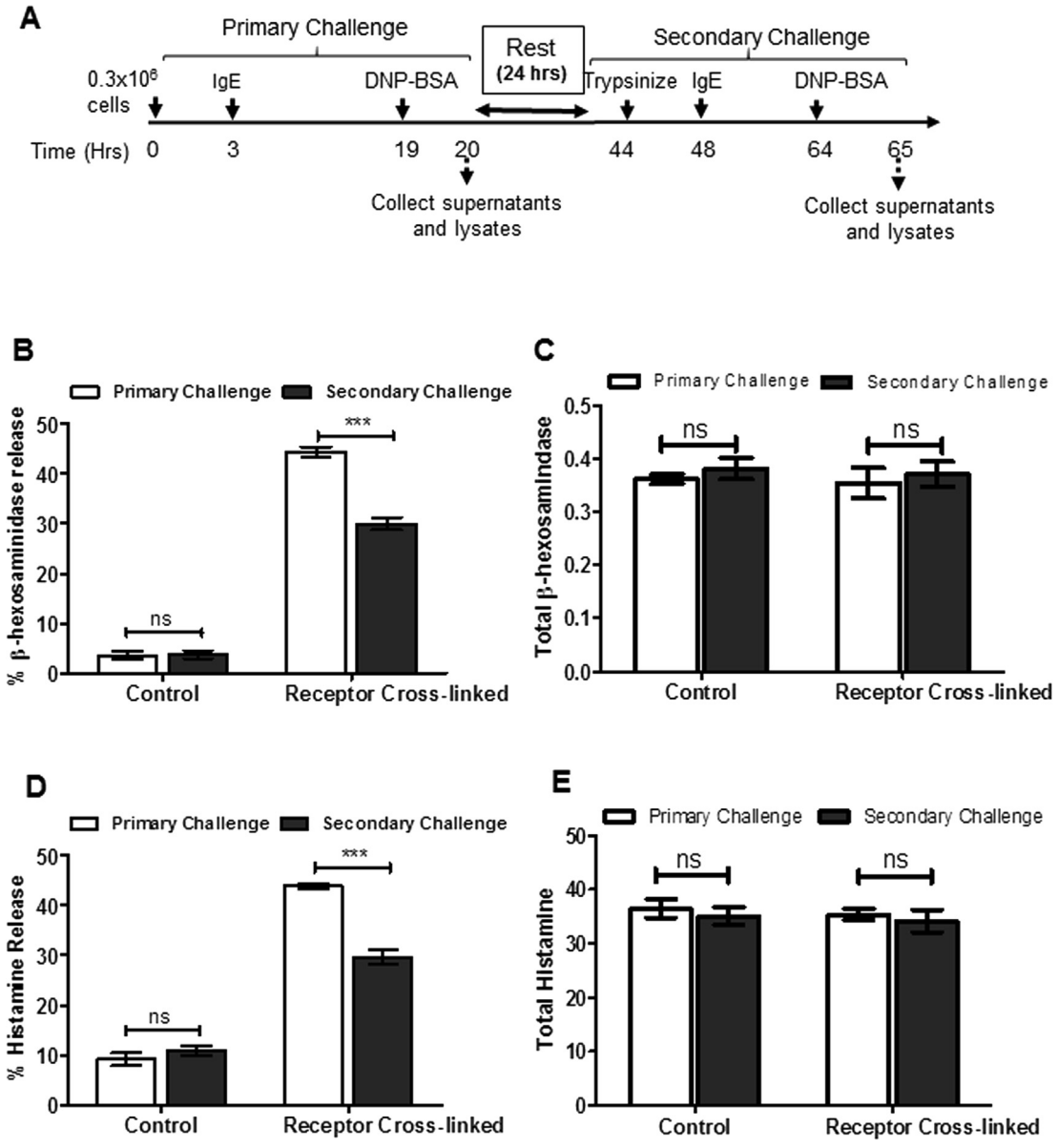
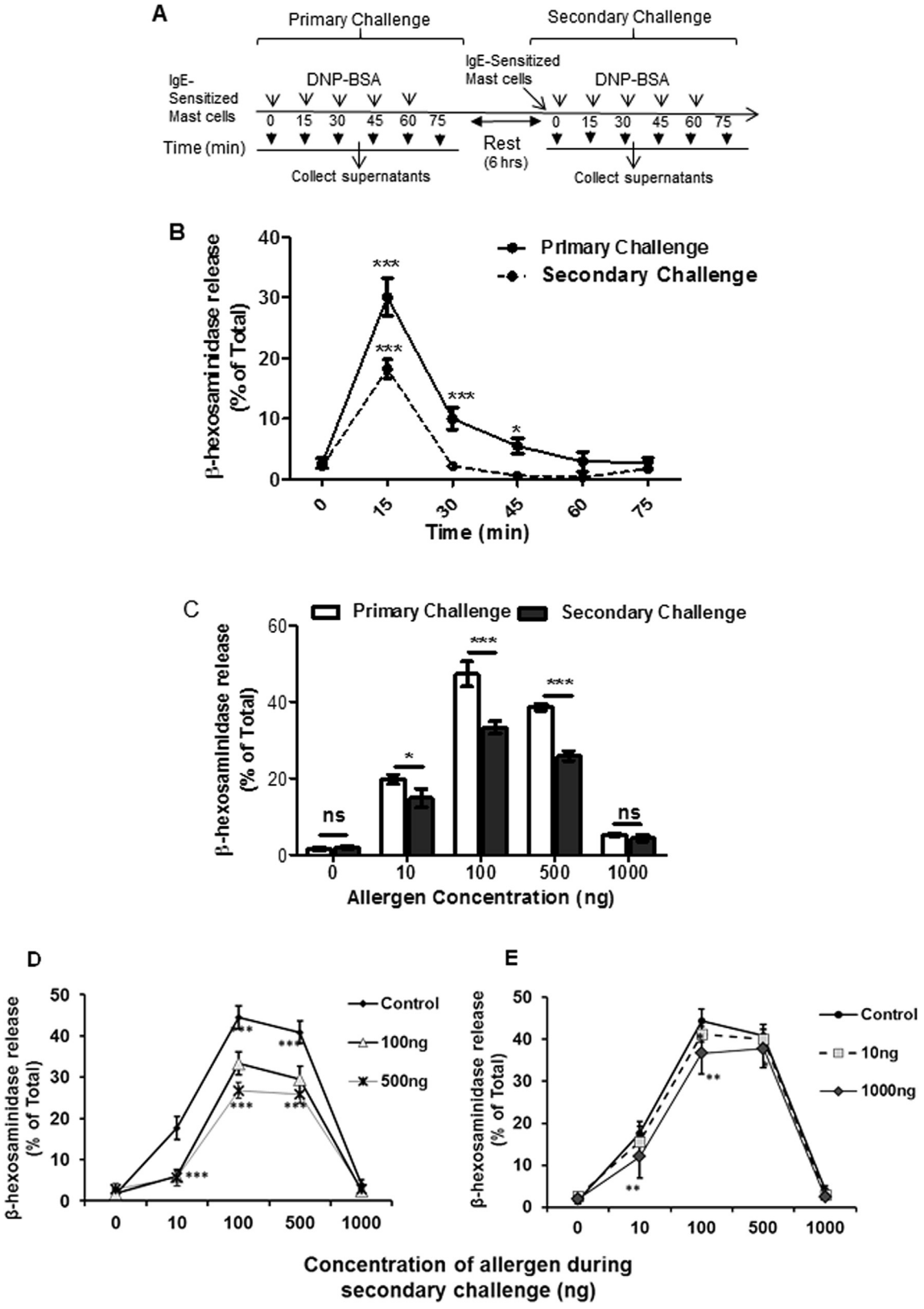


Fig. 3. Mast cell mediator release in response to secondary allergen challenge after 24 hr resting period. Time line of the experiment is shown (A). Cells were sensitized with IgE and next day, challenged with DNP-BSA (100 ng/ml) for 45 min. IgE sensitized cells were taken as control. Supernatants were collected after 45 min of stimulation. One set was lysed immediately after activation and cells in second set were given 24 hr of rest in fresh medium then harvested by trypsin and seeded for secondary challenge maintaining the cell counts similar to primary challenge. 48 hr after the initial activation, cells were sensitized with IgE and activated again similarly as above, percent release and total of β-hexosaminidase (B and C) histamine (D and E) were analyzed after each challenge. Each bar graph represents mean ± SEM of independent experiments in duplicate, and asterisks indicate statistically significant differences in the percentage of β-hexosaminidase release between control and experimental samples (two way ANOVA ***$p \leq 0.001$, ns: Not significant, n = 3).



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case of BMMCs (Fig. 6D and E), internalization was observed more than 90 ± 1.0% of the control during both activations. RBL-2H3 and BMMCs both showed similar results. For second exposure, internalization of IgE-bound FcεRI receptors happens similarly as previous exposure with no significant difference. So, we conclude here that this reduced MC response is not due to any alteration in the initial signalling as indicated by FcεRI internalization.

Fig. 4. β-hexosaminidase release at different time points after activation and in response to various of antigen during primary and secondary challenge. Time kinetics of MC degranulation (A). IgE sensitized RBL cells activated with 100 ng/ml DNP-BSA continuously at 15 min time interval () for 75 min in same well (B) and supernatants were collected at each time point. Dose response (C–E). Cells were first sensitized with anti-DNP-IgE and challenged with doses of DNP-BSA (10 ng/ml, 100 ng/ml, 500 ng/ml and 1000 ng/ml) for 45 min at 37 °C. After 24 hrs, cells were again challenged with each dose to their respective used for primary challenge and percent β-hexosaminidase released during primary and secondary response of each dose was compared (C). For dose response during secondary challenge cells were treated with optimum dose (100 ng), sub-optimum dose (500 ng) Fig. 4D and extreme doses (10 ng and 1000 ng; Fig. 4E) of antigen during primary challenge. Then, for secondary challenge, cells pretreated with above doses, were divided into separate groups and challenged with all the doses (10, 100, 500, and 1000 ng) and compared to control. Legend at top right in figure D and E are the allergen doses used for primary challenge. Each data point represents mean ± SEM of from at least three independent experiments in duplicate, and asterisks indicate statistically significant differences in the percentage of β-hexosaminidase release (student's *t*-test *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.001).

3.5. Phosphoinositide 3-kinase (PI-3K) dependent-signalling pathway has a role in modulation of the pre-stored mediator release from MCs during secondary challenge

PI3K is involved in MC survival, growth, and differentiation, MC migration, cytokine/chemokine production, degranulation and eicosanoid generation [31]. The PI3K specific inhibitor, wortmannin has been widely reported to inhibit antigen-mediated degranulation and cytokine production in both rodent and human MCs [31,32]. To explore any role of PI3K in modulation of MCs response during secondary antigen challenge, cells were treated with wortmannin (100 nM) 30 min before each challenge and analysed for degranulation by β-hexosaminidase and histamine release assay (Fig. 7A). Wortmannin treatment led to a significant inhibition of β-hexosaminidase release when it was present during each challenge (~65% inhibition) with both the cell types (Fig. 7B and C, and data not shown) in comparison to control. Percent reduction was 65.2 ± 3.5% and 64.56 ± 5.3% with RBL-2H3 and BMMCs, respectively. But when it was used only before primary challenge, the inhibition in secretion observed during secondary challenge was completely negated (more than 98%). As a result the secretion was same as that of first time activated control cells in the absence of wortmannin treatment. Similar results were obtained in case of histamine released from RBL-2H3 cells. This implied that PI3K pathway is involved in the dampening of secondary responses.

3.6. Epigenetic changes due to histone demethylation during primary challenge dampen MC pre-stored mediator release on secondary challenge

Epigenetic histone modifications have been shown to be associated with endotoxin tolerance in macrophages and MCs [17,33]. To study role of epigenetic changes in dampening of MCs responses after primary allergen challenge, we used inhibitors of histone modifications (LSD1 specific): pargyline and Tranylcypromine. These inhibitors block demethylation of histone by LSD1 and consequently gene transcription irreversibly. To evaluate the effect of these inhibitors on MC degranulation (pre-stored mediators), Overnight IgE sensitized cells were treated with TCPA (0.25 mM) and Pargyline (1 mM) in complete medium for 1 hr before each activation (Fig. 8A), and analysed for degranulation by β-hexosaminidase release assay. Cells without treatment were taken as control. Use of inhibitors led to a slight inhibition of β-hexosaminidase release when it was present during primary challenge with both the cell types (Fig. 8C, D and E). The inhibition in secretion due to primary antigen challenge was completely negated during secondary challenge when inhibitors were used only before primary challenge. As a result the secretion was same as first time activated cells. TCPA reversed the inhibition (caused by antigen) almost completely (more than 98%) in both cell types (Fig. 8B and D). Other inhibitor pargyline caused complete reversal (more than 99%) in RBL-2H3 whereas partial reversal (approximately 86%) in BMMCs (Fig. 8C and E). The effect of these inhibitors was explored on another pre-stored mediator, histamine release in RBL-2H3 cells (Fig. 8F and G). The results were comparable to what we found with β-hexosaminidase.

4. Discussion

Allergies and related inflammatory disorders are on the rise all over the world including developing countries [1]. New targets to develop drugs and therapies against allergic disorders are being explored widely. Some of them such as chromones, omalizumab and glucocorticoids are being successfully used to treat allergies [19] but, are more expensive than conventional therapies. Therefore, such drugs remain unaffordable for patients belonging to low-income developing countries. Due to their importance in allergic and inflammatory disorders MCs are critical target for immunotherapy against allergies. MCs are unique among innate immune cells, as they are long lived, can proliferate and also totally regenerate after primary challenge.

The aim of this investigation was to evaluate the effect of primary allergen challenge on MCs early phase mediator release, induced tolerance and underlying mechanism. While the recovery of MCs from activation and their ability to re-degranulate has been studied before, responses after next activation have not been explored much. We investigated not only re-activation but comparison of extent of mediator release during primary and secondary allergen challenge. Reactivation of MCs with same antigen used for first activation resulted in decreased secretion in both BMMCs and RBL-2H3 cells. Time kinetics of mediator release further confirmed this inhibition. During our study of time kinetics of MC responses, higher inhibition at later time points could be an indication of exhaustion of the secretory machinery. Some components such as SNAREs which are recycled may not be available later due to a defect in priming [28]. Further, this reduction was not due to absence of bound IgE on cell surface as reported earlier [34] in case of desensitization because in our study the cells were pre-sensitized with IgE before each challenge. Total granule content and surface expression of FcεRI was analysed and compared after each challenge and found to be not affected after primary allergen challenge. There are reports showing that once activated MCs are able to maintain their responsiveness and respond to allergen challenge for next time [13,34], but these studies have not compared the extent of degranulation.

Allergen dose is important to induce proper activation or desensitization of MCs *in vitro* and *in vivo* [35,36]. We showed the effect of amount of antigen on MC secondary responses. Pre-treatment with sub-optimum (500 ng), and optimum (100 ng) dose resulted in comparable reduction. Extreme low (10 ng) or extreme high (1000 ng) dose could induce no or less reduction. Possible explanation for this could be that high dose may cause inappropriate cross-linking by masking FcεRI or binding one antigen molecule to one IgE resulting in monovalent complex formation and further inducing an active turn-off mechanism due to excess bridging by antigen [37]. So, at least in *in vitro* conditions optimum dose of allergen seems best to dampen MC secondary responses unlike low allergen doses in increasing order used in sensitization studies [35]. Overall, pre-treatment of MCs with an antigen resulted in dampening of response when challenged with same antigen again *in vitro* in rodent MCs. These results are similar to earlier report which showed MCs tolerance against LPS results in inhibition in level of pro-inflammatory cytokines released [17]. MCs don't express antigen receptors like B cell receptors and T cell receptors (TCRs). However, they can acquire antigen (Ag) - specificity through binding of Ag-specific IgE to FcεRI expressed on their surface and they maintain this IgE-

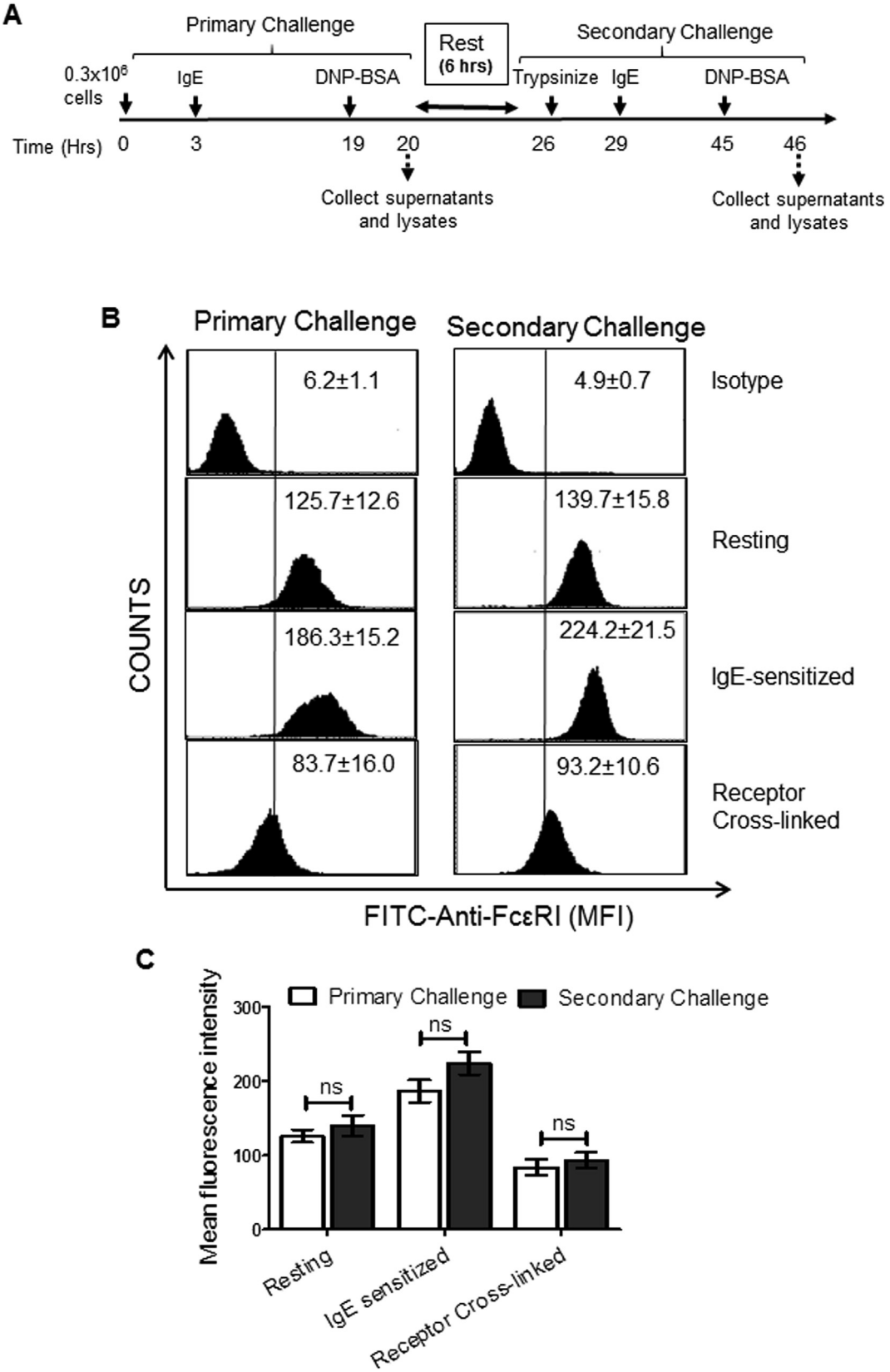

Fig. 5. Changes in FcεRI expression on MC surface during primary and secondary allergen challenge (FcεRI staining). Timeline of the experiment is shown (A). RBL cells were sensitized or not with TIB 142 sup cross-linked DNP-BSA for 45 min, and to stop the reaction ice cold PBS was used. Blocking was done by using 1 μg Fc Block. To detect the surface expression of FcεRI, cells were stained with anti-FcεRI or its isotype control, followed by biotinylated secondary antibody and finally with streptavidin-FITC then analysed by flow cytometry. Histograms depict one representative experiment from more than five independent experiments (B) Shown is the graphical representation of above histograms showing mean fluorescence intensity ± SEM (C, ***$p \leq 0.001$, ns: Not significant, n = 3).

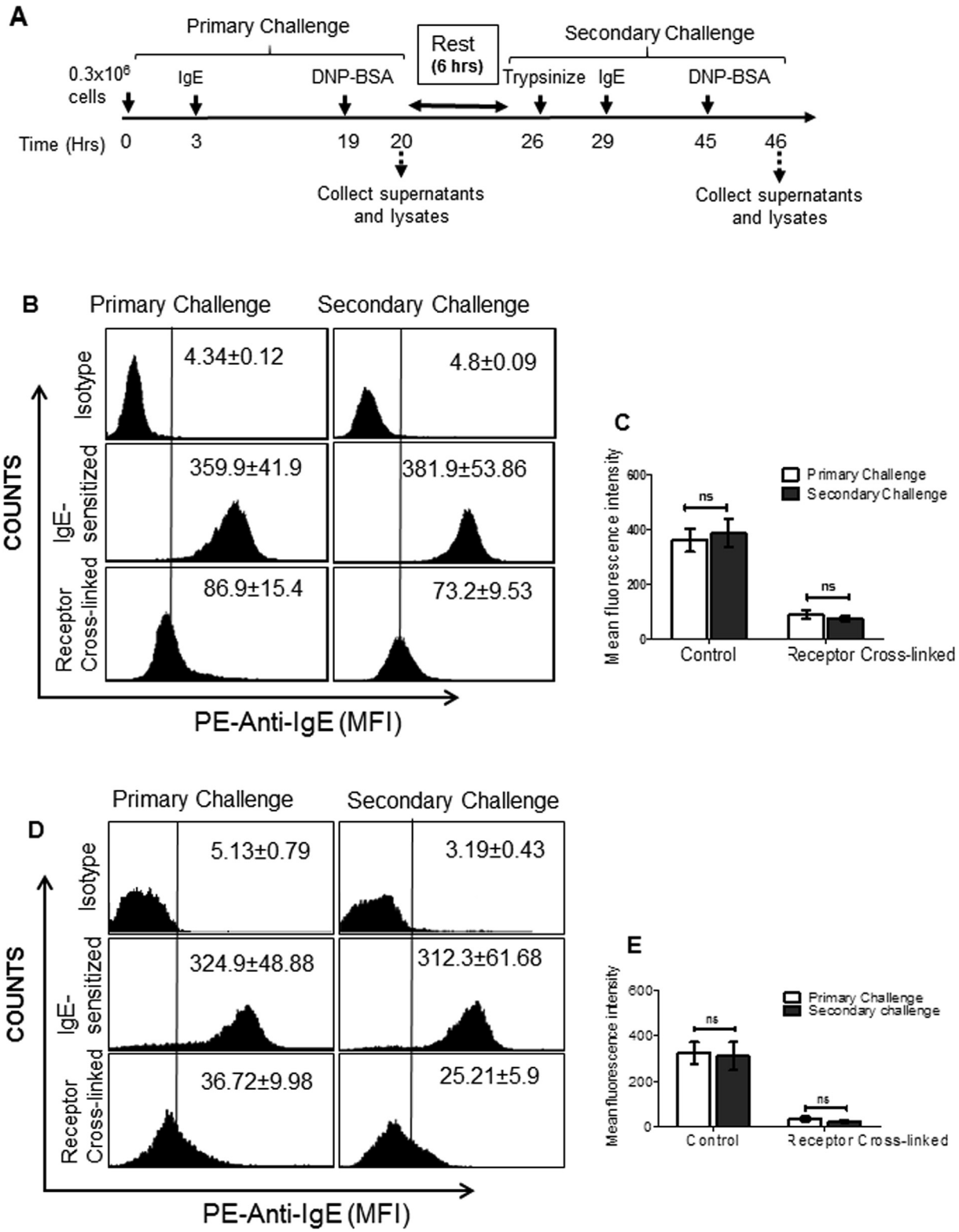

Fig. 6. Changes in IgE-bound-FcεRI expression on MC surface during primary and secondary allergen challenge. Timeline of the experiment is shown (A). RBL (B and C) and BMMC (D and E) were sensitized with TIB 142 sup, cross-linked with 100 ng/ml DNP-BSA for 45 min, and to stop the reaction ice cold PBS was used. Blocking was done by using 1 μg Fc Block. To detect the surface expression of IgE-bound-FcεRI, cells were stained with PE-conjugated anti-IgE antibodies and analysed by flow cytometry. Panel (B) and (D) are showing IgE-bound-FcεRI surface expression (Upper panel: isotype control, Middle panel: IgE sensitized and Lower panel: receptor cross-linked samples) during primary and secondary challenge. Histograms depict one representative experiment from more than five independent experiments (B & D). Shown are the graphical representation of above histograms showing mean fluorescence intensity ± SEM (C and E, ***$p \leq 0.001$, ns: Not significant, n = 5).

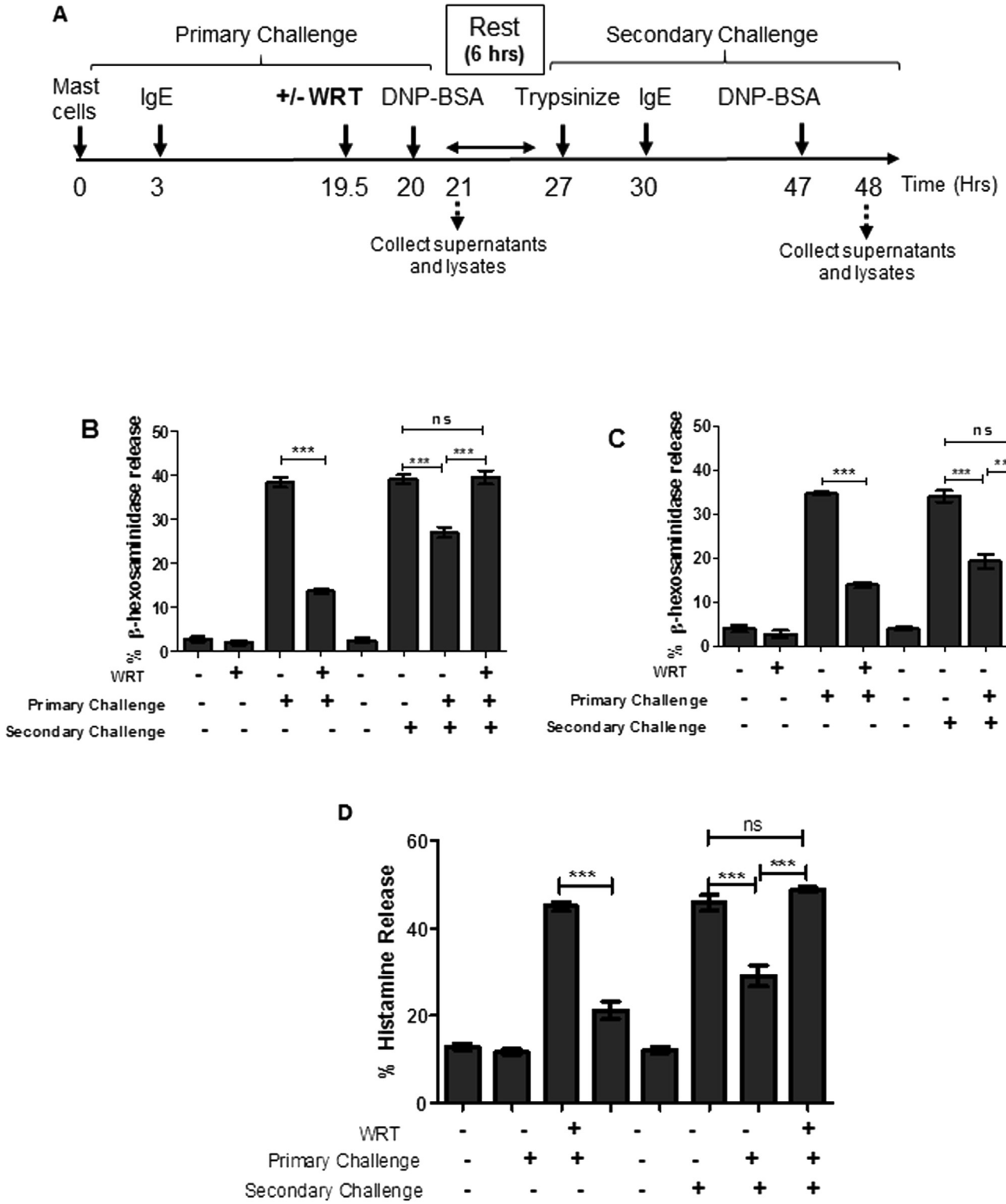

Fig. 7. Effect of pre-treatment with wortmannin on mast cell response after each challenge. Time line of the experiment is shown (A). Cells were sensitized with anti-DNP IgE overnight at 37 °C. Wortmannin was added (+) or not (−) to some wells 30 min before primary challenge. After the initial activation, cells were counted seeded and sensitized with anti-DNP IgE. Next day, cells were treated with or without Wortmannin (100 nM) for 30 min prior to second round of activation with DNP-BSA as done for primary challenge. The amount of β- hexosaminidase and histamine release (in RBL-2H3 cells only, D) was measured for both the challenges in RBL (B) and BMMCs (C). Allergen challenge given is shown by plus (+) whereas minus (−) indicates no allergen challenge only IgE sensitization. Each bar graph represents mean ± SEM of at least three independent experiments in duplicate and asterisks indicate statistically significant differences in the percentage of β-hexosaminidase release (***$p \leq 0.001$) between two different experimental conditions.

mediated memory for a longer period [38].

Primary and secondary challenges involve signalling, internalization and redistribution of FcεRI in similar fashion during both challenges. Dampening of response at single cell level may also require specific signalling or changes during or after primary challenge. Reduced secretion of pre-stored mediator could be due to: 1.) lesser pre-

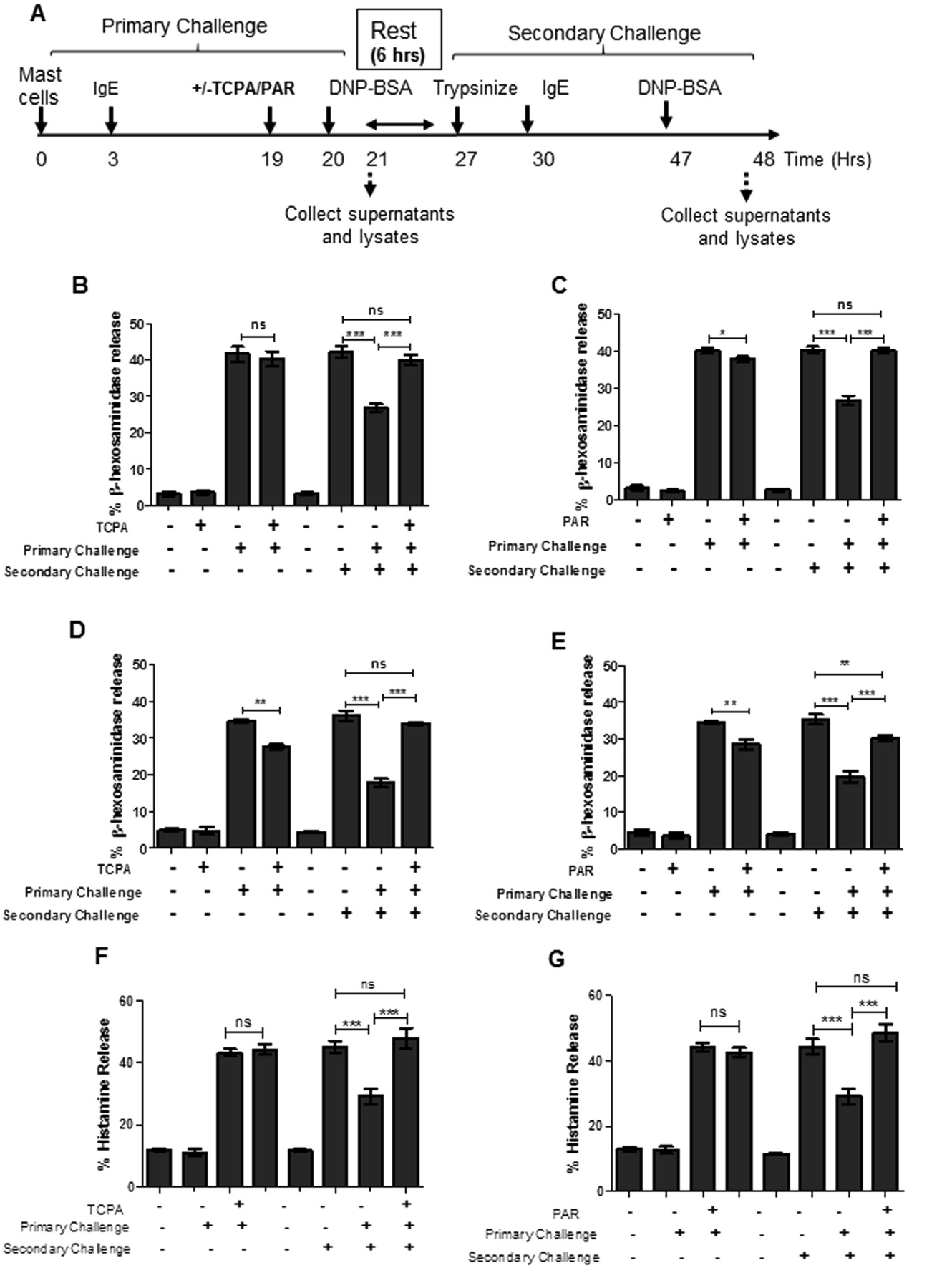

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Fig. 8. Effect of histone modification inhibitors, pargyline and tranylcypromine on mast cell responses. Time line of the experiment is shown (A). Cells were sensitized with anti-DNP IgE. 60 min before activation 1 mM PAR (Pargyline) and 0.25 mM TCPA (Tranylcypromine) was added (except controls) and incubation continued at 37 °C. Supernatants and lysates were collected after 45 min of cross-linking or activation. 24 h after the initial activation, cells were again sensitized, treated with or without inhibitors and activated again with DNP-BSA similarly as during primary challenge. The amount of β- hexosaminidase and histamine release was measured as percent of total and compared for both the challenges in RBL (B, C) and BMMCs (D, E). The effect of inhibitors on histamine release was analysed in RBL-2H3 cells only (F and G). Allergen challenge given is shown by plus (+) whereas minus (−) indicates no allergen challenge only IgE sensitization Each bar graph represents mean ± SEM of three independent experiments and asterisks indicate statistically significant differences in the percentage of β-hexosaminidase release (*p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.001) between two different experimental conditions.

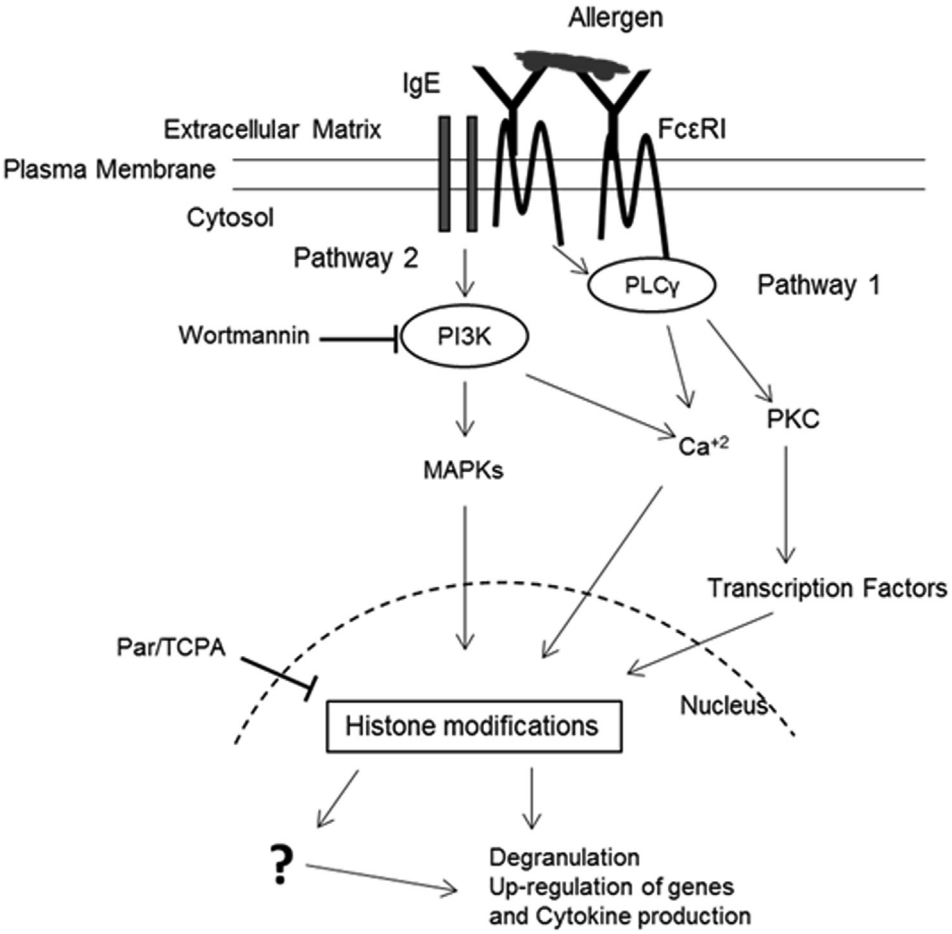

Fig. 9. Proposed model for mechanism of regulation of mast cells' secondary degranulation response. Cross-linking of high-affinity FcεRI receptors on surface through binding of IgE bound to allergen leads to aggregation of FcεRI followed by activation of various downstream signalling pathways. Two pathways have been shown here which work independently leading to degranulation, production and secretion of cytokines. PI3K mediated pathway (Pathway-2) is inhibited by wortmannin whereas PLCγ-mediated (Pathway-1) is not, and remains active. PI3K mediated pathway or its downstream events may be responsible for dampening of secondary mast cell responses. Histone modification may also be involved in such responses by unknown mechanism as use of TCPA and Pargyline during primary challenge led to normal β-hexosaminidase release during secondary allergen challenge.

stored mediators formed and stored after primary challenge, 2.) alteration in FcεRI expression on surface, 3.) alteration in binding of IgE/early signalling events, 4.) specific signalling events may cause some alteration such as unavailability of SNARE proteins, and 5.) epigenetic changes may alter expression of some specific proteins required for degranulation. Dampening of MCs response due to same allergen has been shown in current study. Total granule content, distribution of Fc receptors and internalization remained unaffected during course of the challenges which rule out the possibility of first three reasons to be responsible for these modulations. So, we can conclude that dampening of MC response during secondary challenge is not due to absence of IgE or reduction in FcεRI expression or it's cross-linking.

Further to elucidate the possible mechanisms behind dampening of MCs responses, MCs were treated with inhibitors of signalling pathway and histone modification before activation. PI3K has been well known to be involved in MC degranulation and cytokine production/release [31,32]. Wortmannin treatment led to a complete reversal of dampened β-hexosaminidase release during secondary challenge when used before primary challenge only in both the cell types *in vitro*. Pre-treatment with wortmannin before either of the challenges resulted in more than 50% inhibition in FcεRI-mediated MC degranulation and release of histamine and β-hexosaminidase, as explored previously with RBL-2H3 and mouse BMMCs [39]. This implies that PI3K is involved not only in the signalling leading to release of pre-stored MC mediators but also in the modulation of pre-stored mediator release during secondary responses. The exact role of PI3K as far as its direct or indirect involvement in dampening of pre-stored mediator release is not known. FcεRI signalling cascade in activated MCs involves two pathways: principal phospholipase C γ (PLCγ)-dependent and an alternative PI3K-dependent pathway (Fig. 9). Both the pathways work in parallel to mediate MCs responses such as release of pre-stored mediators and cytokine production involving similar or different downstream signalling molecules [40]. In the absence of PI3K (inhibited by wortmannin), PLC γ pathway might be involved in enhanced degranulation during secondary challenge in inhibitor treated cells (Fig. 9).

Epigenetic changes or histone modifications have been reported to play central role in induction of tolerance in immune cells. For example, histone methylation and acetylation are involved in monocyte trained immunity [33] Use of KDM1 (an enzyme that demethylates both H3k9me3 and H3K4me3) inhibitors, Pargyline and tranylcypromine (TCPA) treatment led to reversal of the inhibition when these inhibitors were used before primary challenge only, followed by secondary

challenge without inhibitors. TCPA reversed the inhibition almost completely whereas pargyline caused partial reversal of inhibition. Taken together these results indicate that histone modifications are possibly involved in modulation of MC pre-stored mediator release during multiple exposures with the same allergen. Previous studies have reported shown that trimethylation of H3K4 (H3K4me3, activating mark) at *Il6* promoter was increased in lipopolysaccharide (LPS) stimulated naïve macrophages, which further was decreased in tolerant macrophages [33]. In another study on MCs, there was transient reduction in trimethylation of H3K9 (H3K9me3, suppressive mark) at *Tnf* and *Il6* promoters as well as in gene bodies in LPS stimulated naïve BMMCs and no change was observed in tolerant cells [17]. All these studies focused on modulation of newly synthesized cytokine release by immune cells, whereas, in the present study, we for the first time show the importance of PI3K signalling pathway and epigenetic changes involving histone demethylations in modulation of pre-stored mediator release during secondary challenge with same allergen in MCs.

In the current study, we have investigated the modulations of MCs responses to induce tolerance against antigen. Such training of innate immune cells like MCs can be a useful common tool for novel immunotherapies for various infectious and non-infectious diseases. MCs are critical mediators of allergies and good targets for therapies. Further, elucidation of the molecular mechanisms involved may provide new affordable global therapeutic avenues for treating allergic disorders.

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Declaration of Competing Interest

None.

Author Contributions

Conceived and designed the experiments: NP. Performed the experiments: RV. Analysed the data: NP, RV. Contributed reagents, materials and analysis tools: NP. Wrote the paper: NP, RV.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2019.103944.

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