

The interaction between Lyn and FcεRIβ is indispensable for FcεRI-mediated human mast cell activation

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FcεRIβ; histamine; human mast cells; immunoreceptor tyrosine-based activation motif; Lyn.

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Abstract

Background: FcεRIβ reportedly functions as an amplifier of the FcεRIγ-mediated activation signal using a reconstitution system. However, the amplification mechanisms in human mast cells (MCs) are poorly understood. We previously reported the hyperexpression of FcεRIβ of MCs in giant papillae from vernal keratoconjunctivitis patients, compared with that in conjunctivae from nonallergic conjunctivitis patients. Elucidation of the molecular mechanisms of the amplification induced by FcεRIβ should provide new targets for novel therapeutic interventions. The aim is to understand in greater details the function of FcεRIβ in human MC FcεRI expression and signaling.

Methods: FcεRIβ and Lyn expression was reduced using a lentiviral shRNA silencing technique. Localization of Lyn and FcεRIβ in cultured MCs was examined by confocal microscopic analysis. Mediators were measured by ELISAs.

Results: The diminution of FcεRIβ significantly downregulated cell surface FcεRI expression and FcεRI-mediated mediator release/production. The downregulation of FcεRI-mediated degranulation was not only due to the decrease in FcεRI expression. The diminution of FcεRIβ inhibited the redistribution of Lyn within the cell membrane following IgE sensitization. The diminution of Lyn in MCs significantly downregulated FcεRI-mediated degranulation. The recombinant cell-penetrating forms of phosphorylated FcεRIβ immunoreceptor tyrosine-based activation motif (ITAM) for intracellular delivery disturbed the interaction between Lyn and phosphorylated endogenous FcεRIβ ITAM, resulted in inhibiting IgE-dependent histamine release from MCs *in vitro* and from giant papillae specimens *ex vivo*.

Conclusion: The interaction between Lyn and FcεRIβ is indispensable for FcεRI-mediated human MC activation, and specific inhibition of the interaction may represent a new therapeutic strategy for the treatment of human allergic diseases.

Key finding

The interaction between Lyn and FcεRIβ is indispensable for FcεRI-mediated human mast cells activation, and disturbance of the interaction between Lyn and phosphorylated FcεRIβ ITAM may represent a new therapeutic strategy for the treatment of allergic diseases in humans.

The high-affinity receptor for IgE (FcεRI) is composed of three distinct protein species: an IgE-binding α-subunit (FcεRIα), a β-subunit (FcεRIβ), and a homodimer of disulfide-linked γ-subunits (FcεRIγ) (1). The human FcεRI can be expressed either as αγ₂ trimers or as αβγ₂ tetramers (2). The structure of the FcεRI αβγ₂ tetramers is unique among Fc receptors, and FcεRI αβγ₂ tetramers are expressed exclusively

on the surface of MCs and basophils, which play pivotal roles in immediate-type and inflammatory allergic reactions (3). We previously reported the hyperexpression of FcεRIβ of MCs in giant papillae from vernal keratoconjunctivitis patients, compared with that in bulbar conjunctivae from conjunctivochalasis or superior limbic keratoconjunctivitis patients (4). We found the preferential localization of FcεRIβ⁺ MCs around epithelium, suggesting that the FcεRIβ⁺ MCs around epithelium in mucosa from allergic patients are easily able to access allergens (4). We therefore hypothesized that FcεRIβ is a target molecule for the treatment of human allergic diseases.

Kinet's group has extensively studied the role of human FcεRIβ *in vitro* and *in vivo* by comparing its function in αγ₂ and αβγ₂ transfectants using a reconstitution system (i.e., co-transfection with spleen tyrosine kinase [Syk] and Lyn) in NIH3T3 cells and by comparing human *FcεRIα* transgenic mice and murine *FcεRIβ* gene-disrupted human *FcεRIα* transgenic mice (5–7). FcεRIβ has been found to increase the intracellular processing of FcεRIα, resulting in the amplification of cell surface FcεRI expression (7). FcεRIβ immunoreceptor tyrosine-based activation motif (ITAM) contains the third tyrosine between the two canonical ones and has a shorter spacer region between the canonical tyrosines (8). FcεRIβ amplifies the intensity of cell activation signals sent by the FcεRIγ with a gain of five- to sevenfold, as measured using co-transfected Syk activation and calcium mobilization (5). However, the amplification mechanisms in human MCs are poorly understood. Elucidation of the molecular mechanisms of the amplification induced by FcεRIβ should provide new targets for novel therapeutic interventions.

Methods

Generation of adult peripheral blood-derived cultured MCs

Lineage-negative (CD4[−], CD8[−], CD11b[−], CD14[−], CD16[−], and CD19[−]) mononuclear cells were selected from the peripheral blood (PB) mononuclear cells and cultured in serum-free Iscove's methylcellulose medium (Stem Cell Technologies Inc., Vancouver, BC, Canada) and Iscove's modified Dulbecco's medium (IMDM) containing recombinant (r) human (h) stem cell factor (SCF; PeproTech EC Ltd., London, UK) at 200 ng/ml, rhIL-6 (PeproTech) at 50 ng/ml, and rhIL-3 (PeproTech) at 1 ng/ml, as previously described (9). On culture day 42, methylcellulose was dissolved in PBS and the cells were resuspended and cultured in IMDM containing 100 ng/ml of rhSCF and 50 ng/ml of rhIL-6 with 2% FCS. The cultured mast cell (MC) numbers and purity were determined by counting after metachromatic staining with toluidine blue (Kimura's stain) (10) or by tryptase staining (clone AA1; DAKOCytomation, Carpinteria, CA, USA).

Activation of cultured human MCs

Cultured MCs were incubated with the indicated concentrations of mouse anti-human FcεRIα (clone CRA1; eBioscience, San Diego, CA, USA), 1 μM calcium ionophore

A23187 (A23187; Sigma-Aldrich, St. Louis, MO, USA), or HEPES alone at 37°C for various times. In some experiments, MCs were sensitized by incubation at 37°C for 30 min or 24 h with 1 μg/ml myeloma IgE (Calbiochem, Gibbstown, NJ, USA). After washing, the cells were challenged with rabbit anti-human IgE antibody (DAKOCytomation) at 37°C for the indicated time (See Online Repository for additional information about the experimental protocols.).

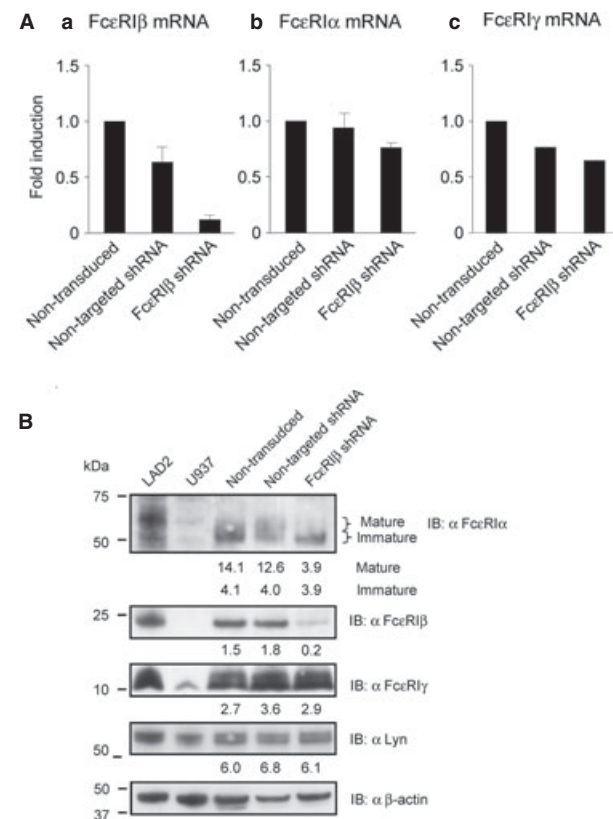


Figure 1 Diminution of FcεRIβ in cultured human mast cells (MCs) using the shRNA technique. (A) The FcεRIβ mRNA (A-a), FcεRIα mRNA (A-b), or FcεRIγ mRNA (A-c), and (B) FcεRIα, FcεRIβ, FcεRIγ, or Lyn protein expression levels in human MCs transduced with FcεRIβ shRNA were examined by real-time RT-PCR and immunoblotting, respectively. The expression levels of FcεRIβ mRNA (A-a), FcεRIα mRNA (A-b), or FcεRIγ mRNA (A-c) in nontransduced MCs were designated as 1. Data of FcεRIβ and FcεRIα mRNA are expressed as the mean ± SEM of three independent experiments using three different donors. Data of FcεRIγ mRNA are expressed as the mean of triplicates using one donor (A). The data are representative of similar results obtained from two independent experiments using two different donors (B). LAD2 cells and U937 cells were used as a positive and a negative control of FcεRIα and FcεRIβ expression, respectively. Relative band intensities of FcεRIα (mature and immature), FcεRIβ, FcεRIγ and Lyn normalized against β-actin are shown. 'Mature' and 'immature' mean the glycosylated FcεRIα protein with the smear around Mw 60–65 kDa and with apparent Mw 50 kDa, respectively.

Results

Diminution of FcεRIβ in cultured human MCs using an shRNA technique

To clarify the roles of FcεRIβ on FcεRI cell surface expression and FcεRI-mediated MC activation, FcεRIβ mRNA and protein were knocked down using a lentiviral shRNA silencing technique. The mRNA expression for FcεRIα and FcεRIγ was not affected (Fig. 1A). The FcεRIβ protein level was decreased to ~10%, but the protein expression of FcεRIγ was not affected (Fig. 1B). The expression levels of the glycosylated FcεRIα protein with apparent Mw 50 kDa (immature FcεRIα) were not much affected by diminution of FcεRIβ, but the smear around 60–65 kDa of glycosylated FcεRIα (mature FcεRIα) was decreased to ~20%. The expression level of Lyn was not affected by diminution of FcεRIβ.

Downregulation of FcεRI cell surface expression and FcεRI-mediated mediator release by diminution of FcεRIβ in cultured human MCs

We performed a flow cytometric analysis using anti-FcεRIα mAb to assess the effect of the diminution of FcεRIβ on the FcεRI cell surface expression level. A statistical analysis revealed that the cell surface FcεRIα expression was significantly decreased by the diminution of FcεRIβ in cultured MCs (Fig. 2A,B). To clarify the effect of the diminution of FcεRIβ on MC activation through FcεRI, we compared the release of the mediators by nontransduced MCs and cells transduced with lentiviral shRNA constructs for nontargeted shRNA or FcεRIβ shRNA. The diminution of FcεRIβ significantly inhibited histamine release (Fig. 2C; closed bars), PGD₂ synthesis (Fig. 2D; closed bars), and cytokine

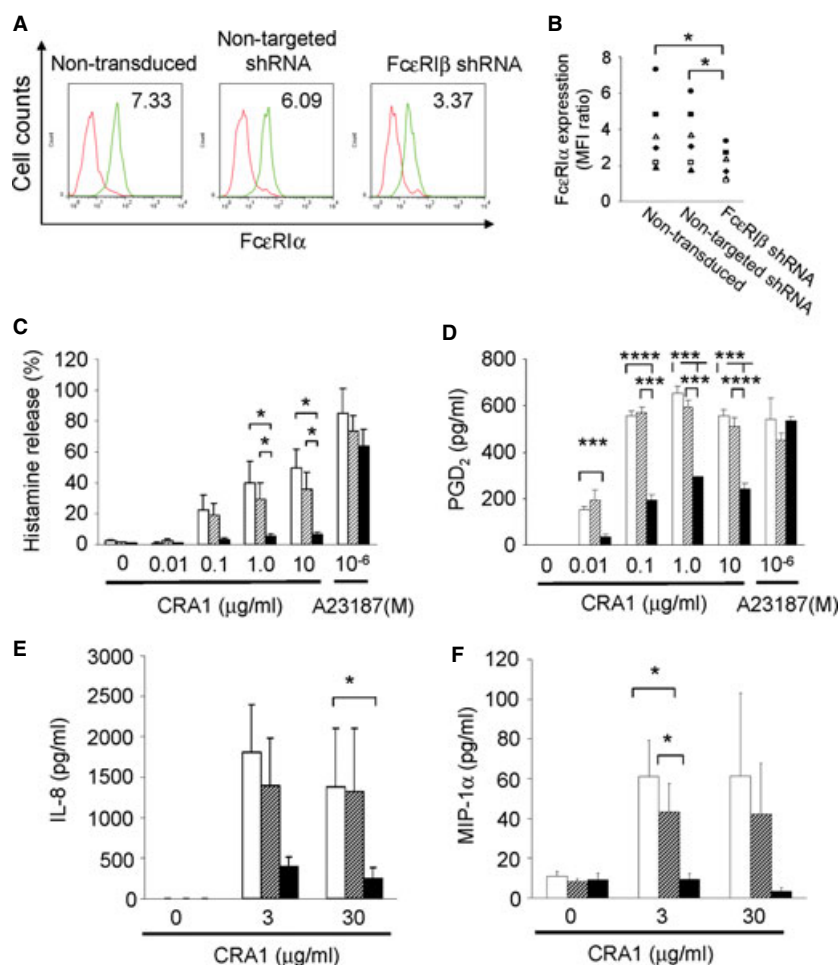


Figure 2 Effect of diminution of FcεRIβ on FcεRI cell surface expression and mediator release by cultured mast cells (MCs) following FcεRI aggregation. (A) The FcεRI cell surface expression of nontransduced MCs and cells transduced with nontargeted shRNA or FcεRIβ shRNA was assessed using FACS. The numbers indicate the MFI ratios. The red and green lines indicate mouse IgG2b (isotype control) and FcεRIα, respectively. (B) Statistical analysis of expression levels of (A). Expression levels shown on the vertical

axis were the MFI ratios ($n = 6$ donors). (C–F) Nontransduced MCs (open bars) and MCs transduced with nontargeted shRNA (hatched bars) or FcεRIβ shRNA (closed bars) were activated with CRA1 or A23187 for 30 min for histamine release (C) and PGD₂ production (D) and for 24 h for IL-8 (E) and MIP-1α production (F). *, ***, and **** indicate $P < 0.05$, 0.005, and 0.001, respectively. The data in C–F are expressed as the mean $\pm$ SEM of three or four independent experiments using three or four different donors.

production (Fig. 2E: IL-8 and Fig. 2F: MIP-1α; closed bars) in the cultured MCs following aggregation of FcεRI but did not affect the mediator release/production by A23187 (Fig. 2C,D).

Downregulation of FcεRI-mediated degranulation is not only due to a decrease in cell surface FcεRI expression

CD63, a secretory lysosomal marker, reportedly localizes on granule membranes of basophils, MCs, and platelets, and expression of CD63 on their cell surfaces is upregulated along with degranulation through granule–plasma membrane fusion (11, 12). To differentiate between the downregulation of FcεRI-mediated degranulation caused by a decrease in cell surface FcεRI expression as a consequence of the knockdown of FcεRIβ and the true inhibition of an ‘amplification pathway’, we compared the number of CD63-positive MCs at the same cell surface and FcεRI expression levels of MCs transduced with nontargeted shRNA and FcεRIβ shRNA using FACS. As can be seen in Fig. 3, the number of CD63-positive MCs, which were transduced with FcεRIβ shRNA, was significantly lower than that in cultured MCs transduced with nontargeted shRNA, even at the same FcεRIα expression levels (red squares in Fig. 3A,B), suggesting that the downregulation of FcεRI-mediated degranulation is not only due to a decrease in FcεRIα expression.

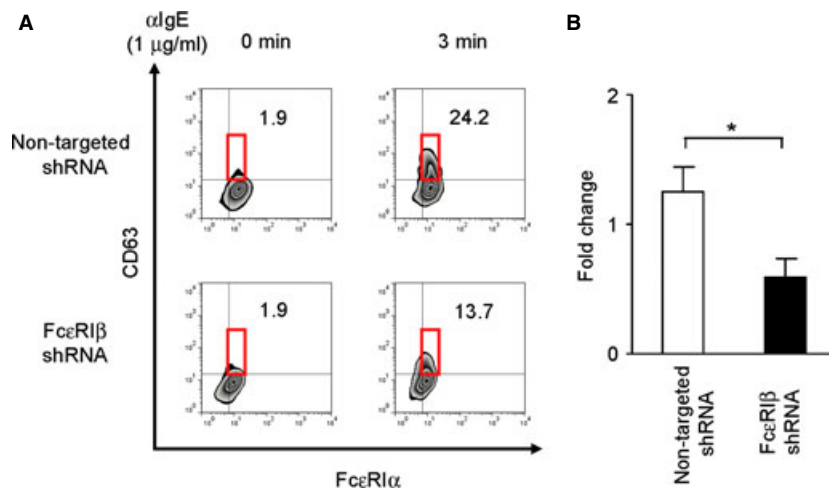


Figure 3 Flow cytometric analysis of cell surface FcεRIα and CD63 expression on cultured mast cells (MCs) transduced with nontargeted shRNA and FcεRIβ shRNA following FcεRI aggregation. (A) Comparison of CD63 expression between MCs transduced with nontargeted shRNA and FcεRIβ shRNA at 3 min after the addition of anti-IgE. The red squares indicate the CD63-positive MCs at the same FcεRIα expression levels between the MCs transduced with nontargeted shRNA and FcεRIβ shRNA. The numbers indicate the percentage of CD63-positive MCs in the red squares. We examined the time course of cell surface FcεRIα and CD63 expression on human MCs following the aggregation of FcεRI. MCs were sensitized with human IgE for 30 min; this sensitization did not affect

Downregulation of redistribution of Lyn within the cell membrane following IgE sensitization by diminution of FcεRIβ in cultured MCs

The redistribution of Lyn into small patches in the plasma membrane following FcεRI aggregation is thought to be an important step in initiating FcεRI phosphorylation and early signaling events (13). We therefore hypothesized that the diminution of FcεRIβ inhibits the recruitment of Lyn within the cell membrane following FcεRI aggregation. To test this hypothesis, we compared the localization of Lyn in cultured MCs transduced with lentiviral shRNA constructs for nontargeted shRNA or FcεRIβ shRNA before and after IgE sensitization or FcεRI aggregation following IgE sensitization (Fig. 4). Endogenous Lyn in the resting MCs was mainly found associated with the plasma membrane, and some Lyn was also localized in the perinuclear region, which is in agreement with Kovářová et al.'s (13) findings (Fig. 4A-c, white arrows). Following IgE sensitization, Lyn was distributed in small patches in the cell membrane, and the intensity of Lyn fluorescence in the cell membrane appeared to increase (Fig. 4A-g, red arrows). Following FcεRI aggregation, FcεRIβ was also distributed in small patches in the cell membrane (Fig. 4A-j). As can be seen in the representative image, the recruitment of Lyn within the plasma membrane decreased following the subsequent sensitization of IgE through the diminution of FcεRIβ (Fig. 4A-s). To examine the co-localization

the expression levels of cell surface FcεRI (data not shown). In MCs in a resting state (0 min: before the addition of anti-IgE), 95% of the MCs expressed FcεRIα (data not shown). At 3 min after the addition of anti-IgE, 91% of the MCs expressed FcεRIα, but 10 min of incubating the MCs with anti-IgE downregulated the cell surface FcεRI expression (80%). Therefore, we decided to compare the frequencies of CD63-positive MCs at 3 min after the addition of anti-IgE. (B) Statistical analysis of expression levels of (B). The percentage of CD63-positive nontransduced MCs at 3 min after the addition of anti-IgE in each experiment was designated as 1. Data are expressed as the mean ± SEM of four independent experiments using four different donors. * indicates $P < 0.05$.

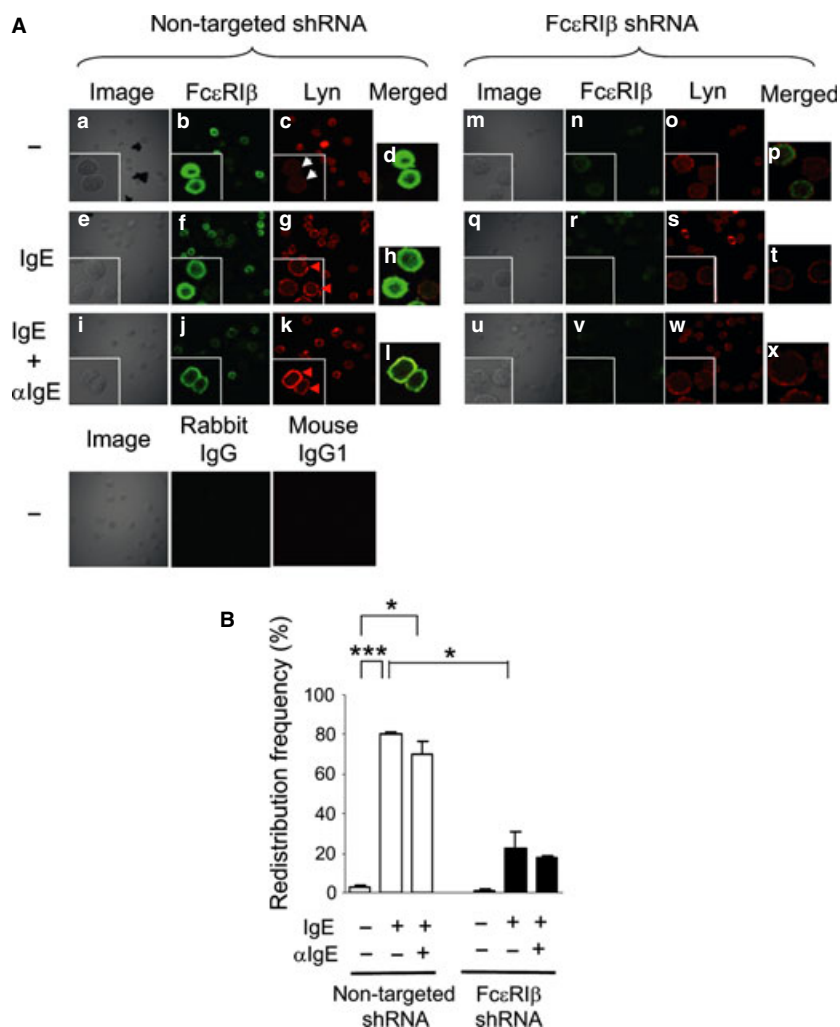


Figure 4 Effect of diminution of FcεRIβ on redistribution of Lyn in cultured mast cells (MCs). (A) Comparison of localization of Lyn in MCs transduced with nontargeted shRNA (left panels) or FcεRIβ shRNA (right panels) before (–) and after IgE sensitization for 24 h (IgE) and 1 min after FcεRI aggregation following IgE sensitization (IgE + αIgE), using confocal microscopy. The green fluorescence and red fluorescence indicate FcεRIβ and Lyn, respectively. The yellow fluorescence indicates double-positive staining. The insets show magnified images of each figure. Figure A–d, h, l, p, t, and x show

merged confocal microscopy images of the insets. MCs, which were treated with isotype control immunoglobulins, are shown as negative controls at the bottom of the panels. (B) Quantitative analysis of the redistribution of Lyn to the cell membrane in MCs shown in (A). The mean ± SEM of the redistribution efficiency were calculated from three independent experiments using three different donors, with more than 100 cells analyzed in each experiment. Ring-like stained cells were counted as positive cells (e.g., the red arrows in Fig. 4A–g,k). *, **, and *** indicate $P < 0.05$, 0.01 , and 0.005 , respectively.

of Lyn and FcεRIβ, images of MCs dual-stained with anti-Lyn and anti-FcεRIβ Abs were merged. Lyn and FcεRIβ appeared to be co-localized with control MCs following FcεRI aggregation, and the co-localized spots were observed in the cell membrane (Fig. 4A–l), whereas the localization of Lyn did not change after FcεRI aggregation with MCs transduced with FcεRIβ shRNA (Fig. 4A–x). A quantitative analysis of the redistribution of Lyn within the cell membrane after IgE sensitization in MCs is shown in Fig. 4B. The redistribution of Lyn within the cell membrane in MCs transduced with FcεRIβ shRNA following IgE sensitization significantly decreased, compared with that in MCs transduced with nontargeted shRNA.

Diminution of Lyn expression in cultured MCs resulted in downregulation of FcεRI-mediated degranulation

To confirm that the diminution of Lyn downregulates FcεRI-mediated degranulation, we suppressed Lyn transcripts in MCs using the lentiviral shRNA silencing technique using two different constructs, and Lyn decreased by ~90% and ~80%, respectively (Fig. 5A, Lyn shRNA #1 and #2). However, no significant difference in the expression levels of PLCγ1, FcεRIβ, or FcεRIγ in MCs transduced with Lyn shRNA was observed. The FcεRI cell surface expression of nontransduced MCs and cells transduced with nontargeted shRNA or Lyn shRNA was assessed using FACS. The

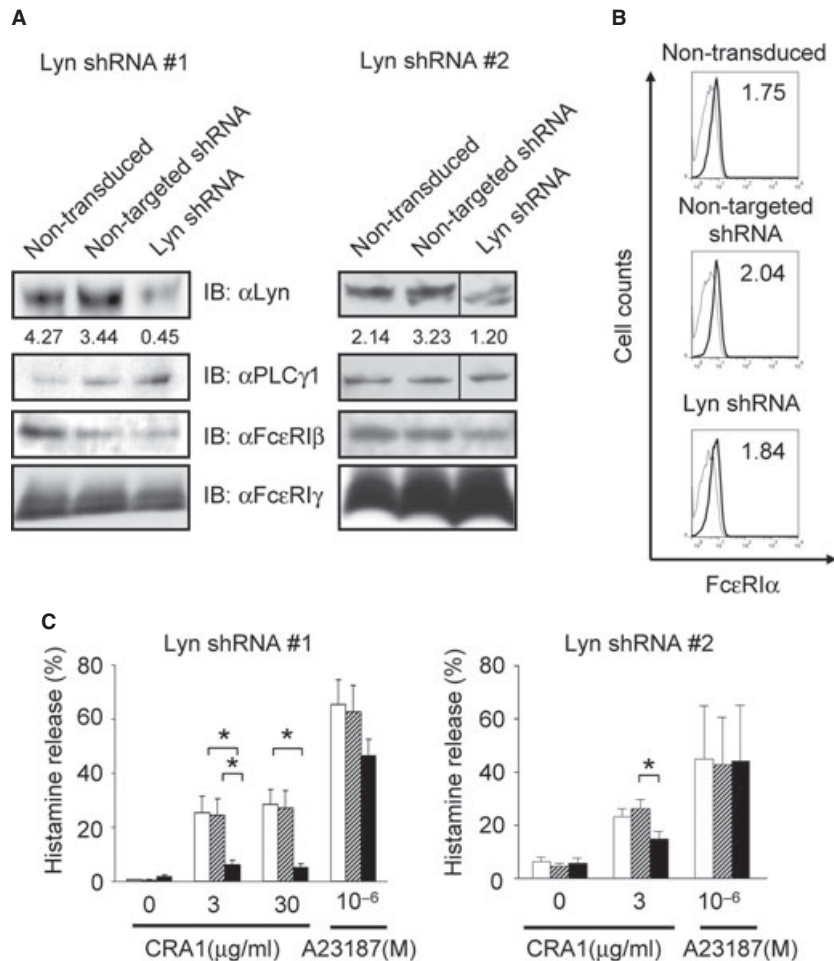


Figure 5 Diminution of Lyn in cultured human mast cells (MCs) using the shRNA technique and effect of diminution of Lyn on FcεRI cell surface expression and IgE-dependent MC degranulation. (A) Lyn expression levels in human MCs transduced with Lyn shRNA using two constructs (#1 and #2) were examined by immunoblotting. Cell lysates were also immunoblotted with anti-PLCγ1, FcεRIβ, or FcεRIγ Abs. The data are representative of similar results obtained from three independent experiments using three different donors. Relative band intensities of Lyn normalized

against PLCγ1 are shown. (B) FACS analysis of the FcεRI cell surface expression of nontransduced MCs and cells transduced with nontargeted shRNA or Lyn shRNA. The numbers indicate the MFI ratios. The dotted and solid lines indicate mouse IgG2b (isotype control) and FcεRIα, respectively. (C) Histamine release from non-transduced MCs (open bars) and MCs transduced with nontargeted shRNA (hatched bars) or Lyn shRNA (constructs #1 and #2, closed bars) following activation with CRA1 or A23187 ($n = 4$ donors, mean $\pm$ SEM). * indicates $P < 0.05$.

expression levels of FcεRI were not affected by the diminution of Lyn (Fig. 5B). The suppression of Lyn resulted in a significant decrease in histamine release by MCs following FcεRI aggregation, compared with that in MCs transduced with nontargeted shRNA (Fig. 5C).

Physical association between phosphorylated FcεRIβ ITAM and Lyn and the effect of FcεRIβ peptides on histamine release of cultured MCs following FcεRI aggregation

To examine whether triphosphorylated human FcεRIβ ITAM peptide (YpYpYp) interacts with Lyn, pull-down assays were performed using biotinylated *N*-terminus of FcεRIβ (E'),

YpYpYp (D'), or nonphosphorylated FcεRIβ ITAM peptide YYY (A', see Table S1), and the interacting proteins were analyzed using immunoblotting with anti-Lyn. The YpYpYp (D') alone showed a physical association with Lyn (Fig. 6A). To examine whether triphosphorylated human FcεRIβ ITAM peptide (YpYpYp) competes with intracellular FcεRIβ in cultured MCs, we developed several recombinant cell-penetrating forms (14) of triphosphorylated tyrosines of FcεRIβ ITAM (YpYpYp, see Table S1). To compare the effect of YpYpYp on the IgE-mediated activation of MCs, nonphosphorylated FcεRIβ ITAM peptide (YYY), monophosphorylated noncanonical tyrosine of FcεRIβ ITAM peptide (YYpY), biposphorylated canonical tyrosines of FcεRIβ ITAM peptide

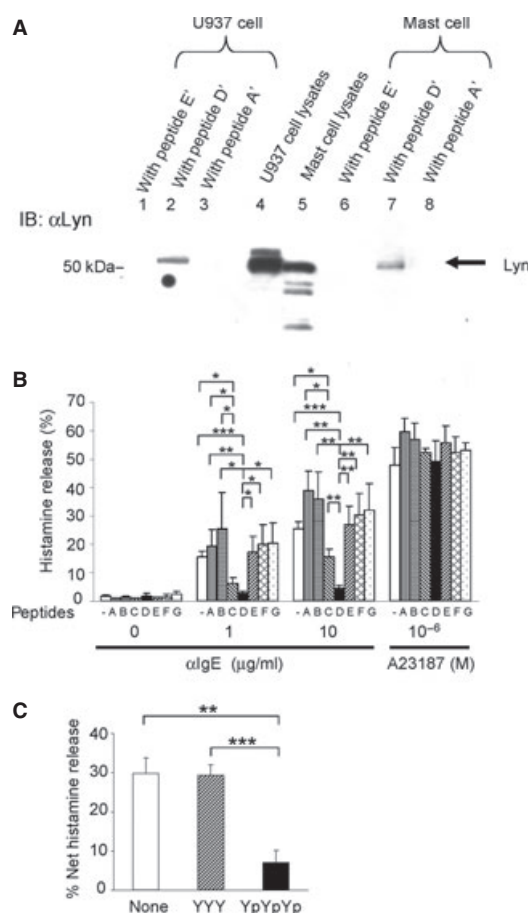


Figure 6 Physical association between phosphorylated FcεRIβ immunoreceptor tyrosine-based activation motif (ITAM) and Lyn and effect of FcεRIβ peptides on histamine release of cultured mast cells (MCs) following FcεRI aggregation. (A) U937 cell lysates (lanes 1, 2, 3) or MC lysates (lanes 6, 7, 8) incubated with biotinylated FcεRIβ *N*-terminus (E'), YpYpYp (D'), or YYY (A') were immobilized with streptavidin sepharose beads, and the interacting proteins were analyzed using immunoblotting with anti-Lyn. U937 cell lysates (lane 4) and MC lysates (lane 5) were immunoblotted with anti-Lyn as positive controls. The data are representative of similar results obtained from two independent experiments using two different donors. (B) Following the incubation of IgE-sensitized MCs with peptides (A–G) for 15 min, the MCs were activated with anti-IgE Ab (αIgE) or A23187 for 30 min for histamine release. The data are expressed as the mean ± SEM of four independent experiments using four different donors. See Table S1 with regard to the sequences of the peptides A–G. '–' indicates the incubation of MCs without any peptides. *, **, and *** indicate $P < 0.05$, 0.01, and 0.005, respectively. (C) Effect of the phosphorylated FcεRIβ ITAM peptide on histamine release from giant papilla following the addition of anti-IgE. The chopped giant papilla were incubated with FcεRIβ ITAM YYY (hatched bar) or YpYpYp (closed bar) or without peptides (open bar) for 15 min, and then activated with anti-IgE. The data are expressed as the mean ± SEM of three independent experiments using three different donors. ** and *** indicate $P < 0.01$ and 0.005, respectively.

(YpYpYp), and the *N*-terminus and C-terminus of FcεRIβ were also used. We confirmed the intracellular localization of these FITC-conjugated peptides using FACS and a confocal laser scanning microscope analysis (Fig. S1A,B).

Peptides C (YpYYp, see Table S1) and D (YpYpYp) significantly inhibited IgE-dependent histamine release but not A23187-induced histamine release (Fig. 6B). Peptide D (YpYpYp) significantly inhibited IgE-dependent PGD₂ synthesis (Fig. S1C). Finally, we examined the effect of YYY and YpYpYp on IgE-dependent histamine release from specimens of giant papillae *ex vivo*. The peptide YpYpYp significantly inhibited FcεRI-mediated histamine release (Fig. 6C).

Downregulation of redistribution of Lyn within the cell membrane in cultured MCs following IgE sensitization by the intracellular introduction of YpYpYp

To analyze the mechanisms of the peptide D (YpYpYp)-mediated inhibition of IgE-dependent histamine release, we compared the effect of the FcεRIβ *N*-terminus and YpYpYp on the redistribution of Lyn to the cell membrane before and after IgE sensitization and FcεRI aggregation. In Fig. 7A,B, the green-positive cells indicate the FITC-conjugated peptides that were successfully introduced to intracellular regions (red arrows; peptide⁺ cells). In Fig. 7B, Lyn was distributed in the cell membrane in the green-negative cells (a ring-like stain for anti-Lyn, white arrows; peptide[–] cells) but the green-positive cells (YpYpYp-positive cells) did not show a ring-like staining of Lyn (red arrows). In contrast, in Fig. 7A, either green-positive (*N*-terminus peptide-positive cells; red arrows) or green-negative cells (white arrows) showed a ring-like stain for anti-Lyn. As all MCs were not activated by anti-IgE, some MCs did not show a ring-like stain for anti-Lyn whatever peptides were introduced to intracellular regions or not. A quantitative analysis of the redistribution of Lyn within the cell membrane after IgE sensitization or FcεRI aggregation in MCs is shown in Fig. 7C. The redistribution of Lyn within the cell membrane in MCs treated with YpYpYp following FcεRI aggregation was significantly lower than that in MCs treated with the *N*-terminus of FcεRIβ.

Discussion

In this study, we found that the diminution of FcεRIβ in cultured MCs induced the downregulation of cell surface FcεRI expression, and FcεRI-mediated mediator release (Fig. 1, and 2). The downregulation of FcεRI-mediated degranulation may not only be due to the decrease in cell surface FcεRI expression (Fig. 3). The diminution of FcεRIβ inhibited the redistribution of Lyn within the cell membrane following IgE sensitization (Fig. 4). We previously showed that, besides Lyn, other signaling protein can bind to phosphorylated mouse FcεRIβ ITAM (15). Although there is a possibility that interactions between several signaling proteins and the phosphorylated FcεRIβ ITAM provide an amplifying function in FcεRI-mediated MC activation, the reduction in Lyn in cultured human MCs significantly downregulated

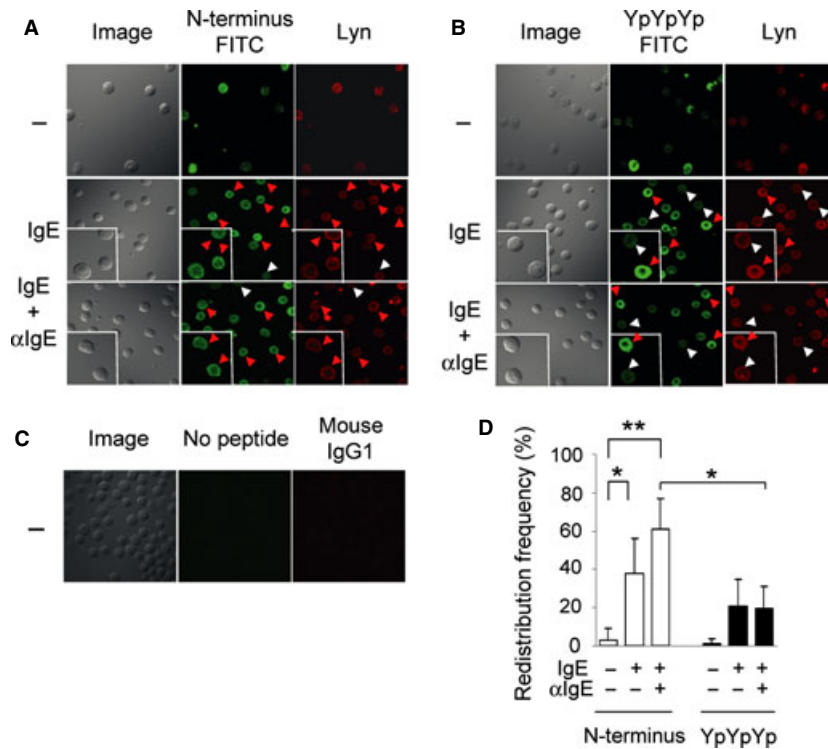


Figure 7 Effect of redistribution of Lyn to the cell membrane in cultured mast cells (MCs) by intracellular introduction of FcεRIβ peptides. (A–B) Comparison of the redistribution of Lyn in MCs treated with FITC-conjugated FcεRIβ N-terminus (A), and phosphorylated FcεRIβ immunoreceptor tyrosine-based activation motif (ITAM, YpYpYp), B) before (–) and after IgE sensitization for 24 h (IgE) and 1 min after FcεRI aggregation following IgE sensitization (IgE + αIgE), using confocal microscopy. The green fluorescence and red fluorescence indicate the intracellular FITC-conjugated peptides and Lyn, respectively. The insets show a magnified image of

each figure. The red and white arrows indicate peptide⁺ (green⁺) cells and peptide[–] (green[–]) cells, respectively. (C) MCs, which were treated without peptides or with mouse IgG1, are shown as negative controls. (D) Quantitative analysis of the redistribution of Lyn in MCs shown in (A) and (B). Ring-like stained cells for anti-Lyn mAb were counted as positive cells. The mean ± SEM of the redistribution efficiency were calculated from three independent experiments using three different donors, with more than 100 cells analyzed in each experiment. * and ** indicate $P < 0.05$ and 0.01 , respectively.

FcεRI-mediated degranulation (Fig. 5). Based on our findings of a physical association between Lyn and phosphorylated FcεRIβ ITAM in humans (Fig. 6A), we concluded that the interaction between Lyn and phosphorylated FcεRIβ ITAM functions as a positive regulator of the FcεRI-mediated human MC activation signal.

The intracellular induction of YpYpYp binds to Lyn in resting MCs (Fig. 6A), and this binding may further inhibit the association between Lyn and phosphorylated ITAM of endogenous FcεRIβ following FcεRI aggregation. Indeed, a confocal microscopic analysis has shown that the intracellular induction of YpYpYp inhibits the redistribution of Lyn in small patches in the cell membrane following FcεRI aggregation (Fig. 7). This may be due to the excess binding of Lyn to the introduced YpYpYp in MCs. The co-redistribution of FcεRI and Lyn requires dual fatty acylation of Lyn, the SH2 domain and/or SH3 domain of Lyn, and Lyn kinase activity (13). Palmitoylation-site-mutated Lyn, which is anchored to the plasma membrane but exhibits reduced sublocalization into lipid rafts, initiates the tyrosine phosphorylation of FcεRI subunits, Syk, and LAT, along with increasing the

concentration of intracellular Ca^{2+} . However, Lyn mutated in both the palmitoylation site and the myristoylation site does not anchor to the plasma membrane and is incapable of initiating FcεRI phosphorylation and early signaling events (13). Thus, Lyn must be anchored to the plasma membrane for proper signaling (13), and intracellularly introduced YpYpYp showed a dominant negative effect.

With regard to Lyn expression, genetic variations in mice have been reported to influence FcεRI-induced MC activation and allergic responses (16); the deficiency of Lyn enhances degranulation in *129/Sv* mice BMMCs (16) but inhibits this response in *C57BL/6* BMMCs (16, 17). In contrast, Lyn reportedly positively regulates degranulation upon low-intensity stimulation of FcεRI with IgE plus anti-IgE, or IgE plus low affinity of antigen, whereas Lyn works as a negative regulator of high-intensity stimulation with IgE plus high affinity of antigen in *C57BL/6* BMMCs (18). Yamashita et al. (16) reported that the diminution of Lyn enhances β-hexosaminidase release from biotinylated IgE-sensitized human MCs following FcεRI aggregation by the addition of streptavidin. However, in our cultured human MC system, the full expres-

sion of Lyn is necessary for FcεRI-mediated degranulation (Fig. 5). Thus, we examined the effect of the diminution of Lyn on histamine release from biotinylated IgE-sensitized human MCs following FcεRI aggregation by the addition of streptavidin. We found that the diminution of Lyn did not enhance the IgE-dependent degranulation (data not shown). The reason for the discrepancy between Yamashita et al.'s (16) and our findings is not clear, although the culture system for human MCs and the Lyn shRNA constructs were different.

In summary, our data demonstrate that the interaction between Lyn and FcεRIβ is indispensable for FcεRI-mediated human MC activation. FcεRIβ is preferentially expressed in mucosa around epithelium of patients with allergic diseases such as vernal keratoconjunctivitis (4). Specific disturbance of the interaction between phosphorylated FcεRIβ ITAM and Lyn may represent a new therapeutic strategy for the treatment of allergic diseases in humans.

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Conflict of interest

The authors declare no conflict of interest.

Supporting Information

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

Data S1. Repository.

Figure S1. Intracellular localization of FcεRIβ peptides and effect of FcεRIβ peptides on PGD₂ production of cultured MCs following FcεRI aggregation.

Table S1. Sequences of phosphorylated FcεRIβ immunoreceptor tyrosine-based activation motif.

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Online Repository

The interaction between Lyn and Fc ϵ RI is indispensable for Fc ϵ RI-mediated human mast cell activation

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Short title: Interaction of Lyn and FcεRIβ in human mast cell activation

Supplemental methods

Ethical considerations

The study was approved by the Ethics Committees of Nihon University, Juntendo University, School of Medicine, and Kyoto Prefectural University 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 were kindly provided by Dr. Kirshenbaum (NIAID, NIH, Bethesda, MD) (1). U937 cells were purchased from American Type Culture Collection (Manassas, VA).

Lentivirus shRNA vector construction and gene transduction

Lentiviral expression plasmids with the sense and antisense oligonucleotide sequences for the construction of two FcεRIβ (GenBank accession number NM_000139) and five Lyn shRNAs (GenBank accession number NM_002350) were purchased from Sigma-Aldrich. To avoid off-target responses with the Lyn shRNA construct, we

examined 5 constructs and found that 4 of them effectively knocked down Lyn expression in human MCs. Two of the 4 constructs downregulated PLC γ 1 expression; thus, we chose the remaining 2 constructs (MISSION[®] shRNA plasmid DNA, cat# TRCN0000010105 and TRCN0000196254). The packaging vector (MISSION[®] lentiviral packaging mix; Sigma-Aldrich) and the lentiviral expression vector (pLKO<-puro) with Fc ϵ RI β shRNA (MISSION[®] shRNA plasmid DNA, cat# TRCN0000029605), Lyn shRNA (see above) or the MISSION[®] non-targeted shRNA control vector (Sigma-Aldrich) or a lentiviral expression vector encoding EGFP were cotransfected into 293T packaging cells with TransIT (TaKaRa Bio Inc., Shiga, Japan), as previously described. (2) After 6 h, the culture supernatants were changed and after 48 h, they were centrifuged at 3000 rpm for 10 min and filtered with a 0.45 μ m filter. The filtered supernatants were centrifuged at 9000 rpm overnight at 4°C, and the released virus was kept on ice for 1 h. This step concentrated the supernatants from 220 mL to 1 ~ 2 mL. The transduction of cultured human MCs (1×10^5) was conducted by resuspending the cells in 100 μ L of virus containing IMDM. Three days after infection, the medium was changed to virus-free IMDM containing rhSCF at 100 ng/mL and rhIL-6 at 50 ng/mL with 2% FCS; antibiotic selection (2 μ g/mL puromycin) was then performed for 7 days.

RNA isolation and real-time quantitative RT-PCR

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

Flow cytometric analyses

Flow cytometric analysis of the cultured MCs was performed as described previously (3). PE- or biotin-conjugated anti-FcεRIα mAb (clone CRA1), PE-conjugated anti-CD63 (clone H5C6; BD Biosciences, San Diego, CA), or FITC-conjugated FcεRIβ ITAM peptides and N- and C-termini of FcεRIβ containing a membrane-translocating motif (4) (Table S1) were used. These peptides were produced by Toray Research

Center, Kanagawa, Japan. PE/Cy5-streptavidin was purchased from Biolegend, San Diego, CA.

Confocal laser scanning microscopy

Confocal microscopic analysis was performed as described previously (3). The cells were fixed and permeabilized and then incubated with rabbit anti-FcεRIβ Ab (5), mouse anti-Lyn mAb (clone LYN-01; Biovendor, Brno, Czech Republic), the isotype control mouse IgG1, rabbit IgG, or FITC-conjugated FcεRIβ peptides (Toray Research Center, Table S1). The cells were then incubated with rhodamine-conjugated goat anti-mouse IgG (Millipore, Billerica, MA) or Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). A model FV500 or FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) was used.

Immunoblotting

To probe whole-cell lysates for FcεRIβ, FcεRIα, FcεRIγ, Lyn, PLCγ1, and β-actin, cell lysates were probed with anti-human FcεRIβ (5), anti-FcεRIα, anti-FcεRIγ, anti-Lyn, and anti-PLCγ1 (Upstate Biotechnology), and anti-β-actin (clone C4, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively. Band intensities were measured by

densitometry analysis using ImageJ.

Pull-down assays

Cell lysates were prepared and the proteins were separated as described previously (6).

The pre-cleared cell lysates were incubated with biotinylated Fc ϵ RI β peptides (Toray Research Center) and then streptavidin immobilized Sepharose beads (GE Healthcare, Uppsala, Sweden) were added to the lysates and the tubes were incubated for 2 h at 4°C. After washing the beads, the recovered proteins were analyzed using western blotting, as described previously (6).

Effect of Fc ϵ RI β peptides on IgE-dependent histamine release from cultured human MCs in vitro and giant papillae obtained from vernal keratoconjunctivitis patients ex vivo

IgE-sensitized human MCs were pre-incubated with each of the Fc ϵ RI β peptides (5 μ M of A, B, C, D, E, F, or G, see Table S1) for 15 min and then activated with rabbit anti-IgE Ab (DAKOCytomation) for 30 min. Giant papillae tissues were resected for therapeutic purposes from vernal keratoconjunctivitis patients as previously described (7). The tissues were cut into 2-mm square pieces and cultured overnight with Stem34 serum free medium (Invitrogen) and 200 ng/mL of rhSCF in a 96-well culture dish. After changing the medium, the tissues were incubated either with triphosphorylated Fc ϵ RI β ITAM (YpYpYp) or the control peptide: non-phosphorylated Fc ϵ RI β ITAM

(YYY, 2 μ M) for 2 h. Then, the specimens were incubated with rabbit anti-IgE antibody (10 μ g/mL, DAKOCytomation) for 30 min. The supernatants and the tissues were collected separately and stored at -80°C until further analysis. All the experiments were performed in triplicate.

Mediator assays

The extent of MC degranulation was monitored by measuring histamine release into the supernatants following Fc ϵ RI aggregation using an enzyme immunoassay kit (Immunotech A Beckman Coulter Co., Marseille, France). The net percentage of histamine release was calculated from the ratio of each sample, with spontaneous release subtracted from the total histamine level. PGD₂ in the supernatants was measured using an enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI). Human IL-8 and MIP-1 α were measured using ELISA kits purchased from R&D Systems Inc., Minneapolis, MN.

Statistical analysis

Differences between two groups of data were analyzed using an unpaired Student *t*-test and were considered significant at $P < 0.05$.

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Supplemental Figure legends

Figure S1. Intracellular localization of FcεRI peptides and effect of FcεRI peptides on PGD₂ production of cultured MCs following FcεRI aggregation.

A-B, FACS and fluorescence confocal laser scanning microscopy showing the intracellular localization of FcεRIβ peptides. Peptides B, C, E, F, and G showed similar patterns of staining (data not shown). The red, green, and blue lines indicate peptide concentrations of 0, 1 (1/1000 dilution), and 2 μM (1/500 dilution), respectively. See Table S1 with regard to the sequences of the peptides A - G. **C**, Following the incubation of IgE-sensitized MCs with peptides (A – G) for 15 min, the MCs were activated with anti-IgE Ab (αIgE) or A23187 for 30 min for PGD₂ production. The data are expressed as the mean ± SEM of four experiments. See Table S1 with regard to the sequences of the peptides A - G. “-” indicates the incubation of MCs without any peptides. *, **, and *** indicate $P < 0.05$, 0.01, and 0.005, respectively.

Fig. S1

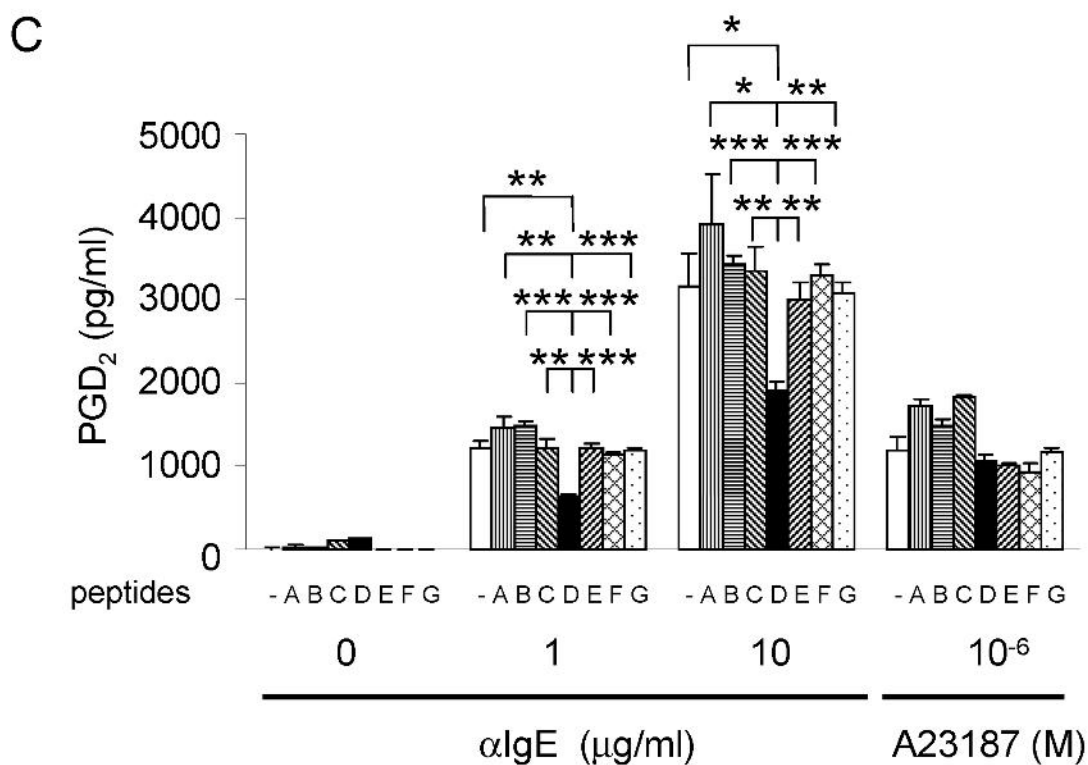
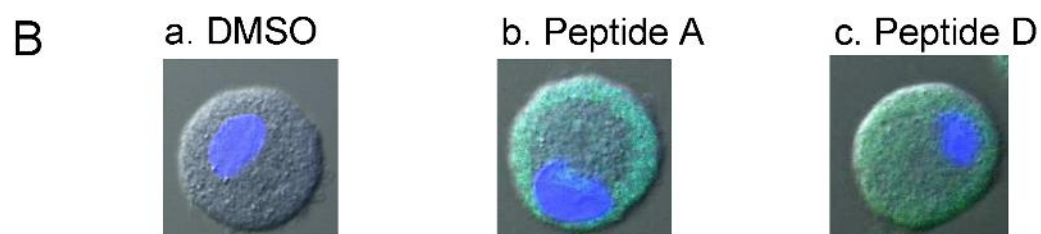
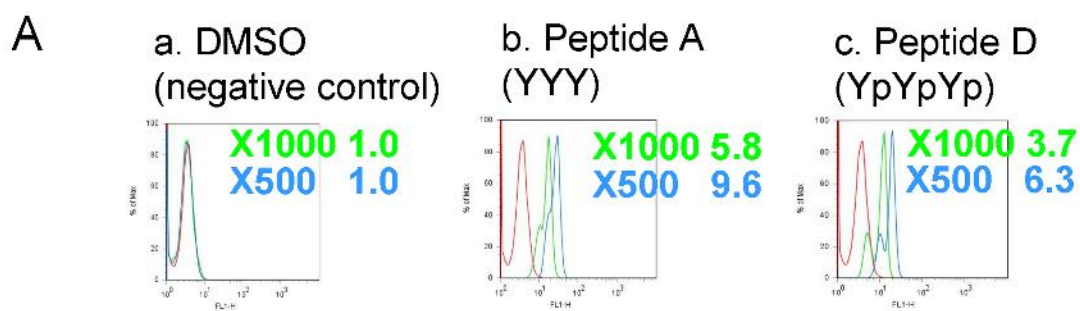


Table S1. Sequences of phosphorylated FcεRIβ immunoreceptor tyrosine-based activation motif

(peptide A) YYY -FITC

AAVLLPVLLAAP-KVPEDRVYEELNIYSATYSELEDPG-FITC

(peptide B) YYpY-FITC

AAVLLPVLLAAP-KVPEDRVYEELNIY(p)SATYSELEDPG-FITC

(peptide C) YpYYp -FITC

AAVLLPVLLAAP-KVPEDRVY(p)EELNIYSATY(p)SELEDPG-FITC

(peptide D) YpYpYp -FITC

AAVLLPVLLAAP-KVPEDRVY(p)EELNIY(p)SATY(p)SELEDPG-FITC

(peptide E) N terminus-FITC

AAVLLPVLLAAP-MDTESNRRANLALPQEPSSVPAFEV -FITC

(peptide F) C terminus 1-FITC

AAVLLPVLLAAP-CGAGEELKGNKVPEDRVYEELNIYS-FITC

(peptide G) C terminus 2-FITC

AAVLLPVLLAAP-EELNIYSATYSELEDPGEMSPPIDL-FITC

(peptide A') biotin-YYY

Biotin-KVPEDRVYEELNIYSATYSELEDPG-NH₂

(peptide D') biotin-YpYpYp

Biotin-KVPEDRVY(p)EELNIY(p)SATY(p)SELEDPG-NH₂

(peptide E') biotin-N terminus

Biotin-MDTESNRRANLALPQEPSSVPAFEV-NH₂

Y(p) ; phospho-Y