

Highly expressed cytoplasmic FcεRIβ in human mast cells functions as a negative regulator of the FcRγ-mediated cell activation signal

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Summary

Background We recently reported that the interaction between Lyn and FcεRIβ is indispensable for FcεRI-mediated human mast cell (MC) activation and that FcεRIβ functions as an amplifier of FcεRI-mediated activation signal. Some of FcεRIβ in cytoplasm appeared not to be co-localized with FcεRIα. The function of FcεRIβ in the cytoplasm remains unknown.

Methods The localization of FcεRIβ and FcεRIα in giant papillae specimens from patients with allergic keratoconjunctivitis and of FcεRIβ, FcεRIα, and Lyn in cultured human MCs was examined using confocal microscopy. FcεRIβ was overexpressed using an adenovirus vector system. Mediators were measured by enzyme immunoassays or enzyme-linked immunosorbent assays.

Results In the subepithelial region, FcεRIβ was mainly localized in the cell membrane of MCs. In the perivascular region, FcεRIβ expression was scattered throughout the cytoplasm and in the cell membrane of MCs. Overexpression of FcεRIβ in MCs mainly increased its cytoplasmic expression and slightly up-regulated cell surface FcεRI expression. However, overexpression of FcεRIβ in MCs resulted in down-regulation of the tyrosine phosphorylation levels of FcεRIβ and Syk and down-regulation of the Ca²⁺ influx soon after FcεRI aggregation and then resulted in down-regulation of degranulation, PGD₂ synthesis, and production of a set of cytokines. This negative regulatory effect may be due to inhibition of the redistribution of Lyn to small patches within the plasma membrane.

Conclusion Cytoplasmic FcεRIβ, which is not co-localized with FcεRIα, may function as a negative regulator, as it can capture important signalling molecules such as Lyn.

Keywords allergic keratoconjunctivitis, cytoplasmic FcεRIβ, histamine, human mast cells, Lyn, perivascular region

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Introduction

The high-affinity receptor for IgE (FcεRI) is composed of three distinct protein species: an α-subunit (FcεRIα), a β-subunit (FcεRIβ), and a homodimer of disulphide-linked γ-subunits (FcRγ) [1]. We reported that the diminution of FcεRIβ in human cultured mast cells (MCs)

induced the down-regulation of cell surface FcεRI expression and FcεRI-mediated mediator release [2]. Thus, FcεRIβ, which is associated with FcεRIα in the membrane, functions as an amplifier of the FcεRI-mediated activation signal. This is in agreement with Kinet et al.'s previous studies [3–5] that FcεRIβ functions as an amplifier of FcεRI expression and the FcRγ-mediated activation signal in FcεRI-transfectant NIH-3T3 cells and murine MCs derived from MS4A2-disrupted human FCER1A transgenic mice.

Y.O. and A.M. contributed equally to the manuscript.

We reported that some FcεRIβ in the cytoplasm was not co-localized with FcεRIα in human MCs [6]. Intracellular FcεRIβ was mainly expressed in the part close to cell membrane and Golgi apparatus, and some of it was expressed in endoplasmic reticulum, but few in the intermediate compartment [6]. The function of cytoplasmic FcεRIβ, which is not co-localized with FcεRIα, in human MCs has not been clarified yet.

The first stage in FcεRI signalling is represented by ligand-dependent receptor aggregation and is followed by receptor phosphorylation mediated by tyrosine kinase of the Src family Lyn [7]. Chemical cross-linking and a yeast two-hybrid system were used to show that a small fraction of Lyn is constitutively associated with FcεRIβ prior to activation [8, 9]. Signal transduction in the rat basophilic leukaemia (RBL)-2H3 cells is in part regulated by the shuttling of limited amounts of Lyn kinase that initiate a cascade of phosphorylation events [10]. It has been reported that the constitutive interaction between the N-terminal unique domain of Lyn and FcεRIβ is a crucial step in the initiation of FcεRI signalling and that Lyn is limiting for FcεRI-induced secretion of inflammatory mediators [11]. We thus hypothesized that intracellular FcεRIβ, which is not associated with FcεRIα, might associate with signalling molecules such as Lyn and regulate FcεRI-mediated MC activation.

Materials and methods

Ethical considerations

The study was approved by the Ethics Committees of Nihon University and Juntendo University, School of Medicine, and all the subjects provided written informed consent in accordance with the Helsinki Declaration of the World Medical Association.

Cell lines

LAD2 cells [12] were kindly provided by Dr Kirshenbaum, NIAID, NIH, Bethesda, MD, USA.

Generation of adult peripheral blood (PB)-derived cultured MCs

All human subjects in this study provided written informed consent, and the study was approved by the ethics review board of all of the hospitals that participated in the study. Lineage-negative mononuclear cells (MNCs) were selected from the PBMNCs and cultured in serum-free Iscove's methylcellulose medium (Stem Cell Technologies Inc., Vancouver, BC, Canada) and IMDM containing recombinant (r) human (h) SCF (PeproTech EC Ltd., London, UK) at 200 ng/mL, rhIL-6 (PeproTech

EC Ltd.) at 50 ng/mL, and rhIL-3 (PeproTech EC Ltd.) at 1 ng/mL as previously described [13].

RNA isolation and real-time quantitative RT-PCR

Total cellular RNA was isolated from MCs, and real-time quantitative PCR was performed as previously described [13]. The primers used for TaqMan analysis were as follows: FcεRIα, sense primer (5'-TGGAATCCCCTACTCTACTGTGTGTA-3'), antisense primer (5'-CCTTAGGTTCTGAGGGACTGC-3'), and probe (5'-FAM-CCTTACTGTTCTTCGCTCCAGATGGCGTGT-TRAM-3'). Human gene-specific primers and probe sets for FcεRIβ and GAPDH were designed by using the Assays-on-Demand™ service (Applied Biosystems, Santa Clara, CA, USA).

Generation of FLAG-tagged FcεRIβ and FcεRIβ without FLAG-tag expression constructs and preparation of recombinant adenovirus

A FLAG-tagged FcεRIβ and FcεRIβ without FLAG tag were generated by PCR using human FcεRIβ cDNA as a template. The following primers were used: a sense primer (5'-GGAAGATCTGATATGGACACAGAAAGTAATAG-3') and an antisense primer (5'-CCCAAGCTTACTACTTGTCATCGTCATCCTTGTTAGTCTAAATCAATGGGAGGAGACA-3'). A FLAG sequence (DYKDDDDK) was added to the N-terminus of FcεRIβ using a sense primer containing the corresponding nucleotide sequence. The following procedure was performed by GenoFunction, Inc., Tsukuba, Japan. To produce recombinant adenovirus, DNAs encoding the tagged FcεRIβ, LacZ, and enhanced green fluorescent protein (EGFP) were introduced into the multiple cloning sites in pShuttle2's expression cassette, which is flanked by two restriction enzyme sites, PI-Sce I and I-Ceu I (BD Biosciences, San Diego, CA). Both sites are also present in the pAd-HM15RGD (GenoFunction). After double digestion of pShuttle2 with PI-Sce I and I-Ceu I, the DNA fragment encoding the tagged FcεRIβ, LacZ, and EGFP was ligated with the viral DNA (pre-digested with PI-Sce and I-Ceu I) and then digested with *Swa* I to remove self-ligated or non-recombinant adenoviral DNA. The ligation mixtures were transformed into *E. coli* strain DH5α to obtain recombinant adenovirus DNAs. A low-passage-number HEK 293-cell line was transduced with each of the recombinant adenovirus DNAs to produce recombinant adenoviruses Ad5 (human adenoviral type 5)/FcεRIβ, Ad5/β-gal, and Ad5/EGFP. Empty vector (Ad5/β-gal; Mock)-transduced cells were used as a control. Recombinant adenoviruses were purified by two rounds of CsCl density centrifugation, dialysed, and stored at -80°C. Titres of infective adenovirus particles were evaluated with an Adeno-X Rapid titer kit (BD Biosciences).

Adenoviral transduction and evaluation of gene expression

PB-derived MCs cultured in 24-well plates were infected with Ad5/Fc ϵ RI β , Ad5/ β -gal, or Ad5/EGFP at a multiplicity of infection (MOI) of 10, 100, 300, or 1000. After 3 days, cells were washed and resuspended with culture medium for 1 day. At day 5, gene transduction efficacy and gene expression intensity were assessed by flow cytometry for Ad5/EGFP and by immunoblotting for Ad5/Fc ϵ RI. Activation of MCs was performed at day 5. As can be seen in Fig. S1, the viabilities of the MCs in the presence of SCF and in the absence of SCF (w/o SCF) after 5 days of incubation were 89.0% and 66.9%, respectively. The viabilities of the mast cells infected with Ad5/Fc ϵ RI β at a MOI of 30, 100, and 300 in the presence of SCF were 69.5%, 74.5%, and 69.1%, respectively. The viabilities of mast cells infected with Ad5/ β -gal (Mock) at MOI of 30, 100, and 300 in the presence of SCF were 72.8%, 67.9%, and 75.3%, respectively. Similar results were obtained in two additional experiments using cells from different donors.

Flow cytometric analyses

Flow cytometric analysis of MCs was performed as described previously [13]. PE-conjugated anti-Fc ϵ RI α mAb (clone CRA1; eBioscience, San Diego, CA, USA), PE-conjugated anti-CD63 (clone H5C6; BD Biosciences), and FITC-conjugated or biotin-conjugated rabbit anti-Fc ϵ RI β polyclonal Ab (242), which recognizes C-terminus of Fc ϵ RI β [14], were used. PE/Cy5-streptavidin was purchased from Biolegend, San Diego, CA, USA. Tyrosine phosphorylation of extracellular-signal-regulated kinase (ERK) 1/2, phospho-Jun amino-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) was quantified by flow cytometric analysis using antiphospho-ERK1/2 mAb (clone 20A; BD Biosciences), anti-JNK mAb (clone 41; BD Biosciences), and antiphospho-p38MAPK mAb (clone 36; BD Biosciences) according to the manufacturer's protocol (BD Biosciences).

Ca²⁺ mobilization

MCs were incubated with 10 μ M indo-1-AM (Molecular Probes, Carlsbad, CA, USA) in the presence of Pluronic F127 (Sigma-Aldrich, St. Louis, MO, USA) and 0.2% FCS at 37°C for 30 min. Cells were washed twice, resuspended in 500 μ L of HBSS containing 1% FCS, and stimulated with DNP-BSA. Ca²⁺ mobilization was analysed in a BD-LSR (BD Biosciences) equipped with UV lasers. Ratiometric analysis of Ca²⁺-bound indo-1 (FL5)/Ca²⁺-free indo-1 (FL4) was performed using CellQuest (BD Biosciences) and FlowJo (Tree Star, San Carlos, CA, USA) software programs.

Immunohistochemical analysis

Giant papillae were resected for therapeutic purposes from five patients, three with atopic keratoconjunctivitis (AKC) and two with vernal keratoconjunctivitis (VKC) during resection surgery after obtaining written informed consent as previously described [15]. The giant papillae specimens were immediately fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 3 h. After washing with 30% sucrose in PBS, the tissues were frozen in optimal cutting temperature (OCT; Sakura Finetek, Tokyo, Japan) compound using liquid nitrogen. Then, 5- μ m frozen sections were made and air-dried. Immunohistochemical staining was performed according to the previously described methods [16]. For mast cell staining, anti-Fc ϵ RI α mAb (clone CRA1), antitrypsin mAb (clone AA1; DakoCytomation Inc., Carpinteria, CA), and rabbit anti-Fc ϵ RI β polyclonal Ab (which recognizes C-terminus: 242) [14] were used as previously described [16]. Alexa 488-, 594-conjugated donkey anti-mouse IgG, and anti-rabbit IgG (all from Invitrogen Corporation, Carlsbad, CA, USA) were used as secondary antibodies. A confocal laser scanning microscopy (Olympus FV-500 or FV-1000, Tokyo Japan) was used for imaging.

Confocal laser scanning microscopy of isolated cells

Confocal microscopic analysis was performed as described previously [13]. The cells were fixed and permeabilized and incubated with mouse anti-Fc ϵ RI α mAb (clone CRA1), rabbit anti-Fc ϵ RI β polyclonal Ab (which recognizes C-terminus, 242) [14], mouse anti-Lyn mAb (clone LYN-01; Biovendor, Brno, Czech Republic), anti-FLAG mAb (Sigma-Aldrich), the isotype control mouse IgG1, or mouse IgG2b. The cells were then incubated with PE-conjugated goat anti-mouse IgG2b (BD Biosciences), PE-conjugated goat anti-mouse IgG (BD Biosciences), Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes), or FITC-conjugated anti-rabbit IgG (BD Biosciences). The stained cells were washed and treated with a SlowFade antifade kit with DAPI (Molecular Probes). A model FV500 and FV1000 confocal laser scanning microscopes (Olympus) were used.

Immunoprecipitation

Cell lysates were prepared, and proteins were separated as described previously [17]. For co-immunoprecipitation studies, anti-phosphotyrosine (clone 4G10; Upstate Biotechnology) or isotype IgG immobilized on Sepharose beads (Pharmacia, Piscataway, NJ, USA) was added to the pre-cleared lysate, and the tubes were incubated for 2 h at 4°C. After washing the beads, the

immunoprecipitates were analysed by Western blotting as described previously [17].

Immunoblotting

Immunoprecipitates were immunoblotted with anti-human Lyn kinase (Upstate Biotechnology), anti-Syk (clone 4D10.1; Upstate Biotechnology), and anti-human FcεRIβ. To probe whole-cell lysates for FcεRIα, FcεRIβ, FcRγ, tyrosine-phosphorylated protein, phosphorylated SHIP-1, and SHIP-1, cell lysates were probed with anti-FcεRIα (Upstate Biotechnology), anti-human FcεRIβ [14], anti-FcRγ (Upstate Biotechnology), anti-phosphotyrosine (clone 4G10), anti-phospho-SHIP-1 (Cell Signaling Technology, Tokyo, Japan), and anti-SHIP-1 (Upstate Biotechnology), respectively. Overexpressed FLAG-tagged FcεRIβ was immunoblotted with anti-FLAG mAb (Sigma-Aldrich).

Mediator assays

The extent of MC degranulation was monitored by measuring histamine release (HR) into the supernatants following FcεRI aggregation using an enzyme immunoassay kit (MBL Co. Ltd., Nagoya, Japan). PGD₂ in the supernatants was measured with an enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI, USA). Human TNF-α and MIP-1α were measured with ELISA kits purchased from R&D Systems Inc., Minneapolis, MN, USA.

Statistical analysis

Differences between two paired groups were analysed by the unpaired Student's *t*-test, and they were considered significant at *P* < 0.05. Values are expressed as means ± SEM.

Results

Localization of FcεRIβ in human MCs

To investigate the localization of FcεRIβ expression in MCs using confocal microscopy, we stained giant papillae specimens from patients with AKC or VKC using anti-FcεRIα and anti-FcεRIβ (Fig. 1). Whether FcεRIβ was localized in the membrane alone or in both the membrane and cytoplasm was determined using images showing the staining pattern for FcεRIβ (Fig. 1A). Cells exhibiting a ringlike staining pattern without scattered cytoplasmic staining were regarded as MCs with membrane-localized FcεRIβ (Fig. 1Aa). A semi-quantitative analysis indicated that 74 ± 12% of the FcεRIβ⁺FcεRIα⁺ cells in the subepithelial region exhibited a ringlike stain for anti-FcεRIβ (Fig. 1Aa, b, and c; white

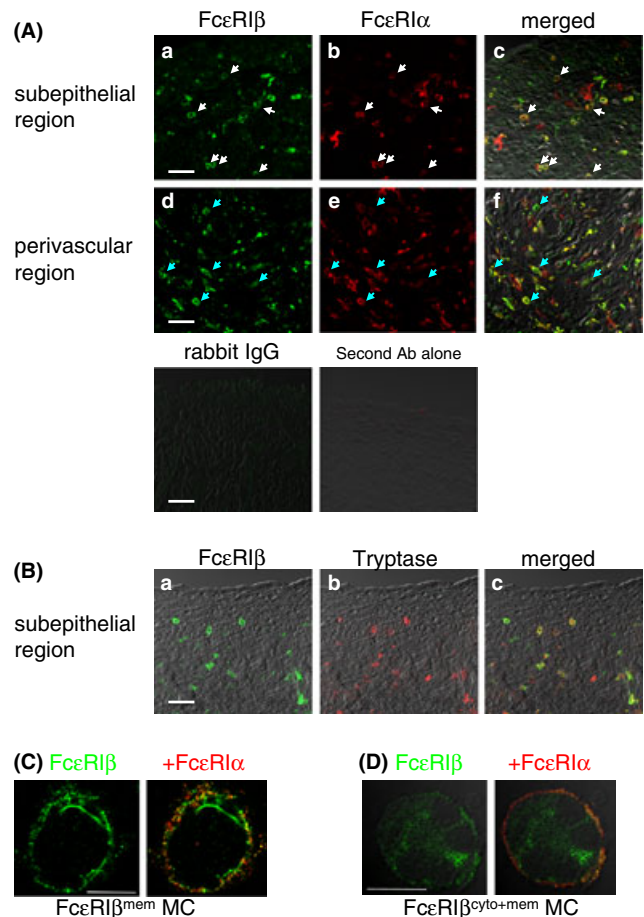


Fig. 1. Localization of FcεRIβ in human MCs and relationship between the expression levels of FcεRIβ. (A) Localization of FcεRIβ in MCs of the subepithelial (a, b, c) and perivascular regions (d, e, f) in giant papillae. Giant papillae specimens were immunostained with anti-FcεRIβ Ab (green, a and d) or anti-FcεRIα mAb (red, b and e). The white arrows indicate the ringlike staining pattern of FcεRIβ. The blue arrows indicate the cytoplasmic staining pattern of FcεRIβ. As controls, staining with rabbit IgG and secondary Abs alone is shown. (B) The giant papillae specimens were immunostained using anti-FcεRIβ Ab (green, a) and antitrypsin mAb (red, b). Scale bars indicate 50 μm. (C and D) Immunocytochemical staining of human peripheral-blood-derived MCs with anti-FcεRIα Ab (red) and anti-FcεRIβ Ab (green). C shows the ringlike staining pattern of FcεRIβ (FcεRIβ^{mem}). D shows the ringlike staining and cytoplasmic scattering staining patterns of FcεRIβ (FcεRIβ^{cyto+mem}). Scale bars indicate 10 μm. Original magnification, ×200.

arrows, *n* = 5 donors). On the other hand, FcεRIβ was localized in both the cytoplasm and the cell membrane of 79 ± 12% of the FcεRIβ⁺ cells in the perivascular region (Fig. 1Ad, e and f; blue arrows, *n* = 4 donors). The frequency of MCs showing a cytoplasmic staining pattern for FcεRIβ differed significantly between the subepithelial and perivascular regions (*P* < 0.05). Some cytoplasmic FcεRIβ was not co-localized with FcεRIα (Fig. 1f). Multiple images showing a Z-stack through the depth of a section revealed that the localization of

Fc ϵ RI β is comparable throughout the depth of the MCs in the subepithelial and perivascular regions (Movies S1 and S2, respectively). Figure 1B shows that all the Fc ϵ RI β ⁺ cells were tryptase⁺ cells. We then examined the localization of Fc ϵ RI α and Fc ϵ RI β in PB-derived cultured MCs (Figs 1C and D). Fc ϵ RI α was mainly located on the surface of MCs. There were two patterns of localization of Fc ϵ RI β in the cells. In the first, Fc ϵ RI β was localized in the cell membrane (Fig. 1C). In the second, Fc ϵ RI β expression was scattered throughout the cytoplasm and in the cell membrane (Fig. 1D). We confirmed that some of Fc ϵ RI β in cytoplasm was not co-localized with Fc ϵ RI α in PB-derived MCs.

Up-regulation of Fc ϵ RI β expression and marginal up-regulation of surface Fc ϵ RI α expression in cultured MCs by Ad5/Fc ϵ RI β (Fc ϵ RI β) transduction

We performed a flow cytometric analysis with Ad5/EGFP to assess the efficiency of foreign gene transduction and expression in PB-derived MCs. As shown in Fig. S2, the transduction efficiency and the expression intensity of the EGFP gene in MCs increased in MOI-dependent manner from 10 to 300.

Next, we quantitatively analysed the expression levels of Fc ϵ RI β mRNA in human MCs using Ad5/Fc ϵ RI β (Fig. 2a). Fc ϵ RI β mRNA expression increased by about 50- and 100-fold at an MOI of 100 and 300, respectively. By contrast, the expression level of Fc ϵ RI α mRNA was unaffected by Fc ϵ RI β transduction. To examine the expression level of Fc ϵ RI β protein in MCs, we next performed a Western blotting of Fc ϵ RI β -transduced MCs using anti-human Fc ϵ RI β Ab and an anti-FLAG mAb (data not shown). The FLAG-tagged, overexpressed Fc ϵ RI β (β_0) was about 1 kDa larger than endogenous Fc ϵ RI β (β_e , Fig. 2b). An alternative splice variant of Fc ϵ RI β was about 10 kDa smaller than the endogenous Fc ϵ RI β (truncated β ; t-Fc ϵ RI β) [18]. A Western blotting analysis confirmed the increased expression of Fc ϵ RI β in an MOI-dependent manner, and a densitometrical analysis revealed that the expression level of Fc ϵ RI β also increased by 1.9-fold as a result of Fc ϵ RI β transduction at MOI 300 (data not shown). The expression level of t-Fc ϵ RI β did not change. Although the expression level of t-Fc ϵ RI β appeared to increase as a result of Fc ϵ RI β transduction at MOI 30 shown in Fig. 2b, similar data could not be obtained in two additional experiments. The smear bands around 66 kDa were mainly endoglycosidase H insensitive and represented mature α (α_{Golgi}), and the 54-kDa bands represented the immature core-glycosylated Fc ϵ RI α in ER (α_{ER}) (Fig. 2b and Fig. S3). Fc ϵ RI β transduction caused the expression levels of α_{Golgi} and α_{ER} to increase by 1.3- and 2.1-fold, respectively, at MOI 300. When evaluated by MFI normalized to the isotype control, the

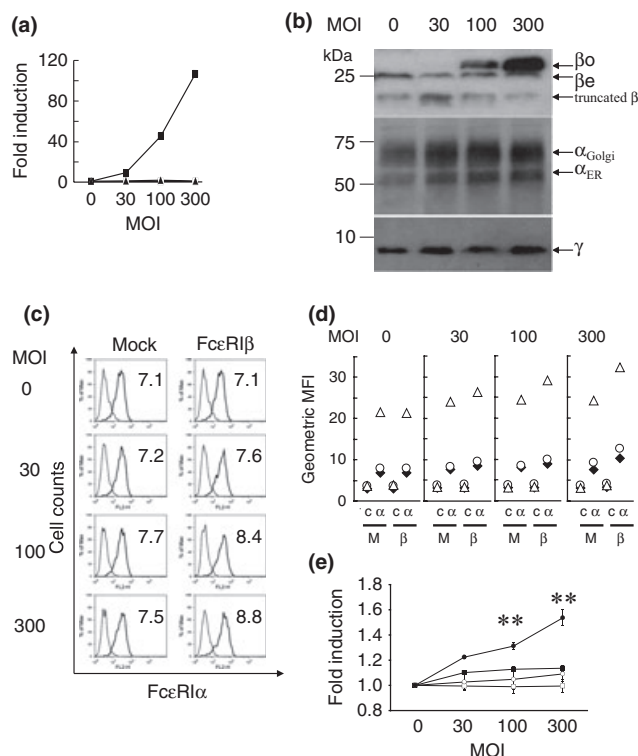


Fig. 2. Up-regulation of Fc ϵ RI β mRNA and protein and surface Fc ϵ RI α expression in human MCs by Fc ϵ RI β gene transfer. (a) Quantitative analysis of the level of expression of Fc ϵ RI β mRNA (closed squares) and Fc ϵ RI α mRNA (closed triangles) in human MCs following the transduction of Fc ϵ RI β at multiplicity of infections (MOIs) ranging from 0 to 300. The open symbols indicate the level of expression in Mock-transduced MCs. The expression levels of Fc ϵ RI β and Fc ϵ RI α mRNA in non-transduced human MCs (MOI = 0) have been set equal to 1. The results of one experiment that is representative of the three that were conducted are shown. (b) Effect of expression of Fc ϵ RI β protein and Fc ϵ RI α protein by MCs following transduction of Fc ϵ RI β . Western blotting analysis of Fc ϵ RI β , Fc ϵ RI α , and Fc γ was performed in Fc ϵ RI β -transduced MCs with anti-human Fc ϵ RI β , anti-Fc ϵ RI α , and anti-Fc γ Abs. β_e , β_0 , and truncated β indicate endogenous Fc ϵ RI β , overexpressed FLAG-tagged Fc ϵ RI β , and truncated form of Fc ϵ RI β , respectively. α_{Golgi} , mature α ; α_{ER} , immature α . (c) FACS analysis of the cell surface expression levels of Fc ϵ RI α on MCs by transduction of either Fc ϵ RI β (right panels) or Mock (left panels). The numbers in the histograms indicate the MFI ratio of Fc ϵ RI α (thick line) to the isotype control (mouse IgG2b, thin line). The results of one experiment that is representative of the three conducted are shown. (d) Cell surface Fc ϵ RI α expression levels shown on the vertical axis are the geometric MFI (n = three donors; each symbol indicates one donor). C and α indicate control mouse IgG2b- and anti-Fc ϵ RI α mAb-treated MCs, respectively. M and β indicate Mock- and Fc ϵ RI β -transduced MCs, respectively. (e) Fold induction of cell surface expression levels of Fc ϵ RI α on MCs as a result of transduction of Fc ϵ RI β (closed circles). The expression level (geometric MFI) of MCs at MOI 0 is assumed to be 1. Closed squares indicate the intensity of Fc ϵ RI α expression by Mock-transduced MCs. Open circles and squares indicate the isotype controls on Fc ϵ RI β -transduced MCs and Mock-transduced MCs, respectively. Data are expressed as means $\pm$ SEM of three experiments. ** P < 0.01 for comparison of Mock- and Fc ϵ RI β -transduced MCs.

surface expression level of FcεRIα increased up to 1.6-fold in an MOI-dependent manner from 0 to 300 (Figs 2c, d and e). The fold induction of the MFI for anti-FcεRIα Ab in FcεRIβ-transduced cells was significant at MOIs of 100 and 300, but the increase was marginal (Fig. 2c and d).

FcεRIβ-transduced cultured MCs can be used as a model of MCs expressing the cytoplasmic FcεRIβ

To examine the localization of FcεRIβ in FcεRIβ-transduced MCs, we performed confocal laser scanning microscopy of MCs treated with Mock (Fig. 3a) and Ad5/FcεRIβ (Fig. 3b) using anti-FcεRIβ Ab. Expression of FcεRIβ and FcεRIα increased in the cell membrane and in the cytoplasm of MCs treated with Ad5/FcεRIβ (Fig. 3b). The majority of the FcεRIβ was co-localized with FcεRIα in the cell membrane, while some of the FcεRIβ was co-localized with FcεRIα in the cytoplasm (yellow in Fig. 3b), but it should be noted that some FcεRIβ appeared to be expressed without co-expression with FcεRIα. When FcεRIβ-positive MC populations

were analysed following transduction of FcεRIβ at the MOI of 0 and 100, the proportion of FcεRIβ-positive MCs increased from ~60% to 80% (Fig. 3c). Thus, transduction of FcεRIβ increased both the number of FcεRIβ-positive MCs and the level of FcεRIβ protein expression in each MC. Therefore, FcεRIβ-transduced MC can be used as a model of MCs expressing the cytoplasmic FcεRIβ.

Regulation of mediator release by cultured MCs by FcεRIβ transduction

To clarify the effect of overexpressed FcεRIβ on FcεRI-mediated MC activation, we compared the release of the mediators by Mock- and FcεRIβ-transduced MCs. To avoid the effect of the FLAG tag, we used FcεRIβ without FLAG-tag expression constructs for transduction. The overexpression of FcεRIβ significantly inhibited HR (Fig. 4a), PGD₂ synthesis (Fig. 4b), and TNF-α production by MCs, but not the production of MIP-1α (Fig. 4c). IL-5 and IL-3 production was also inhibited by FcεRIβ transduction (data not shown). The time course of HR by Mock- and FcεRIβ-transduced MCs following FcεRI aggregation was compared (Fig. 4d), and inhibition of HR by FcεRIβ-transduced MCs was observed up to 60 min. Calcium ionophore A23187-induced histamine and PGD₂ release was unaffected by FcεRIβ transduction (Figs 4a and b).

Comparison between tyrosine phosphorylation of proteins following FcεRI aggregation in Mock- and FcεRIβ-transduced cultured MCs

We examined how overexpression of FcεRIβ affects the tyrosine phosphorylation pattern in MCs following FcεRI aggregation. Activation of MCs treated with Mock and FcεRIβ by FcεRI aggregation for 1, 5, and 15 min resulted in *de novo* tyrosine phosphorylation of a number of proteins that were generally similar in MCs treated in both ways (Fig. 5a). However, as there were some clear quantitative differences in the phosphorylation of ~72-kDa proteins at 1 min after FcεRI aggregation, we examined the tyrosine phosphorylation levels of FcεRIβ, Lyn, and Syk at 1 min after FcεRI aggregation (Fig. 5b). As the anti-FcεRIβ Ab was not good for immunoprecipitation, anti-phosphotyrosine Ab was used for immunoprecipitation. The results showed down-regulation of phosphorylation of FcεRIβ and Syk, but not of Lyn in the FcεRIβ-transduced MCs at 1 min after FcεRI aggregation. We then compared tyrosine phosphorylation of SHIP-1, ERK1/2, JNK, and p38 MAPK in MCs treated with Mock and FcεRIβ following FcεRI aggregation (Figs 5c and d). Densitometrical analysis revealed that intensity of tyrosine-phosphorylated SHIP-1 was not significantly up-regulated in MCs treated with

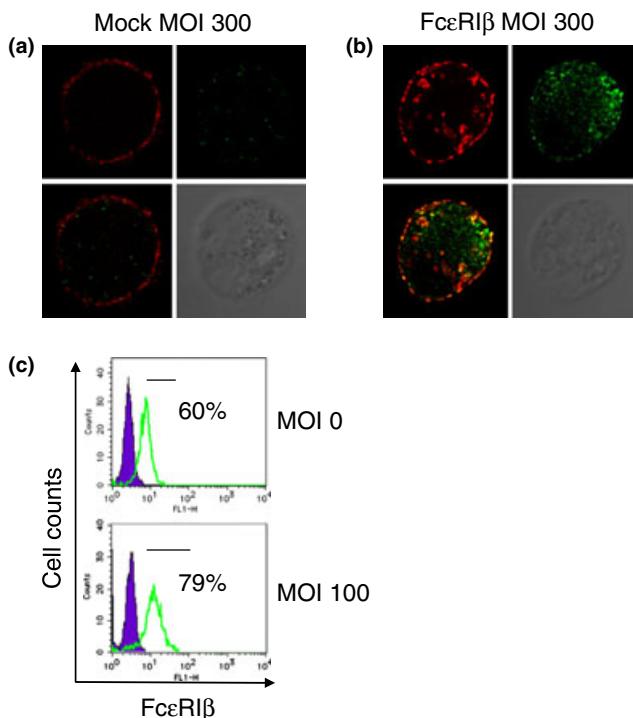


Fig. 3. The FcεRIβ-transduced MCs can be used as a model of FcεRIβ^{cyto+mem} MCs. (a and b) Confocal laser scanning analysis of FcεRIβ and FcεRIα in MCs following transduction of either FcεRIβ (b) or Mock (a), using anti-human FcεRIβ (green) and anti-FcεRIα Abs (red). MCs were transduced with an multiplicity of infection (MOI) of 300. Original magnification, ×800. The results of one experiment that is representative of the three that were conducted are shown. (c) Transduction of FcεRIβ increased the number of FcεRIβ⁺ MCs. FACS analysis of FcεRIβ expression following transduction of FcεRIβ at an MOI of 0 (top) and 100 (bottom).

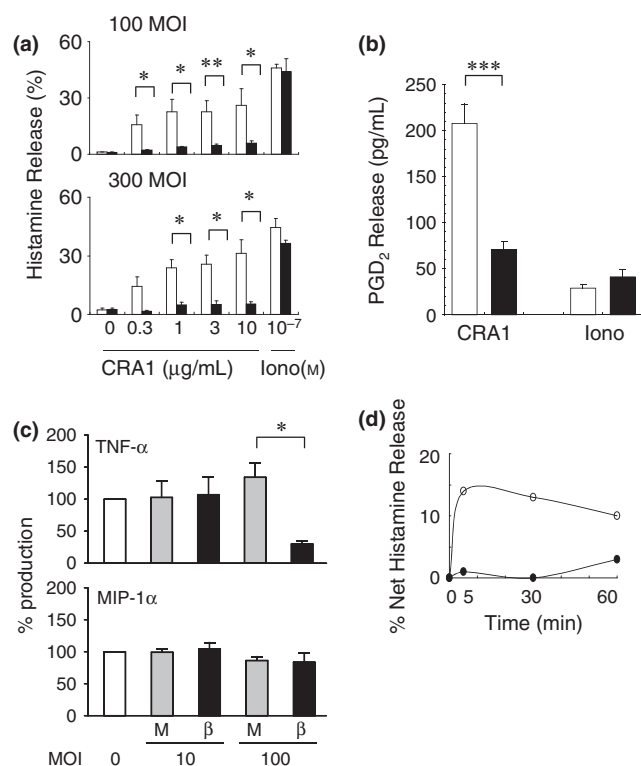


Fig. 4. FcεRIβ modulates mediator release by MCs following FcεRI aggregation. (a) Comparison of histamine release (HR) by Mock-transduced (open bars) and non-FLAG-tagged FcεRIβ-transduced MCs (closed bars) following FcεRI aggregation with an anti-FcεRIα mAb (CRA1) or stimulation with calcium ionophore A23187 (Iono). Data are expressed as means ± SEM of five experiments. (b) Inhibition of PGD₂ release by human MCs following FcεRI aggregation by FcεRIβ gene transduction. Open and closed bars indicate Mock-transduced and FcεRIβ-transduced MCs, respectively. MCs were transduced with an multiplicity of infection (MOI) of 100. Data are expressed as means ± SEM of three experiments. (c) Effect of FcεRIβ transduction on FcεRI-mediated TNF-α and MIP-1α production. Mock-transduced MCs (grey bars) and FcεRIβ-transduced MCs (β, closed bars) were incubated with an anti-FcεRIα mAb (CRA1) for 6 and 24 h for TNF-α ELISA and MIP-1α ELISA, respectively. Open bars indicate non-transduced MCs. Cytokine production by non-transduced MCs following the aggregation of FcεRI was defined as being equal to 100%. The production of TNF-α and MIP-1α by non-transduced MCs in response to CRA1 was 35.1 ± 17.4 and 705 ± 556 pg/mL/ 5×10^5 MCs, respectively. The spontaneous production of TNF-α and MIP-1α by non-transduced MCs was under the detection limits. The spontaneous production of TNF-α and MIP-1α was not affected by the transduction of Mock and FcεRIβ (data not shown). Data are expressed as the mean ± SEM of the % production (three–four experiments). (d) Time course of HR by Mock-transduced (open circles) and FcεRIβ-transduced MCs (closed circles) following FcεRI aggregation. MCs were transduced with an MOI of 100. Data are expressed as the means of two experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Ad5/FcεRIβ at 1 min after aggregation of FcεRI. Tyrosine phosphorylation of the MAPKs was down-regulated in FcεRIβ-transduced MCs in the following order: p38MAPK>ERK1/2>JNK. To confirm the negative

regulatory signal induced by a low level of tyrosine-phosphorylated FcεRIβ and Syk, we measured intracellular Ca²⁺ events in Mock- and FcεRIβ-transduced MCs following activation by a calcium ionophore and activation via FcεRI. The early phase of Ca²⁺ influx into MCs following FcεRI aggregation, but not of calcium ionophore, was down-regulated in FcεRIβ-transduced MCs, compared with Mock-transduced MCs (Fig. 5e).

Down-regulation of redistribution of Lyn following FcεRI aggregation in FcεRIβ-overexpressing cultured MCs

The redistribution of Lyn into small patches within the plasma membrane following FcεRI aggregation is thought to be an important early signal step in initiating FcεRI phosphorylation and early signalling events [19]. We therefore hypothesized that overexpressed FcεRIβ inhibits the recruitment of Lyn within the cell surface following FcεRI aggregation. To test this hypothesis, we compared localization of Lyn in Mock- and FcεRIβ-transduced MCs before and after FcεRI aggregation (Fig. 6). Endogenous Lyn in the resting MCs was mainly found associated with the plasma membrane, and some Lyn was also localized in the cytoplasm (Figs 6a and c, mock, 0 min). Following aggregation of FcεRI Lyn was distributed in small patches within the cell membrane, and the intensity of Lyn fluorescence in the cell membrane appeared to increase (Figs 6a and c, mock, 1 min). Recruitment of Lyn to plasma membrane decreased following FcεRI aggregation in FcεRIβ-transduced MCs (Figs 6a and c, FcεRIβ, 1 min). Quantitative analysis of the redistribution of Lyn within the cell membrane after FcεRI aggregation in MCs is shown in Fig. 6b. The redistribution of Lyn in Mock-transduced MCs was significantly increased following the aggregation of FcεRI, but not in FcεRIβ-transduced MCs ($P < 0.05$). To examine the co-localization of Lyn and FcεRIβ, MCs were dual-stained with anti-Lyn and anti-FcεRIβ Abs (Fig. 6c). Lyn and FcεRIβ were localized very close to each other in resting Mock-transduced MCs, but were not co-localized. Many co-localized spots were observed in FcεRIβ-transduced MCs, especially in the cell membrane (Fig. 6c, FcεRIβ, 0 min, yellow arrows). Following FcεRI aggregation, the co-localized spots remain in the same location in the cell membrane (Fig. 6c, FcεRIβ, 1 min, yellow arrows).

Discussion

In this article, we have reported that MCs, in which FcεRIβ expression is scattered throughout the cytoplasm in addition to being found in the cell membrane, are preferentially localized in perivascular regions. We mimicked an MC type, in which FcεRIβ is expressed

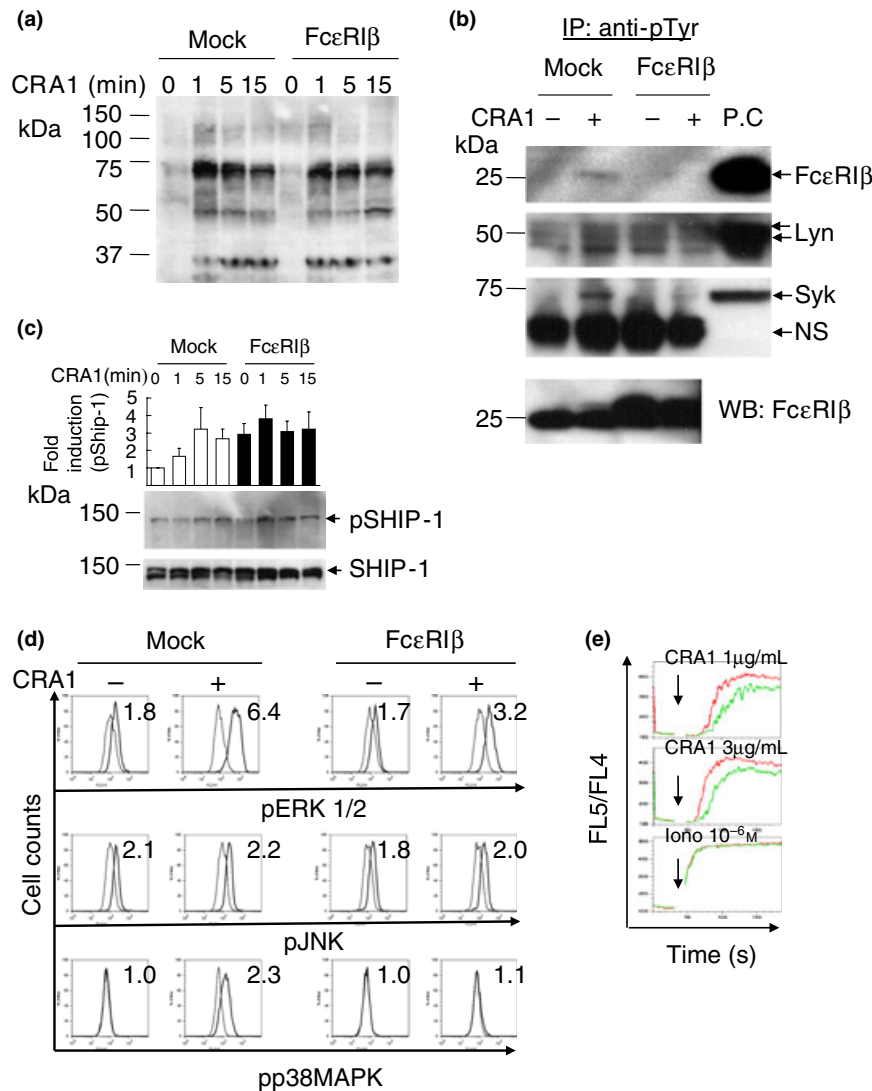


Fig. 5. Comparison between tyrosine phosphorylation of proteins and Ca²⁺ influx in Mock-transduced and in FcεRIβ-transduced mast cells (MCs) following FcεRI aggregation. Tyrosine phosphorylation in Mock-transduced and FcεRIβ-transduced MCs following FcεRI aggregation. MCs were transduced with an multiplicity of infection (MOI) of 100. (a) Total tyrosine-phosphorylated proteins, (b) tyrosine-phosphorylated FcεRIβ, Lyn, and Syk, (c) tyrosine-phosphorylated SHIP1 and total SHIP1 and (d) ERK1/2, Jun amino-terminal kinase, and p38MAPK tyrosine phosphorylation. FcεRI was aggregated for 0, 1, 5, and 15 min (a and c), 1 min (b), or 15 min (d). Cell lysates were probed for phosphotyrosine (a) and tyrosine-phosphorylated SHIP1 and SHIP1 (c). (b) Cells were lysed, and proteins were immunoprecipitated with an antiphosphotyrosine Ab and then probed for FcεRIβ, Lyn, and Syk. In (B), some part of the cell lysates were directly immunoblotted with anti-FcεRIβ (WB: FcεRIβ). NS indicates a non-specific band. P.C. indicates the positive control, which consists of LAD2 cell lysates. The results of one experiment that is representative of the four that were conducted are shown. (c) Fold induction of tyrosine phosphorylation level of SHIP-1 was determined from four individual experiments by densitometry. The tyrosine phosphorylation level of Mock-transduced MCs at 0 min is calculated as 1. (d) Cells were stained for phospho-ERK1/2, phospho-JNK, and phospho-p38MAPK and then analysed for FACS. The number indicates the MFI ratio of phosphorylated mitogen-activated protein kinase (MAPK) to the isotype control. (e) Comparison of Ca²⁺ influx between Mock-transduced (red lines) and FcεRIβ-transduced MCs (green lines) at an MOI of 100 after activation with CRA1 (1 μg/mL: top, or 3 μg/mL: middle) or calcium ionophore stimulation (Iono, 10⁻⁶ M: bottom). The results of one experiment that is representative of the 3–5 that were conducted are shown.

both in the cytoplasm and in the cell membrane, by the FcεRIβ transducing into PB-derived cultured MCs (Fig. 3). Although we were unable to perform a quantitative comparison of the FcεRIβ expression levels in different cell compartments, such as the cell membrane and cytoplasm following FcεRIβ transduction because of the limited availability of human MCs, a Western

blotting analysis of whole-cell lysates, a FACS analysis, and a confocal laser scanning analysis clearly showed that FcεRIβ transduction up-regulated FcεRIβ, FcεRIα, and FcεRIγ expression in both the membrane and the cytoplasm (Figs 2b, c, 3a and b). FcεRIβ transduction up-regulated the surface expression of FcεRIα by 1.6-fold at maximum (Fig. 2); however, overexpression

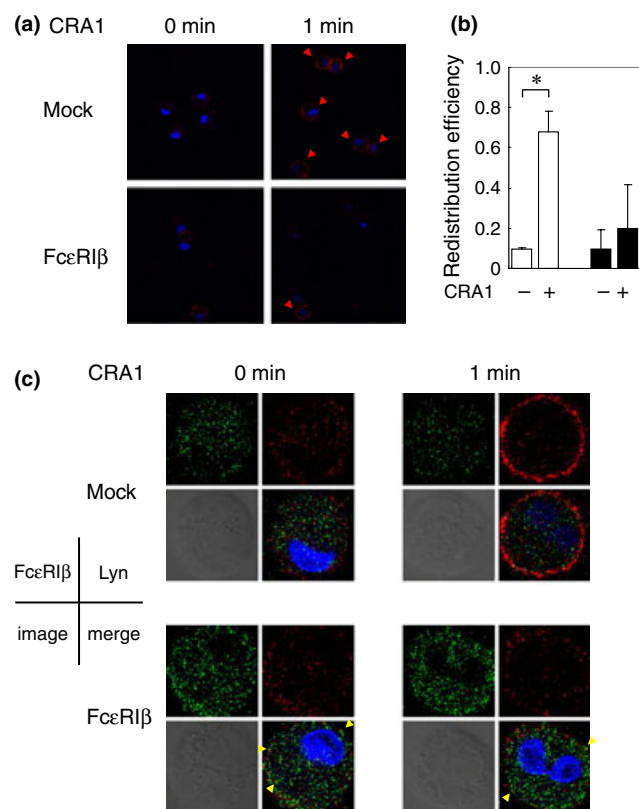


Fig. 6. Down-regulation of redistribution of Lyn in FcεRIβ-transduced MCs following FcεRI aggregation. MCs were transduced with a multiplicity of infection (MOI) of 300. (a) Comparison of localization of Lyn (red) in Mock-transduced (upper panels) and FcεRIβ-transduced MCs (lower panels) before (0 min) and 1 min after FcεRI aggregation, by confocal microscopy. The MCs were fixed and permeabilized and then stained with anti-Lyn mAb and PE-conjugated goat anti-mouse IgG. Original magnification, $\times 200$. (b) Quantitative analysis of redistribution of Lyn following aggregation of FcεRI in Mock-transduced and FcεRIβ-transduced MCs. Means $\pm$ SEM of redistribution efficiency was calculated from three independent experiments, with about 100 cells analysed in each experiment. Ringlike staining cells were counted as positive cells (red arrows in Fig. 6A). $*P < 0.05$ (c) Representative confocal microscopy images of the subcellular localization of Lyn in Mock-transduced (upper panels) and FcεRIβ-transduced MCs (lower panels) before (0 min) and 1 min after FcεRI aggregation. The green fluorescence and red fluorescence indicate FcεRIβ and Lyn, respectively. The yellow arrows point to a couple of double-positive cells (yellow). Original magnification, $\times 800$.

of FcεRIβ down-regulated FcεRI-mediated degranulation, PGD₂ synthesis, and production of a set of cytokines (Fig. 4). We found that at 1 min after FcεRI aggregation, tyrosine-phosphorylated FcεRIβ and Syk and the early phase of Ca²⁺ influx into MCs following FcεRI aggregation were significantly down-regulated in MCs treated with Ad5/FcεRIβ (Fig. 5). We therefore conclude that highly expressed cytoplasmic FcεRIβ functions as a negative regulator of the Fcγ-mediated human MC activation signal.

As loss of Syk expression abrogates FcεRI-mediated cell activation [20], down-regulation of Syk activation soon after FcεRI aggregation may explain the reduction in early Ca²⁺ influx and down-regulation of degranulation, PGD₂ synthesis, and production of a set of cytokines, including TNF-α, which requires stronger activation signals to be expressed than to other cytokines, such as MIP-1α. This is in agreement with the previous finding [21] that weak stimulation of mouse MCs, which caused minimal degranulation, led to a half-maximal chemokine secretory response and a weaker secretory response of cytokines with the exception of TNF-α. Moreover, the heterogeneous down-regulation of MAPKs can be explained by difference in requirement of the strength of activation signals. Activation of p38MAPK may require a stronger activation signal than activation of ERK1/2 does. This finding in human MCs is almost the opposite of the findings in mouse MCs, where weak stimulation favoured the phosphorylation of p38MAPK and ERK2 phosphorylation was induced by a stronger stimulus [21].

The question is why the increase in cytoplasmic FcεRIβ in human MCs caused down-regulation of the tyrosine phosphorylation of FcεRIβ. We confirmed that the FLAG tag had no effect on the inhibition of FcεRI-mediated MC activation using FcεRIβ without FLAG-tag gene transduction (Fig. 4). We confirmed using high-density oligonucleotide probe arrays that expression of ER-stress-inducible genes such as *Grp78*, *Grp94*, and *ERp72*, was not induced in MCs treated with Ad5/FcεRIβ (data not shown), indicating that the negative regulation of the Fcγ-mediated human MC activation signal by FcεRIβ appeared not to be due to dysfunction of the MCs in response to ER stress. The mechanism of down-regulation of tyrosine phosphorylation of FcεRIβ may be inhibition of association between Lyn and FcεRIαβγ₂ complexes following FcεRI aggregation. Yeast two-hybrid studies [9] have indicated that Lyn can directly bind to the unphosphorylated C-terminal cytoplasmic domain of FcεRIβ, while peptide-binding studies [22, 23] have indicated that Lyn binds to phosphorylated ITAMs of FcεRIβ. Moreover, chemical cross-linking studies have demonstrated that 5% of all intracellular Lyn in RBL-2H3 cells is bound to resting, unphosphorylated receptors [8]. Therefore, overexpressed FcεRIβ protein may bind to Lyn in resting MCs, and the binding may inhibit further association between Lyn and phosphorylated ITAMs of FcεRIβ in αβγ₂ complexes following FcεRI aggregation. Although the co-precipitation of FcεRIβ and Lyn in FcεRIβ-transduced MCs was not feasible due to the limited number of human MCs, a confocal microscopic analysis has shown the co-localization of Lyn and FcεRIβ in FcεRIβ-transduced MCs (Fig. 6C). Indeed, a confocal microscopic analysis has shown that overexpression of

FcεRIβ inhibits redistribution of Lyn to small patches within the plasma membrane following FcεRI aggregation. This may be due to excess binding of Lyn to overexpressed FcεRIβ in the cell membrane; however, single FcεRIβ protein is not thought to exist in rafts. We found that increased expression of FcεRIβ led to an unbalanced increase in FcεRIβ compared with FcεRIα in MCs. Lyn must be anchored to the plasma membrane for proper signalling, but recruitment of Lyn into lipid rafts is unnecessary for FcεRI-induced phosphorylation [19]. The constitutive and functional association between Lyn and FcεRI can occur outside the lipid rafts [19]. We also observed that the level of SHIP-1 phosphorylation in unstimulated MCs was already high, but the reason for this high level of phosphorylation is unknown. Whether the up-regulation of the SHIP-1 phosphorylation level in unstimulated MCs affects the activation of MCs is unclear.

We reported that the diminution of FcεRIβ significantly down-regulated cell surface FcεRI expression and FcεRI-mediated mediator release/production [2]. The diminution of FcεRIβ inhibited the redistribution of Lyn within the cell membrane following IgE sensitization. Our present findings appeared to contradict our previous findings. We examined HR by FcεRIβ-transduced MCs at MOIs of 0, 10, 30, and 100 following FcεRI aggregation. The results showed that the inhibition of HR by FcεRIβ-transduced MCs was MOI dependent, at least at MOIs of 10–100 (Fig. S4). As the transduction efficiency of the EGFP gene with an MOI of 10 in MCs was 39% (Fig. S2) and MCs transduced with FcεRIβ could not be isolated, the HR could not be accurately analysed using MCs when small amounts of Ad5/FcεRIβ (MOIs of 0.01, 0.1, 1, or 10) were transduced. Theoretically, however, Fcγ-mediated MC activation signal should be up-regulated by the increased expression of FcεRIβ, if the increased FcεRIβ leads to a balanced increase in FcεRIβ, compared with FcεRIα, in MCs.

A truncated splice variant of FcεRIβ (t-FcεRIβ) that lacks exon3, which codes the first two transmembrane domains, has been identified in human MCs [18]. These two transmembrane domains are required for binding to FcεRIα and receptor trafficking [24]. Therefore, t-FcεRIβ is not transported to the plasma membrane and instead has a diffuse distribution in the nuclear membrane and perinuclear region [18]. The anti-FcεRIβ Ab (242) used in this study, which recognizes the C-terminus of FcεRIβ [14], reacts with both full-length FcεRIβ and t-FcεRIβ. A Western blotting analysis also revealed the presence of t-FcεRIβ, which is about 10 kDa smaller than endogenous FcεRIβ (Fig. 2B). As the cytoplasmic FcεRIβ in the MCs examined in this study might include t-FcεRIβ, we developed another FcεRIβ Ab (Ex3) that recognizes the peptide CDISHIEGDIFSSFKAGY coded by exon 3 and attempted

to distinguish full-length FcεRIβ⁺ MCs and t-FcεRIβ⁺ MCs. Ex3 should recognize full-length FcεRIβ, but not t-FcεRIβ. As can be seen in Fig. S5, Ex3 staining showed some non-specific binding in tissues, but some cells were stained with 242, but not Ex3, indicating that the cytoplasmic FcεRIβ in the tissue MCs included t-FcεRIβ (red arrows).

The overexpression of full-length FcεRIβ has been shown to inhibit antigen-induced degranulation in the LAD2 human MC line, which is in agreement with our findings, while the overexpression of t-FcεRIβ potentiates degranulation [25], indicating that cytoplasmic full-length FcεRIβ and t-FcεRIβ might play different roles in antigen-induced MC degranulation. Thus, the ability of full-length FcεRIβ to inhibit MC mediator release when overexpressed does not necessarily mean that this occurs *in vivo*, as the very high overexpression levels obtained *in vitro* might excessively 'mop up' important signalling molecules (i.e. Lyn). However, our results provide two lines of evidence suggesting that we successfully mimicked a tissue MC, in which FcεRIβ is expressed both in the cytoplasm and the cell membrane, using the transduction of the FcεRIβ into PB-derived cultured MCs. First, immunohistochemical studies demonstrated that the localization of FcεRIβ in tissue MCs appeared to be similar to that of FcεRIβ-overexpressed MCs. Multiple images showing a Z-stack through the depth of the section revealed that the localization of FcεRIβ is scattered throughout the cytoplasm and in the membrane of MCs located in the subepithelial and perivascular regions (Movies S1 and S2, respectively). FcεRIβ expression in non-transduced, PB-derived MCs was scattered throughout the cytoplasm and in the cell membrane, exhibiting a punctate staining pattern (Fig. 1D). This localization is similar to that observed in FcεRIβ-overexpressed MCs, which also showed punctate staining (Fig. 3B). Second, an immunohistochemical study of giant papillae using Ex3 revealed that FcεRIβ was localized in both the cytoplasm and the cell membrane, indicating that the cytoplasmic FcεRIβ in tissue MCs includes full-length FcεRIβ (Fig. S5, blue arrows). The role of perivascular MCs in allergic reactions could be further clarified if perivascular MCs could be shown to be less IgE-responsive than their subepithelial counterpart using isolated MCs. Such a study, however, is not feasible at the moment.

In summary, cytoplasmic FcεRIβ, which is not co-localized with FcεRIα, may function as a negative regulator, as it can capture important signalling molecules such as Lyn. We observed that MCs in the perivascular regions of skin tissues from patients with atopic dermatitis exhibited high expression levels of cytoplasmic FcεRIβ (Fig. S6). Recently, the perivascular position of skin MCs has been reported to allow the selective uptake of IgE from the blood [26]. Perivascular

MCs acquire IgE by extending their cell processes across the vessel wall to capture luminal IgE [26]. These data suggest that perivascular MCs have different functions from MCs located in the subepithelial region.

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Conflict of interest

The authors have no financial conflict.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Viability of human MCs following infection with Ad5/β-gal (Mock: upper panels) or Ad5/FcεRIβ (lower panels) at a multiplicity of infection (MOI) of 30, 100, or 300 in the presence of SCF.

Figure S2. Efficiency of gene transduction into human MCs by a fibre-mutant adenovirus vector.

Figure S3. Up-regulation of immature and mature FcεRIα in human MCs by FcεRIβ transduction.

Figure S4. Multiplicity of infection-dependent inhibition of histamine release by FcεRIβ-transduced MCs (closed circles) at 30 min after FcεRI aggregation.

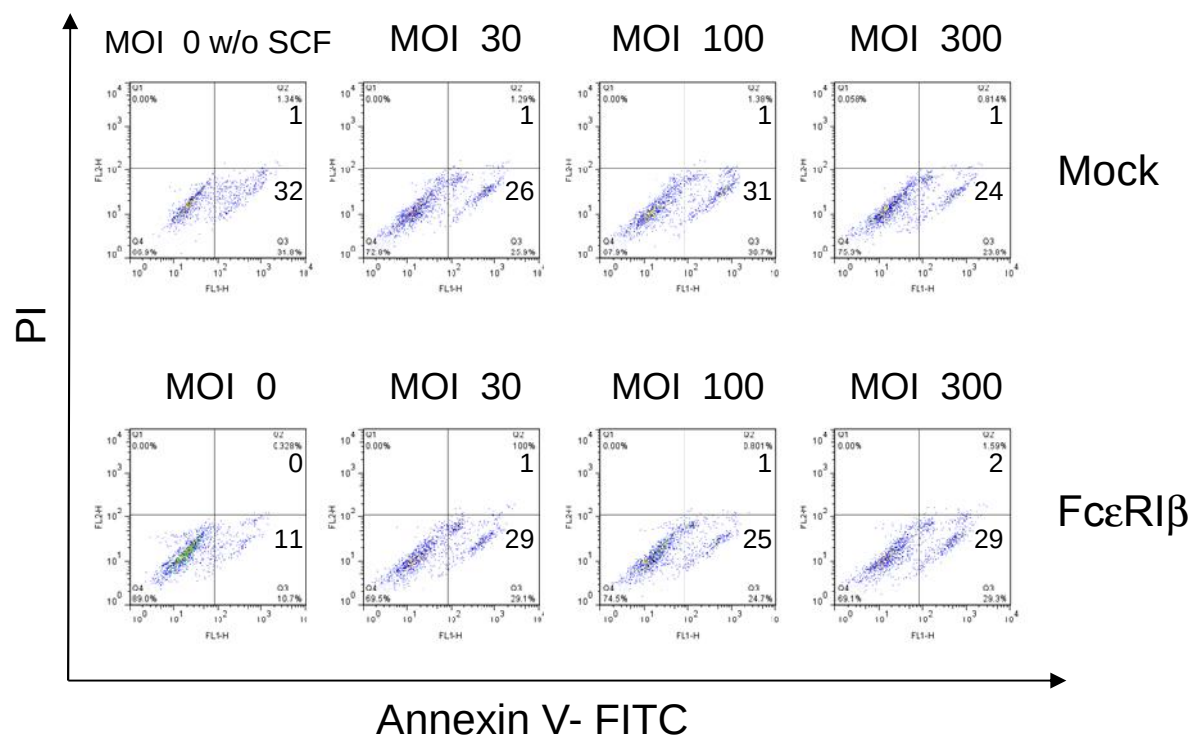
Figure S5. Comparison of anti-FcεRIβ Ab, 242⁺, and Ex3⁺ MCs in sequential sections of giant papillae.

Figure S6. Expression of cytoplasmic FcεRIβ in MCs of the perivascular regions in the skin from a patient with atopic dermatitis.

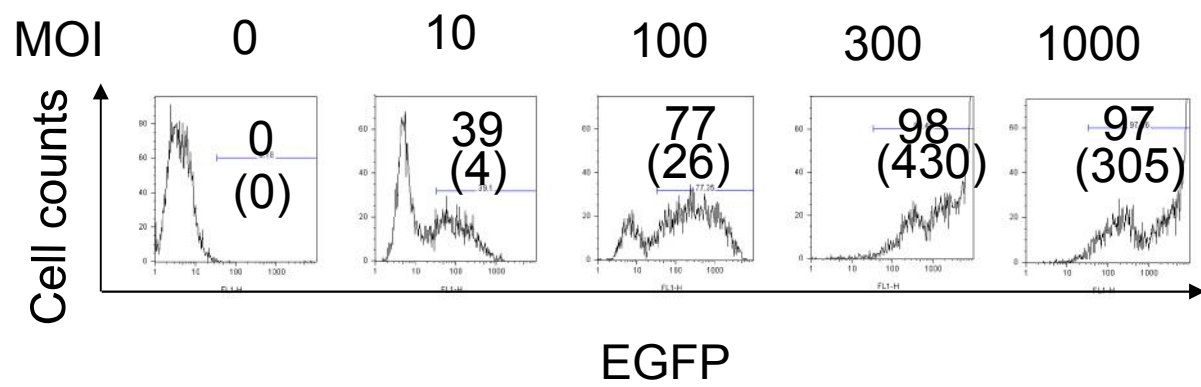
Movie S1. FcεRIβ (green) immunostaining of a human MCs of the subepithelial region in a giant papilla. Vertical (Z) images were reconstructed by accumulating horizontal plane (X-Y) images. Blue: nucleus staining using DAPI. Original magnification, ×200.

Movie S2. FcεRIβ (green) immunostaining of a human MCs of the perivascular region in a giant papilla. Vertical (Z) images were reconstructed by accumulating horizontal plane (X-Y) images. Blue: nucleus staining using DAPI. Original magnification, ×200.

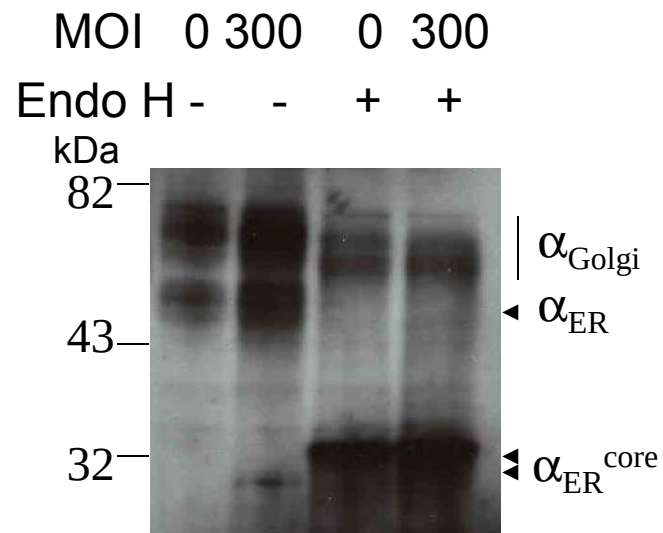
Supplemental Figure 1



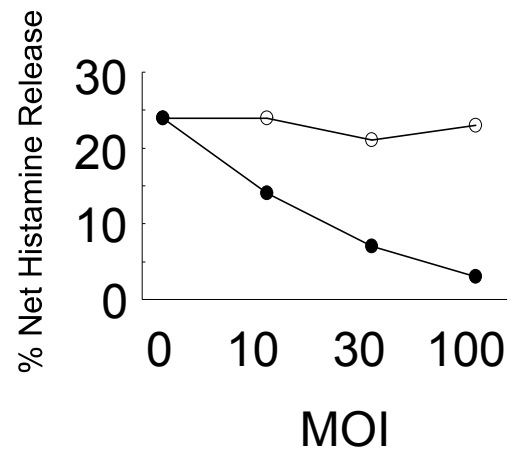
Supplemental Figure 2



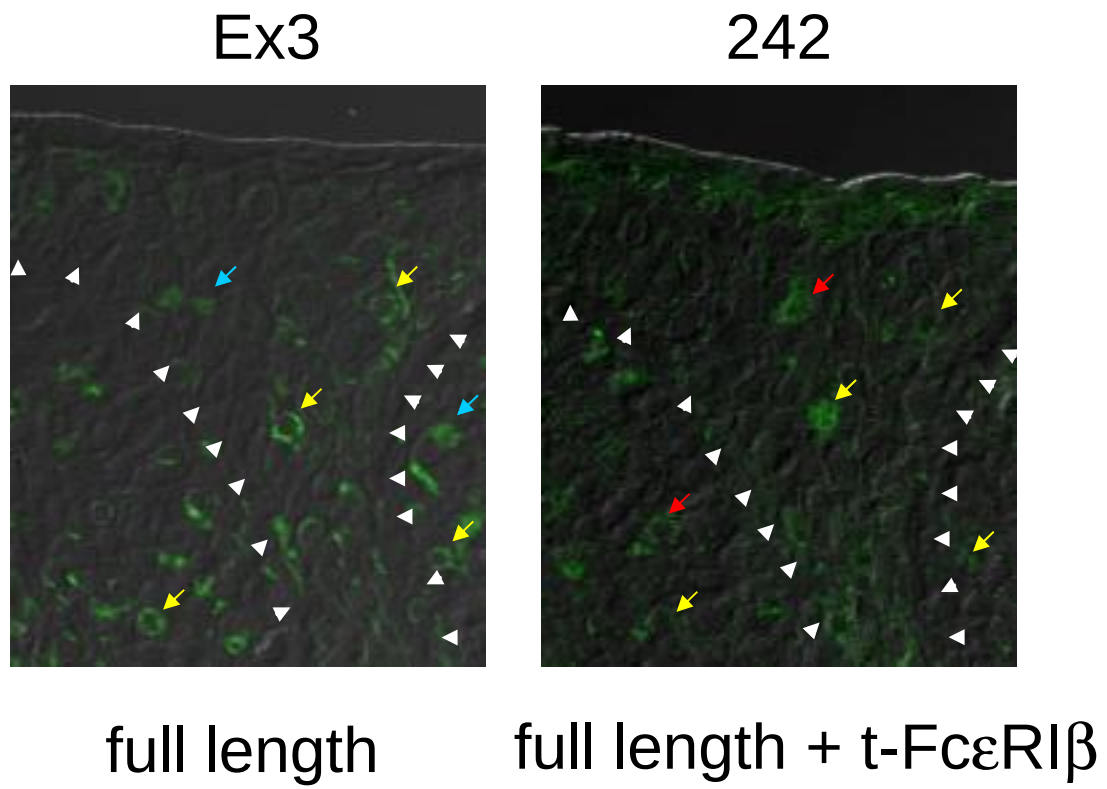
Supplemental Figure 3



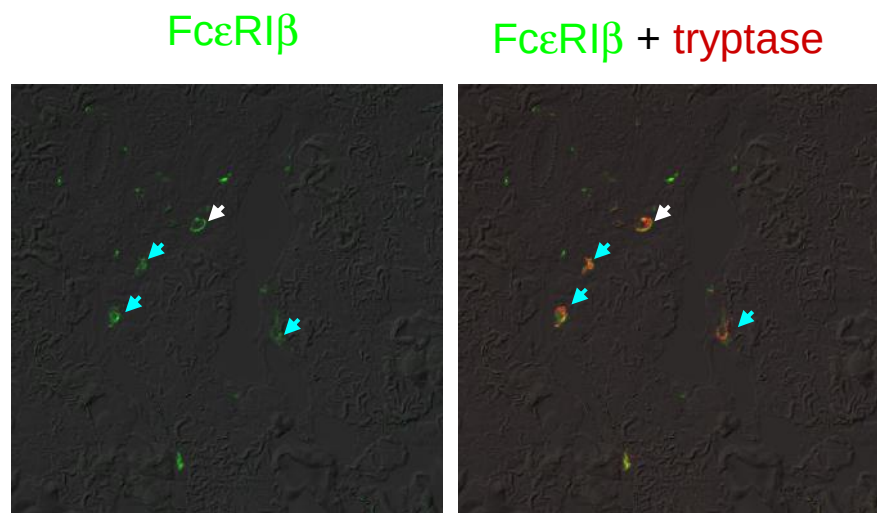
Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6



Supplemental Movie 1



epithelial mast DAPI.mov

The movies have been sent to the editors by e-mail. Please get the movies from the editors.

Supplemental Movie 2



vascular mast.mov

The movies have been sent to the editors by e-mail. Please get the movies from the editors.

Online Repository

Highly expressed cytoplasmic FcεRI in human mast cells functions as a negative regulator of the FcRγ-mediated cell activation signal

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Short title: Role of cytoplasmic FcεRIβ in human mast cells

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Supplemental Figure legend

Supplemental Figure 1. Viability of human MCs following infection with Ad5/ -gal (Mock: upper panels) or Ad5/Fc ϵ RI (lower panels) at a multiplicity of infection (MOI) of 30, 100, or 300 in the presence of SCF. MCs were stained with propidium iodide (PI) and Annexin V. PI⁺ and/or Annexin V⁺ cells were counted as dead cells (the numbers indicate the percentage of cells with either positivity). “w/o SCF” means MCs in the absence of SCF.

Supplemental Figure 2. Efficiency of gene transduction into human MCs by a fiber-mutant adenovirus vector. The efficiency of foreign gene transduction and expression in PB-derived MCs was assessed by FACS analysis with Ad5/EGFP. The numbers in the histograms indicate transduction efficiency (percentage of EGFP-positive cells). The numbers in brackets indicate the MFI ratio of EGFP to the control. The results of one experiment representative of the two that were conducted are shown.

Supplemental Figure 3. Upregulation of immature and mature Fc ϵ RI~ in human MCs by Fc ϵ RI transduction. MCs transduced with Fc ϵ RI β at an MOI of 0 and 300 were lysed, and the Fc ϵ RI α was immunoblotted with anti-Fc ϵ RI α Ab in the presence or

absence of endoglycosidase H (endo H). α_{ER} , immature α ; α_{Golgi} , mature α ; α_{ER}^{core} , the expected molecular weight of the core protein.

Supplemental Figure 4. MOI-dependent inhibition of histamine release by

Fc ϵ RI -transduced MCs (closed circles) at 30 min after Fc ϵ RI aggregation. Open circles indicate histamine release by Mock-transduced MCs. Data are expressed as the means of two experiments.

Supplemental Figure 5. Comparison of anti-Fc ϵ RI Ab, 242⁺, and Ex3⁺ MCs in sequential sections of giant papillae. The anti-Fc ϵ RI β Ab designated as 242 recognizes both full-length and truncated Fc ϵ RI β , whereas the anti-Fc ϵ RI β Ab designated as Ex3 only recognizes full-length Fc ϵ RI β . The yellow arrows indicate the cells that are stained with both 242 and Ex3. The red arrows indicate the cells that are stained with 242 alone. The blue arrows indicate the cytoplasmic staining pattern of Fc ϵ RI β when stained with Ex3. The white arrowheads indicate the boundary line between epithelium and substantia propria.

Supplemental Figure 6. Expression of cytoplasmic FcεRI in MCs of the perivascular regions in the skin from a patient with atopic dermatitis. Skin specimens were immunostained with anti-FcεRIβ Ab (green) or anti-tryptase mAb (red). The white arrows indicate the ring-like staining pattern of FcεRIβ. The blue arrows indicate the cytoplasmic staining pattern of FcεRIβ.

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Highly expressed cytoplasmic FcεRI in human mast cells functions as a negative regulator of the FcRγ-mediated cell activation signal

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Short title: Role of cytoplasmic FcεRIβ in human mast cells

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Supplemental Figure legend

Supplemental Figure 1. Viability of human MCs following infection with Ad5/ -gal (Mock: upper panels) or Ad5/Fc ϵ RI (lower panels) at a multiplicity of infection (MOI) of 30, 100, or 300 in the presence of SCF. MCs were stained with propidium iodide (PI) and Annexin V. PI⁺ and/or Annexin V⁺ cells were counted as dead cells (the numbers indicate the percentage of cells with either positivity). “w/o SCF” means MCs in the absence of SCF.

Supplemental Figure 2. Efficiency of gene transduction into human MCs by a fiber-mutant adenovirus vector. The efficiency of foreign gene transduction and expression in PB-derived MCs was assessed by FACS analysis with Ad5/EGFP. The numbers in the histograms indicate transduction efficiency (percentage of EGFP-positive cells). The numbers in brackets indicate the MFI ratio of EGFP to the control. The results of one experiment representative of the two that were conducted are shown.

Supplemental Figure 3. Upregulation of immature and mature Fc ϵ RI~ in human MCs by Fc ϵ RI transduction. MCs transduced with Fc ϵ RI β at an MOI of 0 and 300 were lysed, and the Fc ϵ RI α was immunoblotted with anti-Fc ϵ RI α Ab in the presence or

absence of endoglycosidase H (endo H). α_{ER} , immature α ; α_{Golgi} , mature α ; α_{ER}^{core} , the expected molecular weight of the core protein.

Supplemental Figure 4. MOI-dependent inhibition of histamine release by

Fc ϵ RI -transduced MCs (closed circles) at 30 min after Fc ϵ RI aggregation. Open circles indicate histamine release by Mock-transduced MCs. Data are expressed as the means of two experiments.

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