



Next-Generation T Cell Isolation Kits, Human

Higher performance, less working time

► Learn more now



The FcR β - and γ -ITAMs Play Crucial but Distinct Roles in the Full Activation of Mast Cells Induced by IgE κ and Protein L

This information is current as of March 22, 2012

Satoshi Nunomura, Yuko Kawakami, Toshiaki Kawakami and Chisei Ra

J Immunol; Prepublished online 19 March 2012;

doi:10.4049/jimmunol.1102796

<http://www.jimmunol.org/content/early/2012/03/19/jimmunol.1102796>

Subscriptions	Information about subscribing to <i>The Journal of Immunology</i> is online at http://www.jimmunol.org/subscriptions
Permissions	Submit copyright permission requests at http://www.aai.org/ji/copyright.html
Email Alerts	Receive free email-alerts when new articles cite this article. Sign up at http://www.jimmunol.org/etoc/subscriptions.shtml/

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include the digital object identifier (DOIs) and date of initial publication.

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
Copyright ©2012 by The American Association of
Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



The FcR β - and γ -ITAMs Play Crucial but Distinct Roles in the Full Activation of Mast Cells Induced by IgE κ and Protein L

Satoshi Nunomura,* Yuko Kawakami,[†] Toshiaki Kawakami,[†] and Chisei Ra*

Previous studies suggested that Protein L (PpL), the bacterial Ig-binding protein, activates mast cells. PpL presumably performs the activation by interacting with membrane-bound IgE κ , but the underlying mechanisms behind the process remain unclear. In the current study, we found that cell-surface Fc ϵ RI expression is a critical factor participant in PpL-mediated full activation of murine mast cells, which includes cytokine production, the degranulation response, and leukotriene C₄ (LTC₄) release, and that engagement of the Fc ϵ RI with IgE κ and PpL is enough to induce tyrosine phosphorylation of ITAM in the FcR β - and γ -signaling subunits. Introduction of mutations in two canonical tyrosine residues (Y47F/Y58F) of the FcR γ -ITAM completely abolished the above-mentioned mast cell functions, with the exception of LTC₄ release. Importantly, the FcR β -ITAM acts as a signal transducer that is responsible for LTC₄ release independently of the FcR γ -ITAM. Taken together, our results suggest crucial and distinct functions for the FcR β - and γ -ITAMs in the Fc ϵ RI-dependent full activation of mast cells induced by IgE κ and PpL. *The Journal of Immunology*, 2012, 188: 000–000.

Mast cells express the Fc ϵ RI on their cell surface, which is composed of a tetramer of the IgE-binding α -chain and two signaling subunits, a β -subunit and a disulfide-linked homodimer of γ -subunits (1, 2). Fc ϵ RI-dependent mast cell activation leads to allergic inflammation. IgE-bound Fc ϵ RI is engaged by specific multivalent Ags, resulting in immediate activation of Fc ϵ RI signaling. Engagement of the Fc ϵ RI elicits rapid tyrosine phosphorylation of the ITAM of the FcR β - and γ -subunits, which in turn leads to activation of subsequent downstream signals, such as Syk, phospholipase C γ (PLC γ), Grb2-associated binder 2 (Gab2), and MAPK family members, and calcium influx (3). Activation of these intracellular signals is essential for the release of diverse inflammatory mediators, including histamine, arachidonate metabolites, and cytokines from mast cells upon Fc ϵ RI cross-linking.

In addition to functions connected with allergic inflammation, previous studies suggested that mast cells act as innate immune cells that protect their host from infections by opportunistic pathogens, such as *Candida albicans*, *Staphylococcus aureus*, and *Escherichia coli*, by expressing a variety of receptors

(e.g., TLR2, -4, and CD48) against the cell wall components of these pathogens (4–9). *Finexgoldia magna* (formerly *Peptostreptococcus magnus*) is one of the major anaerobic bacteria in the oral cavity and the gastrointestinal and urogenital tracts (10). *F. magna* has also been reported to be an opportunistic pathogen that is frequently isolated from patients with clinical infections. Representative infections associated with *F. magna* include bacterial vaginosis and wound and bone/joint infections (10, 11). Earlier studies reported that many strains of *F. magna* isolated from patients with bacterial vaginosis express a surface protein, protein L (PpL), with a high affinity for variable regions of the Ig κ L chains ($K_a = \sim 1 \times 10^9 \text{ M}^{-1}$) (12, 13). Interestingly, purified PpL and *F. magna*-expressing PpL induce the degranulation response and leukotriene C₄ (LTC₄) release in IgE κ -sensitized human mast cells but not in nonsensitized cells (14–16). It is thought that PpL probably engages the IgE κ -Fc ϵ RI complex in human mast cells, because PpL possesses multiple domains that bind to Ig κ L chains (17), which would imply that mast cells can respond to *F. magna* independent of the typical innate immune receptors responsible for defense against pathogens.

Mast cell activation through Fc ϵ RI leads to diverse responses, depending on the quality of the stimulation (18). For example, stimulation of Fc ϵ RI with highly cytokinergic IgE elicits considerable cytokine production but not degranulation (19, 20). Like highly cytokinergic IgE, the interaction of a low-affinity Ag with cell surface-bound IgE, or the aggregation of small numbers of Fc ϵ RI, results in preferential activation of cellular signaling and cytokine production but not in degranulation (21–24). By contrast, a high-affinity Ag or an aggregation of large numbers of Fc ϵ RI induces robust cellular signals that can effectively induce the degranulation response (23, 24). However, it is unclear whether cell-surface Fc ϵ RI expression is responsible for mast cell activation via PpL and IgE κ and whether the combination of PpL and IgE κ actually activates Fc ϵ RI-dependent signaling. Namely, the biological functions of the FcR β - and γ -signaling subunits in PpL-mediated mast cell cytokine production, the degranulation response, and LTC₄ secretion are still unknown.

*Division of Molecular Cell Immunology and Allergology, Advanced Medical Research Center, Nihon University Graduate School of Medical Science, Tokyo 173-8610, Japan; and [†]Division of Cell Biology, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121

Received for publication September 27, 2011. Accepted for publication February 13, 2012.

This work was supported by grants-in-aid for private universities from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to C.R.).

Address correspondence and reprint requests to Prof. Chisei Ra, Division of Molecular Cell Immunology and Allergology, Advanced Medical Research Center, Nihon University Graduate School of Medical Science, 30-1 Oyaguchikami-cho Itabashi-ku, Tokyo 173-8610, Japan. E-mail address: ra.chisei@nihon-u.ac.jp

Abbreviations used in this article: BMMC, bone marrow-derived mouse mast cell; [Ca²⁺]_i, intracellular calcium ion; DNS, dansyl; Gab2, Grb2-associated binder 2; LTC₄, leukotriene C₄; MFI, mean fluorescence intensity; PLC γ , phospholipase C γ ; PpL, protein L; RBL, rat basophilic leukemia; SCF, stem cell factor; TNP, trinitrophenyl.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/\$16.00

Elucidation of the mechanisms involved in the inflammatory responses mediated by mast cells against the superantigen-like protein from *F. magna* could deepen our understanding of mast cells function and the innate immune system. In the current study, therefore, we sought to clarify the underlying mechanisms behind PpL-mediated mast cell activation.

Materials and Methods

Abs and other reagents

Commercially available IgEκ mAbs were used in this study (BD Biosciences, San Diego, CA, for the 15.3.2, C48-2, and 27.72 clones, anti-trinitrophenyl [TNP], or anti-dansyl [DNS]; Santa Cruz Biotechnology, Santa Cruz, CA, for the H1-ε-26.82 clone, anti-DNP). The anti-FcRβ mAb (clone JRK, the hybridoma for which was a kind gift from Dr. Juan Rivera, National Institutes of Health) was prepared in our laboratory. The rabbit anti-FcRγ and anti-Gab2 Abs were from Millipore (Billerica, MA). The mouse anti-phosphotyrosine HRP-conjugated mAb (PY20) was purchased from GE Healthcare Biosciences (Little Chalfont, U.K.).

The recombinant PpL and PpL conjugated with HRP were purchased from Sigma-Aldrich (St. Louis, MO). The PpL-FITC, TNP-BSA (5 M TNP per mole BSA), and DNS-BSA (6 M DNS per mole BSA) were prepared in our laboratory, and the recombinant murine IL-3 and stem cell factor (SCF) were purchased from PeproTech (Rocky Hill, NJ). All the Abs to phosphorylated proteins and the Abs against Syk, PLCγ1, ERK1/2, and p38 MAPK were purchased from Cell Signaling Technology (Beverly, MA).

Animals

FcRγ^{-/-} (C57BL6 background) (25), FcRβ^{-/-} (C57BL6 background) (26), and FcRβ^{-/-}γ^{-/-} mice were bred in the animal facility of the Nihon University School of Medicine under specific pathogen-free conditions. For some experiments, thighbones from FcεRIα^{-/-} and FcεRIα^{+/+} mice were kindly provided by Dr. Toshiaki Kawakami (La Jolla Institute of Allergy and Immunology). C57BL6/J mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). Following the approval of the animal experimentation committee at Nihon University, all of the experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Nihon University.

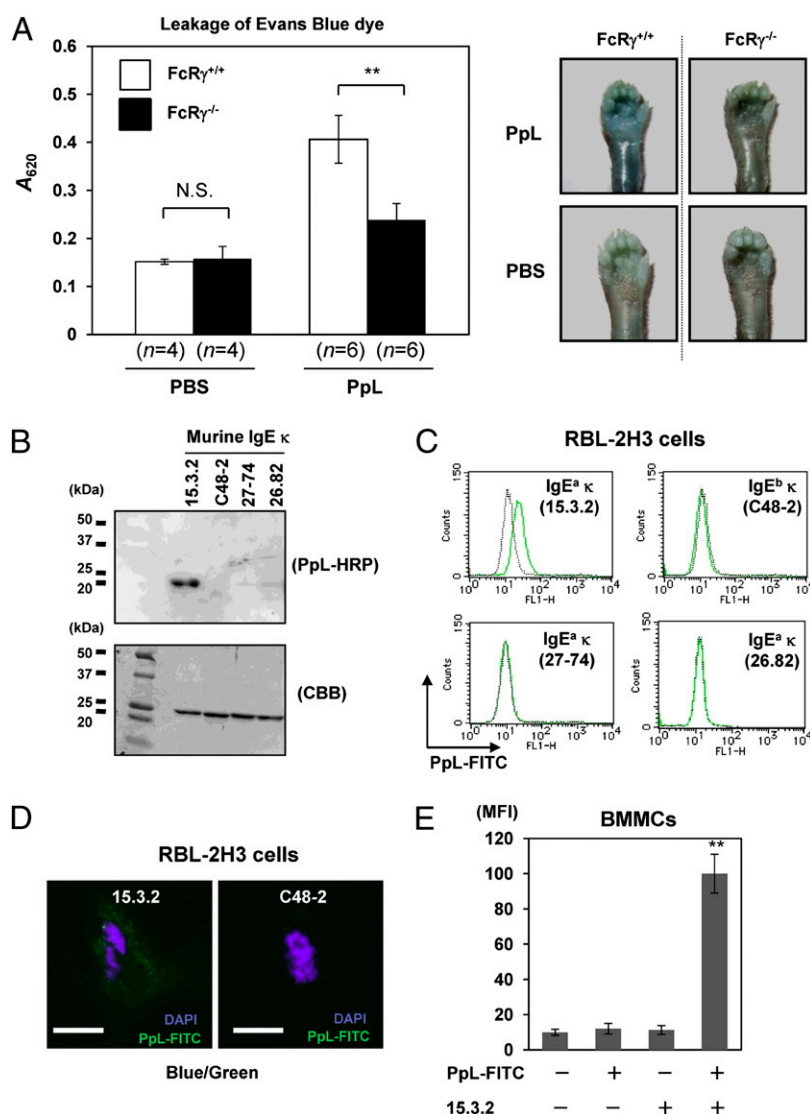


FIGURE 1. 15.3.2, a murine IgEκ clone, is reactive to PpL. **(A)** Increased vascular leakage of the Evans blue dye by PpL administration. The data are shown as the mean ± SE. ***p* < 0.01 (Student *t* test). **(B)** The far-Western blotting. The PpL-reactive IgEκ clone was visualized with PpL-HRP. Coomassie brilliant blue (CBB) staining showed comparable sample loading. **(C)** Binding of PpL-FITC to RBL-2H3 cells sensitized with or without IgEκ clones. IgE^a and IgE^b indicate the Ig allotype of each murine IgEκ clone. Green lines indicate intensity of fluorescence of PpL-FITC. Grey lines indicate intensity of fluorescence of nonlabeled cells. **(D)** Confocal microscopic analyses for binding of PpL-FITC to RBL-2H3 cells sensitized with 15.3.2 or C48.2. Scale bars, 20 μm. Original magnification ×240 for all panels. **(E)** Binding of PpL-FITC to BMMCs sensitized with 15.3.2 or without 15.3.2. The cells were analyzed on a flow cytometer. The results shown in (B)–(D) are representative of three independent experiments with similar results. In (E), data shown are the mean ± SD of samples obtained from four independent experiments. ***p* < 0.01 (Tukey's test with one-way ANOVA).

Cell culture

Bone marrow-derived mouse mast cell (BMMC) cultures were prepared from the femurs of 4–8 wk-old mice, as previously described (27). The ecotropic retrovirus packaging cell line PLAT-E, which was a kind gift from Dr. Toshio Kitamura (Tokyo University), was maintained in DMEM supplemented with 10% (v/v) FBS, 1 $\mu\text{g ml}^{-1}$ puromycin (BD Clontech, San Jose, CA), and 10 $\mu\text{g ml}^{-1}$ blasticidin S (Kaken Pharmaceutical, Tokyo, Japan). Rat basophilic leukemia (RBL)-2H3 cells were maintained in DMEM supplemented with 10% (v/v) FBS.

Retroviral transfection

Retroviral gene transduction was performed as previously described (27). Briefly, pMX-puro plasmids harboring $\text{Fc}\gamma_{\text{YY}}$, $\text{Fc}\gamma_{\text{FF}}$, $\text{Fc}\gamma_{\text{YY}}$, or $\text{Fc}\gamma_{\text{FF}}$ cDNA were transfected into the PLAT-E cells to generate recombinant retroviruses. Bone marrow cells were infected or coinfect with the retroviruses for 24 h in the presence of 10 $\mu\text{g ml}^{-1}$ polybrene (Sigma-Aldrich). The gene-transduced cells were selected with 1.2 $\mu\text{g ml}^{-1}$ puromycin for 10 d. The viable cells (10–20% of the bone marrow cells cultured with retroviruses) were expanded for several weeks. The $\text{Fc}\gamma$ transfectants were grown in a medium containing IL-3 (5 ng ml^{-1}).

The $\text{Fc}\gamma$ cotransfectants were grown in a medium containing IL-3 (5 ng ml^{-1}) and SCF (10 ng ml^{-1}) to obtain a sufficient number of $\text{Fc}\gamma$ -positive cells. The puromycin-resistant $\text{Fc}\gamma$ transfectants, which expressed cell-surface $\text{Fc}\gamma$ at comparable levels, were used for the experiments. The $\text{Fc}\gamma^+$ cells (1 to 2%) were purified from the puromycin-resistant $\text{Fc}\gamma$ cotransfectants using an EasySep FITC Positive Selection kit (StemCell Technologies) and an anti-mouse IgE mAb conjugated with FITC.

Analysis of vascular permeability

PBS containing 0.5% Evans blue dye or PBS containing 100 μg PpL and 0.5% Evans blue dye was i.v. injected into naive $\text{Fc}\gamma^{+/+}$ ($n = 4-6$) and $\text{Fc}\gamma^{-/-}$ mice ($n = 4-6$). The vascular leakage of the Evans blue dye into the skin after 30 min was quantified as the absorbance at 620 nm (A_{620}) of the fluid extracted with formamide.

Assay of degranulation, LTC_4 release, and cytokine production

Mast cells ($1 \times 10^6 \text{ ml}^{-1}$) were sensitized with each 0.5 $\mu\text{g ml}^{-1}$ IgE clone overnight. In some experiments, unsensitized mast cells were also analyzed. The IgE-sensitized and unsensitized mast cells (2×10^5)

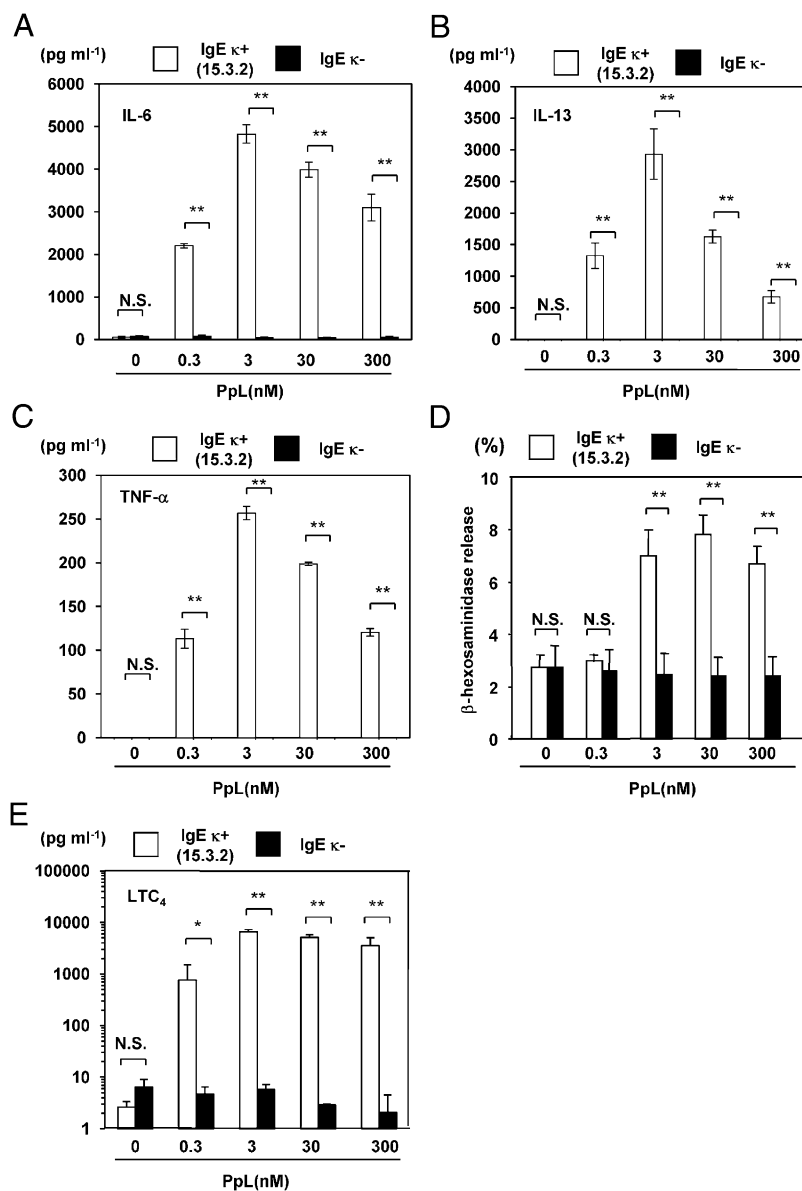
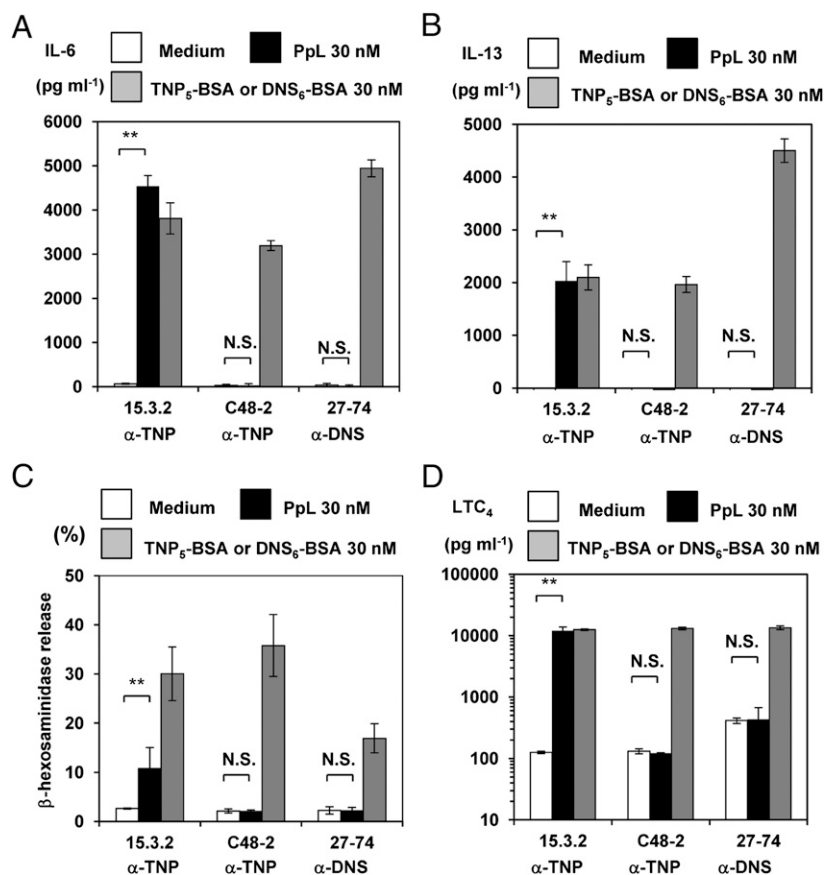


FIGURE 2. PpL loading induces cytokine production, degranulation, and LTC_4 release in BMMCs sensitized with 15.3.2. IgEκ (15.3.2)-sensitized and unsensitized BMMCs were stimulated with or without PpL at the indicated concentrations for 0.5 h (for degranulation and LTC_4) or 8 h (for cytokine production). IL-6 (**A**), IL-13 (**B**), and TNF-α (**C**) production. (**D**) Degranulation. (**E**) LTC_4 production. Data shown are the mean $\pm$ SD of quadruplicates. Similar results were obtained in three independent experiments. * $p < 0.05$, ** $p < 0.01$ (Student *t* test).

FIGURE 3. C48.2 and 27-74 do not mediate mast cell activation upon PpL loading. BMBCs were sensitized with 15.3.2 (anti-TNP IgE), C48.2 (anti-TNP IgE), and 27.74 (anti-DNS IgE). The IgE κ -sensitized mast cells were stimulated with or without 30 nM PpL for 0.5 h (for degranulation and LTC₄) or 8 h (for IL-6 and IL-13 production). Ags (TNP₅-BSA and DNS₆-BSA) were used as immunological positive controls in place of PpL. IL-6 (**A**) and IL-13 (**B**) production. (**C**) Degranulation. (**D**) LTC₄ synthesis. Data shown are the mean $\pm$ SD of quadruplicates. Similar results were obtained in three independent experiments. ** $p < 0.01$ (Student t test).



were washed twice with PBS and then stimulated with or without PpL at the indicated concentrations for 0.5 h (for degranulation and LTC₄) or 8 h (for cytokine production). In some experiments, cells

were stimulated in Ca²⁺-free medium containing 1 mM EGTA for complete depletion of extracellular Ca²⁺. In place of PpL, Ags (TNP₅-BSA and DNS₆-BSA) and A23187 were used as immunological and

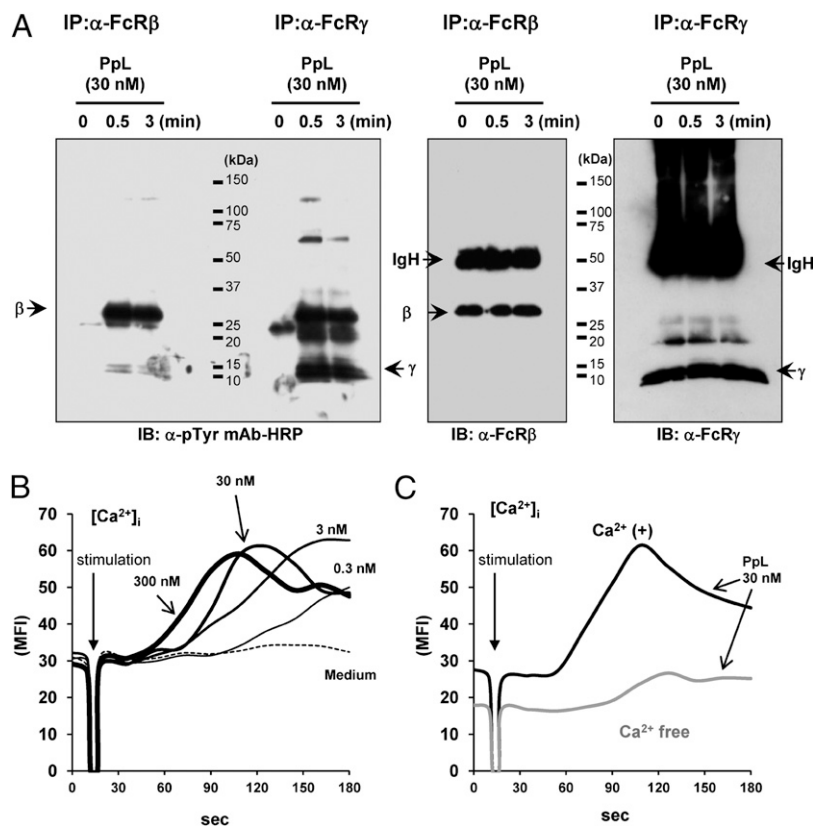


FIGURE 4. PpL loading elicits tyrosine phosphorylation of the FcR β and γ -subunits and intracellular calcium mobilization in BMBCs sensitized with 15.3.2. (**A**) BMBCs sensitized with 15.3.2 were stimulated with 30 nM PpL for 0.5 and 3 min. Total cell lysates were incubated with anti-FcR β or anti-FcR γ Abs. The immunoprecipitates (IP) were analyzed by immunoblotting with anti-phosphotyrosine HRP-conjugated Ab (PY20). (**B** and **C**) PpL-mediated calcium mobilization in the 15.3.2-sensitized BMBCs. Changes in the Fluo-3 fluorescence were determined employing flow cytometry after PpL stimulation in the presence (**B**) or absence (**C**) of extracellular Ca²⁺. The [Ca²⁺]_i mobilization is expressed as the MFI of Fluo-3. The results shown are representative of three (A and B) or four (C) independent experiments with similar results.

nonimmunological positive controls, respectively. Degranulation was determined by β -hexosaminidase release, as described previously (27). The percentage of β -hexosaminidase release was calculated as follows: (supernatant OD of cells)/(total cell lysate OD of cells) $\times$ 100. The LTC₄ production was analyzed with an LTC₄ EIA kit (Cayman Chemical, Ann Arbor, MI). The IL-6, IL-13, and TNF- α productions were analyzed with specific ELISA kits (Invitrogen/BioSource International, Camarillo, CA).

Measurement of mobilization of intracellular calcium ions

Mast cells (1×10^6 ml⁻¹) were sensitized with 0.5 μ g ml⁻¹ 15.3.2 overnight. The IgE-sensitized cells (1×10^6 ml⁻¹) were washed twice with PBS and then loaded with 4 μ M Fluo-3-AM (Dojindo, Kumamoto, Japan) for 30 min at 37°C. The cells were resuspended in 1 $\times$ Tyrode's buffer and stimulated with or without PpL. In some experiments, assays were performed in Ca²⁺-free 1 $\times$ Tyrode's buffer containing 1 mM EGTA for complete depletion of extracellular Ca²⁺. A23187 (1 μ M) was used as a nonimmunological positive control in place of PpL. Changes in dye fluorescence upon addition of stimuli were monitored at 200-ms intervals by a flow cytometer (FACSCalibur; BD Biosciences). The mobilization of intracellular calcium ions ([Ca²⁺]_i) was expressed as the mean fluorescence intensity (MFI). The graph was generated by plotting values of MFI.

Immunoblotting

Mast cells (1×10^6 ml⁻¹) were sensitized with 0.5 μ g ml⁻¹ 15.3.2 overnight. The IgE-sensitized cells were washed twice with PBS and then stimulated with 30 nM PpL for the indicated time periods. The stimulated mast cells ($1-5 \times 10^6$) were washed twice with ice-cold PBS and lysed for 30 min on ice in a lysis buffer (TBS containing 1% Nonidet P-40, 2 mM PMSF, 10 μ g ml⁻¹ aprotinin, 2 μ g ml⁻¹ leupeptin and pepstatin A, 50 mM NaF, and 1 mM sodium orthovanadate). The lysates were centrifuged for 15 min at 15,000 $\times$ g. For immunoprecipitation assays, the cell lysates were incubated with Ab-bound protein G Sepharose for 3 h on ice. The samples were then boiled, separated on a 12% SDS-PAGE gel, and transferred to an Immobilon-P membrane (Millipore). The membrane was incubated with a primary Ab and an appropriate secondary HRP-conjugated Ab. The signals were detected by ECL (GE Healthcare Biosciences). The immunoreactive bands were scanned to produce digital images that were quantified using the SCION Image software, and then the phosphorylation was calculated from the amount of phospho-protein relative to the corresponding non-phospho-loading control.

Far-Western blotting

Four mouse IgE κ proteins (5 μ g/lane) were separated on a 12% SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore). The membrane

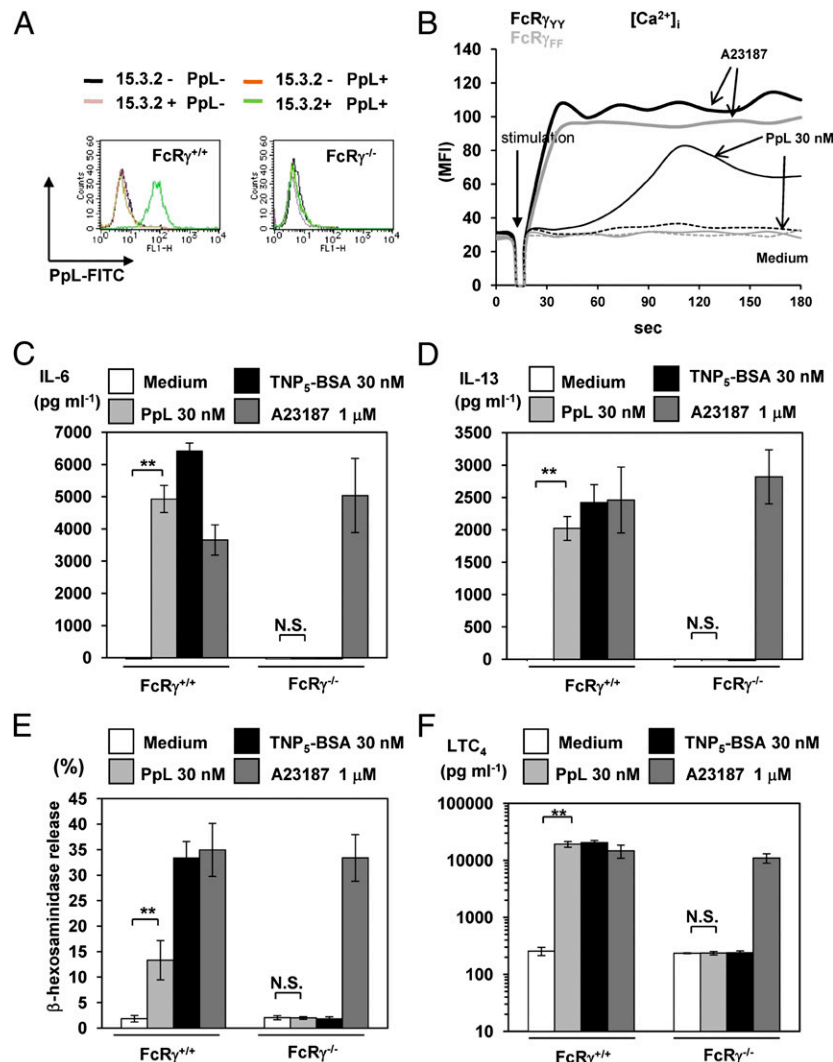


FIGURE 5. Cell-surface Fc ϵ RI is indispensable for PpL-induced mast cell activation. **(A)** PpL does not bind to Fc $\gamma^{-/-}$ mast cells sensitized with 15.3.2. **(B)** PpL-mediated calcium mobilization in the 15.3.2-sensitized Fc $\gamma^{+/+}$ and Fc $\gamma^{-/-}$ mast cells was analyzed by flow cytometry. [Ca²⁺]_i mobilization is expressed as the MFI of Fluo-3. The results shown are representative of three (A) or four (B) independent experiments with similar results. The 15.3.2-sensitized mast cells were stimulated with 30 nM PpL for 0.5 h (for degranulation and LTC₄) or 8 h (for IL-6 and IL-13 production). In place of PpL, TNP₅-BSA, and A23187 were used as immunological and nonimmunological positive controls, respectively. IL-6 **(C)** and IL-13 **(D)** production. **(E)** Degranulation. **(F)** LTC₄ synthesis. Data shown are the mean $\pm$ SD of quadruplicates. Similar results were obtained in three independent experiments. ** $p < 0.01$ (Student t test).

was incubated with PpL-HRP (1:2000) for 1 h. The signals were detected by ECL. Coomassie brilliant blue staining showed comparable sample loading.

Confocal microscopy

RBL-2H3 cells ($2 \times 10^5 \text{ ml}^{-1}$) were sensitized with $0.5 \mu\text{g ml}^{-1}$ 15.3.2 or C48-2 on culture coverglass for 1 h. The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min and then permeabilized in PBS containing 0.1% Triton X-100 for 15 min at room temperature. The cells were washed twice with 1 ml PBS and stained with FITC-conjugated PpL (1:1000) for 60 min in the dark. The coverglasses were washed with PBS and then mounted with Vectashield mounting medium containing DAPI (Molecular Probes/Invitrogen Life Technologies). Confocal microscopy was performed using a FV1000 system and Fluoview software (Olympus, Tokyo, Japan).

Flow cytometric analyses

For analyses of PpL-FITC binding to mast cells, RBL-2H3 cells ($2 \times 10^5 \text{ ml}^{-1}$) and murine mast cells ($2 \times 10^5 \text{ ml}^{-1}$) were sensitized with or without each $0.5 \mu\text{g ml}^{-1}$ IgEκ clone. These cells were washed twice with PBS and labeled with PpL-FITC (1:1000) at 4°C for 30 min. The labeled

cells were analyzed with FACSCalibur (BD Biosciences). The receptor internalization was defined as changes in cell-surface FcεRI expression after PpL stimulation. Briefly, mast cells ($1 \times 10^6 \text{ ml}^{-1}$) were sensitized with $0.5 \mu\text{g ml}^{-1}$ 15.3.2 overnight. The cells were washed twice with PBS and stimulated with or without 30 nM PpL for 20 min. The stimulated cells were labeled with $0.1 \mu\text{g ml}^{-1}$ anti-mouse IgE mAb-FITC for 15 min on ice. The labeled cells were analyzed with FACSCalibur (BD Biosciences).

Statistical analysis

The data shown are the mean $\pm$ SE or SD. The statistical analyses were performed using the Student *t* test or Tukey's test in a one-way ANOVA model. The *p* values < 0.05 were considered to indicate statistically significant differences.

Results

A reactive murine IgEκ clone to PpL (15.3.2 clone)

First, we employed FcRγ^{+/+} and FcRγ^{-/-} mice to examine whether PpL elicits an in vivo inflammatory response through the murine Igs and FcRs. We i.v. injected PpL (100 μg) and Evans

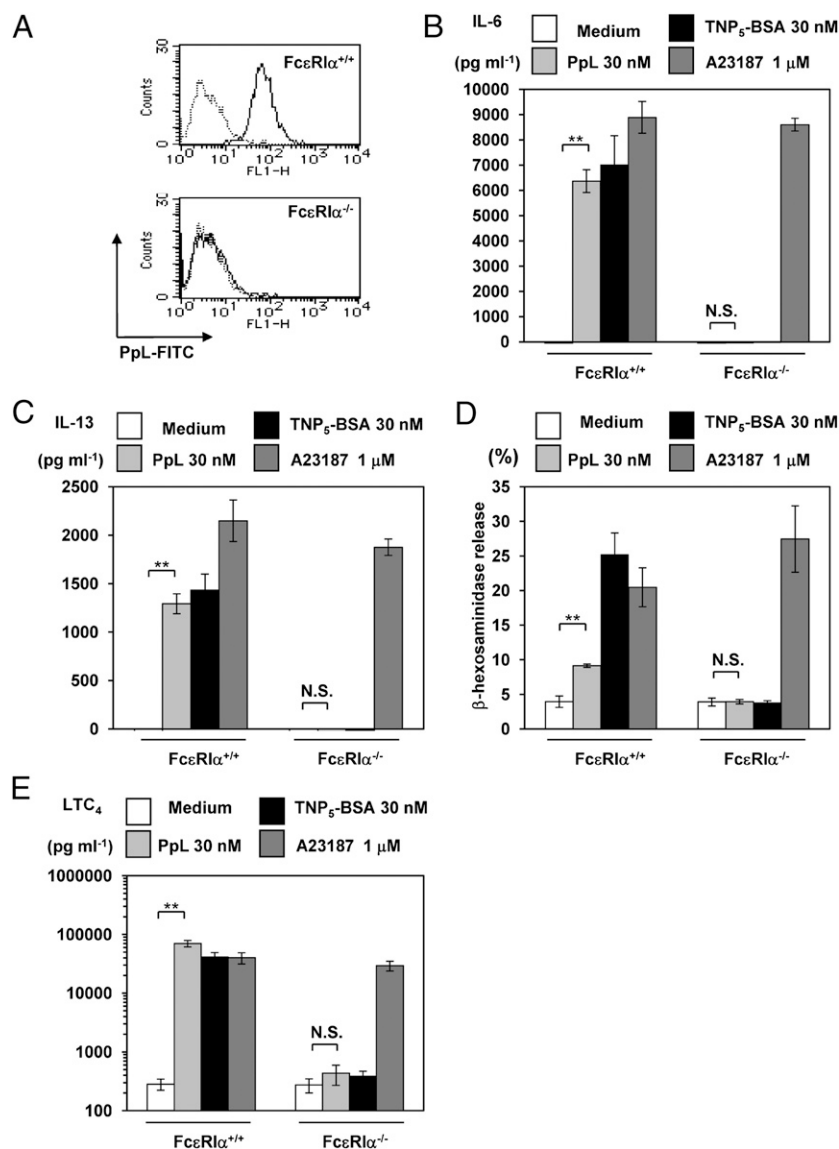


FIGURE 6. FcεRIα^{-/-} mast cells sensitized with 15.3.2 fail to induce cell response upon PpL loading. (A) PpL does not bind to FcεRIα^{-/-} mast cells sensitized with 15.3.2. The results shown are representative of three independent experiments with similar results. The 15.3.2-sensitized FcεRIα^{-/-} and FcεRIα^{+/+} mast cells were stimulated with 30 nM PpL for 0.5 h (for degranulation and LTC₄) or 8 h (for IL-6 and IL-13 production). In place of PpL, TNP₅-BSA and A23187 were used as immunological and nonimmunological positive controls, respectively. IL-6 (B) and IL-13 (C) production. (D) Degranulation. (E) LTC₄ synthesis. Data shown are the mean $\pm$ SD of quadruplicates. Similar results were obtained in three independent experiments. ***p* < 0.01 (Student *t* test).

blue dye into naive $\text{Fc}\gamma^{+/+}$ and $\text{Fc}\gamma^{-/-}$ mice to visualize changes in vascular permeability. As shown in Fig. 1A, administration of PpL increased leakage of the Evans blue dye. Compared to the $\text{Fc}\gamma^{+/+}$ mice, the $\text{Fc}\gamma^{-/-}$ mice showed less dye leakage. These data suggest that the murine endogenous Igs and FcRs are sufficiently reactive to PpL and that murine IgE κ can elucidate PpL-triggered, Ig κ -dependent mast cell activation. Thus, we examined the bindability of murine IgE κ clones to PpL. Far-Western blotting showed that PpL preferentially binds to the light chains of 15.3.2 clone among the four IgE κ clones studied (15.3.2; anti-TNP, C48.2; anti-TNP, 27.74; anti-DNS, H1- ϵ -26.82; anti-DNP) (Fig. 1B). Two clones, 27.74 and H1- ϵ -26.82, were slightly reactive to PpL. Conversely, no significant binding between PpL and C48.2 was detected. Next, we used RBL-2H3 cells to investigate the ability of PpL to bind to the intact IgE κ protein. RBL-2H3

cells were incubated with or without the IgE κ clones and then labeled with FITC-conjugated PpL. Fig. 1C shows that PpL-FITC binds to 15.3.2 but not the other IgE κ clones. Confocal microscopy analyses indicated that PpL localizes to the cell surface of RBL-2H3 cells sensitized with 15.3.2 (Fig. 1D). Similarly, 15.3.2 was essential for the binding of PpL to BMMCs (Fig. 1E). Together, these results indicate that 15.3.2 is the most reactive murine IgE κ clone to PpL.

15.3.2 clone mediates mast cell activation upon PpL loading

To examine whether murine mast cells produces inflammatory mediators in response to PpL in a 15.3.2-dependent manner, BMMCs were sensitized with or without 15.3.2. As shown in Fig. 2, PpL loading elicited cytokine production (IL-6, IL-13, and TNF- α), the degranulation response, and LTC $_4$ release in the

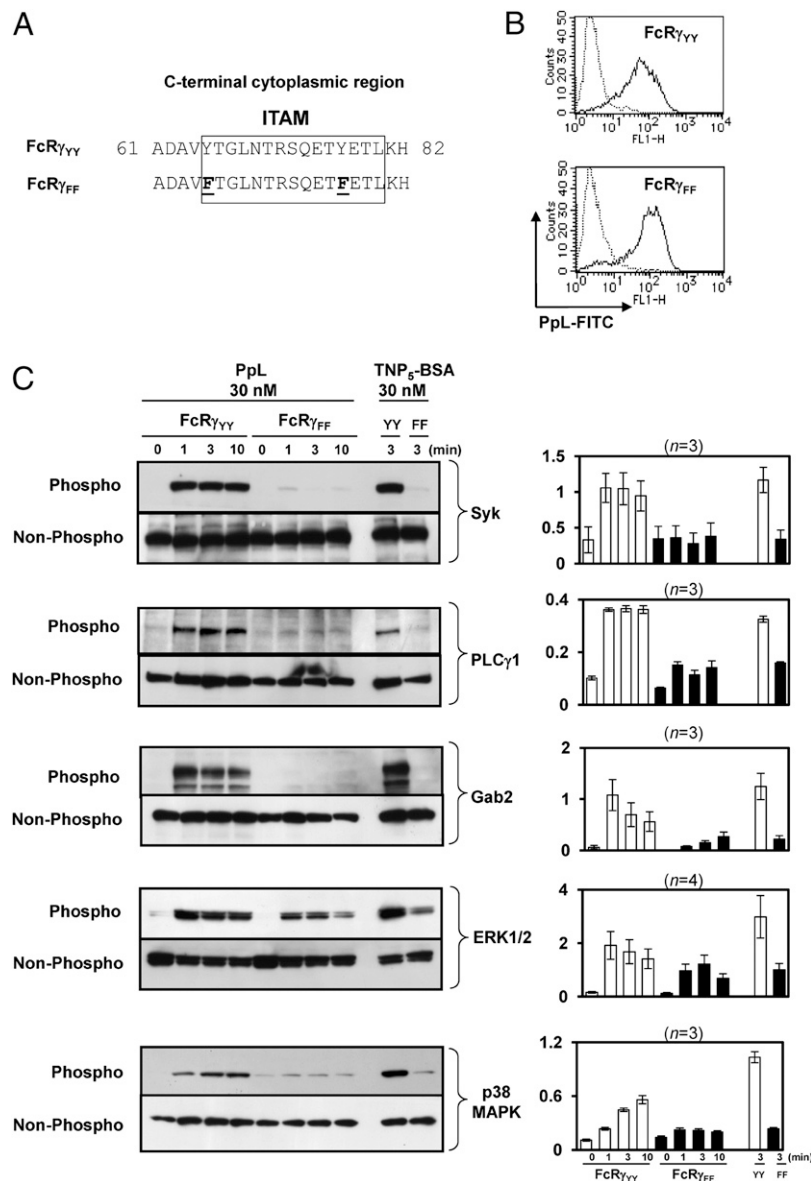


FIGURE 7. Introduction of mutations in tyrosine residues of the Fc γ -ITAM decreases Fc ϵ RI-dependent proximal and distal signals elicited by 15.3.2 and PpL. **(A)** Wild-type Fc γ (Fc γ_{YY}) and mutant Fc γ -ITAM (Fc γ_{FF}), with two tyrosine residues in the ITAM replaced with phenylalanine. **(B)** Introduction of the Fc γ genes into the Fc $\gamma^{-/-}$ mast cells restored their defective PpL binding. **(C)** Fc γ -ITAM transfectants sensitized with 15.3.2 were stimulated with 30 nM PpL for the indicated times. In place of PpL, TNP $_5$ -BSA was used as an immunological positive control. The total cell lysates were analyzed by immunoblotting with anti-phospho-Syk (Tyr ^{352}), PLC γ 1 (Tyr ^{783}), Gab2 (Tyr ^{452}), ERK1/2 (Thr ^{202} /Tyr ^{204}), and p38 MAPK (Thr ^{180} /Tyr ^{182}) Abs. The blots were reprobed with each anti-nonphospho-Abs. The results shown are representative of three or four independent experiments with similar results. The densitometric data (right panels) are shown as mean $\pm$ SD.

BMMCs sensitized with 15.3.2. The PpL concentration for inducing the degranulation response differed from that for other cellular responses. A degranulation response required 3 nM of PpL. Next, we examined the effect of two other IgE κ clones (C48.2, 27.74) on PpL-mediated mast cell activation (Fig. 3). We omitted H1- ϵ -26.82 from our analyses because this highly cytokinergic IgE κ clone itself has the ability to activate mast cells in the absence of Ags (20, 28, 29). Fig. 3 shows that neither C48.2 nor 27.74 induced PpL-dependent mast cell activation but that these IgE clones became capable of mast cell activation upon loading of their specific Ags (TNP₅-BSA or DNS₆-BSA).

PpL loading elicits tyrosine phosphorylation of the Fc ϵ RI signaling subunits and calcium influx in mast cells sensitized with 15.3.2

To elucidate whether PpL triggers aggregation of the IgE κ -Fc ϵ RI complex, we examined the tyrosyl phosphorylation state of the

FcR β - and γ -subunits in 15.3.2-sensitized mast cells. As shown in Fig. 4A, PpL induced rapid and significant tyrosine phosphorylation of both of the FcR signaling subunits of the BMMCs sensitized with 15.3.2. We next examined the mobilization of $[Ca^{2+}]_i$, which is an essential second messenger for cytokine production and chemical mediator release. Fig. 4B shows that PpL induced increases in $[Ca^{2+}]_i$ mobilization in a dose-dependent manner. This $[Ca^{2+}]_i$ mobilization was considerably reduced under calcium-free extracellular conditions, suggesting that calcium influx was activated by the PpL loading (Fig. 4C).

Cell-surface Fc ϵ RI is required for 15.3.2- and PpL-induced mast cell activation

To clarify the role of cell-surface Fc ϵ RI in the mast cell activation induced by 15.3.2 and PpL, we prepared FcR $\gamma^{-/-}$ BMMCs. The FcR $\gamma^{-/-}$ and FcR $\gamma^{+/+}$ BMMCs were incubated with 15.3.2. Flow cytometric analyses showed no binding of PpL to the 15.3.2-

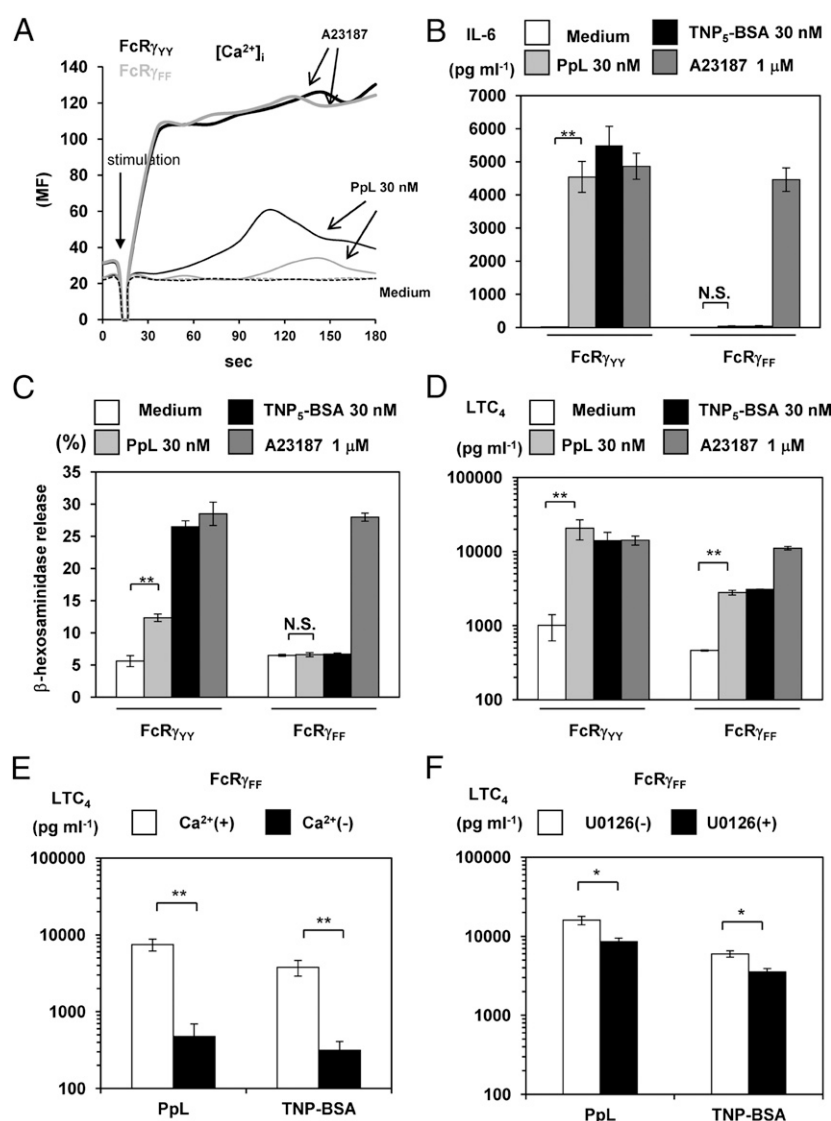


FIGURE 8. FcR γ_{FF} mast cells can release LTC₄. (A) PpL-mediated calcium mobilization in 15.3.2-sensitized FcR γ_{YY} and FcR γ_{FF} mast cells was analyzed as shown in Fig. 4B. In place of PpL, A23187 (1 μ M) was used as a nonimmunological positive control for the calcium response. The results shown are representative of four independent experiments with similar results. The 15.3.2-sensitized FcR γ_{YY} and FcR γ_{FF} mast cells were stimulated with or without 30 nM PpL for 0.5 h (for degranulation and LTC₄) or 8 h (for IL-6 production). In place of PpL, TNP₅-BSA and A23187 were used as immunological and nonimmunological positive controls, respectively. (B) IL-6 production. (C) Degranulation. (D) LTC₄ production. (E and F) The net LTC₄ production. The 15.3.2-sensitized FcR γ_{FF} mast cells were stimulated with or without 30 nM PpL in the presence or absence of extracellular calcium (E) or in the presence or absence of 5 μ M U0126 (F). In (B)–(F), data shown are the mean $\pm$ SD of quadruplicates. Similar results were obtained in three independent experiments. * p < 0.05, ** p < 0.01 (Student t test).

sensitized $\text{Fc}\gamma^{-/-}$ BMMCs, suggesting that cell-surface $\text{Fc}\epsilon\text{RI}$ expression is a critical element in the binding of PpL to BMMCs (Fig. 5A). Consistent with this result, $\text{Fc}\gamma^{-/-}$ BMMCs sensitized with 15.3.2 failed to induce $[\text{Ca}^{2+}]_i$ mobilization (Fig. 5B), cytokine production (IL-6 and IL-13), the degranulation response, and LTC_4 secretion upon PpL loading (Fig. 5C–F). Like $\text{Fc}\gamma^{-/-}$ BMMCs, $\text{Fc}\epsilon\text{RI}\alpha^{-/-}$ BMMCs sensitized with the 15.3.2 lacked the bindability to PpL and failed to induce PpL-dependent cytokine production, the degranulation response, and LTC_4 secretion (Fig. 6). These data indicate that cell-surface $\text{Fc}\epsilon\text{RI}$ is responsible for mast cell activation mediated by IgE κ and PpL.

$\text{Fc}\gamma_{\text{FF}}$ mast cells exhibit decreased activation of proximal and distal signaling molecules elicited by 15.3.2 and PpL

Fig. 4 raises the possibility that tyrosine phosphorylation of the $\text{Fc}\gamma$ subunit plays an important role in PpL-mediated mast cell activation. We therefore prepared BMMCs with or without mutations (Y47F/Y58F) in two canonical tyrosine residues of the $\text{Fc}\gamma$ -ITAM by using retroviral gene transfer to introduce the $\text{Fc}\gamma$ -ITAM constructs into the $\text{Fc}\gamma^{-/-}$ BMMCs (Fig. 7A). Introduction of the $\text{Fc}\gamma$ -subunit genes restored the defective PpL binding seen in the $\text{Fc}\gamma^{-/-}$ BMMCs (Fig. 7B). Next, we examined the activation of representative proximal and distal

signaling molecules in the $\text{Fc}\gamma_{\text{YY}}$ and $\text{Fc}\gamma_{\text{FF}}$ BMMCs. Fig. 7C shows that PpL induced tyrosine phosphorylation of Syk, PLC γ 1, and Gab2 in the $\text{Fc}\gamma_{\text{YY}}$ BMMCs sensitized with 15.3.2. In contrast, the $\text{Fc}\gamma_{\text{FF}}$ BMMCs had decreased phosphorylation of these proximal signaling molecules. Phosphorylation of the distal signaling molecules ERK1/2 and p38 MAPK was also induced following $\text{Fc}\epsilon\text{RI}$ stimulation with 15.3.2 and PpL. Consistent with our proximal signaling data, the level of p38 MAPK phosphorylation was significantly decreased in the $\text{Fc}\gamma_{\text{FF}}$ BMMCs compared with $\text{Fc}\gamma_{\text{YY}}$ BMMCs. To our surprise, we observed apparent ERK1/2 phosphorylation in the $\text{Fc}\gamma_{\text{FF}}$ BMMCs.

LTC_4 secretion, but not cytokine production and the degranulation response, is inducible in $\text{Fc}\gamma_{\text{FF}}$ BMMCs

As demonstrated in Fig. 7C, the phosphorylation of $\text{Fc}\epsilon\text{RI}$ -dependent signaling molecules was reduced in the $\text{Fc}\gamma_{\text{FF}}$ BMMCs but did not completely disappear. Therefore, we examined whether PpL-mediated activation of the $\text{Fc}\epsilon\text{RI}$ -dependent signal in the 15.3.2-sensitized $\text{Fc}\gamma_{\text{FF}}$ BMMCs was sufficient to induce mast cell activation. Upon PpL loading, detectable $[\text{Ca}^{2+}]_i$ mobilization was observed in the 15.3.2-sensitized $\text{Fc}\gamma_{\text{FF}}$ BMMCs, although the level of mobilization was much lower than

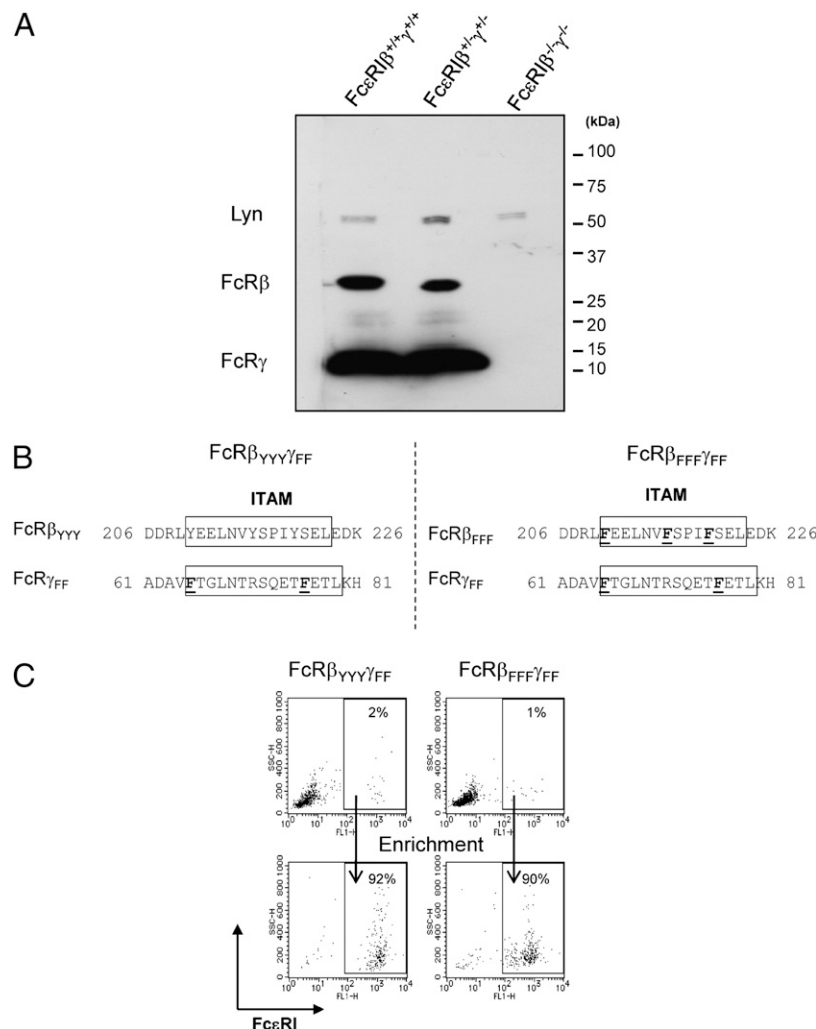


FIGURE 9. Preparation of the $\text{Fc}\beta\gamma$ -ITAM cotransfectants. **(A)** Defective expression of the $\text{Fc}\beta$ and γ proteins in BMMCs from the $\text{Fc}\beta\gamma$ double-knockout mice. **(B)** Wild-type $\text{Fc}\beta$ ($\text{Fc}\beta_{\text{YY}}$) and mutant $\text{Fc}\beta$ -ITAM ($\text{Fc}\beta_{\text{FF}}$), with three tyrosine residues in the ITAM replaced with phenylalanine. **(C)** The $\text{Fc}\epsilon\text{RI}^{+}$ cells were purified from puromycin-resistant $\text{Fc}\beta\gamma$ cotransfectants. In **(A)** and **(C)**, similar results were observed in three independent experiments.

in the 15.3.2-sensitized $\text{Fc}\gamma\text{R}_{\text{YY}}$ BMMCs (Fig. 8A). Fig. 8B and 8C show that introducing mutations into the $\text{Fc}\gamma\text{R}$ -ITAM completely abolished IL-6 production and the degranulation response in the mast cells stimulated with 15.3.2 and PpL. In contrast, LTC_4 release was sufficiently induced, even in the $\text{Fc}\gamma\text{R}_{\text{FF}}$ BMMCs (Fig. 8D). Taken together, PpL-mediated activation of the $\text{Fc}\epsilon\text{RI}$ -dependent signaling in the 15.3.2-sensitized $\text{Fc}\gamma\text{R}_{\text{FF}}$ BMMCs was able to induce LTC_4 release but not cytokine production and the degranulation response. Fig. 8E shows that calcium influx plays a crucial role in LTC_4 release in $\text{Fc}\gamma\text{R}_{\text{FF}}$ BMMCs sensitized with 15.3.2. U0126, a potent inhibitor of MEK1/2, also inhibited

LTC_4 release (Fig. 8F), suggesting that MEK1/2-dependent ERK1/2 activation contributed to the release of this lipid mediator.

FcR β is a signal transducer responsible for LTC_4 release in the absence of a functional $\text{Fc}\gamma\text{R}$ -ITAM

A previous study employing a chimera of the IL-2R α subunit (Tac) and $\text{FcR}\beta$ suggested that the $\text{FcR}\beta$ ITAM lacks signaling capacity (30). However, we re-evaluated the possibility that $\text{FcR}\beta$ itself acts as a signal transducer that leads to LTC_4 secretion in the absence of a functional $\text{Fc}\gamma\text{R}$ -ITAM. To prove this hypothesis, we generated mice deficient in both $\text{FcR}\beta$ and $\text{Fc}\gamma\text{R}$. The $\text{FcR}\beta^{-/-}$

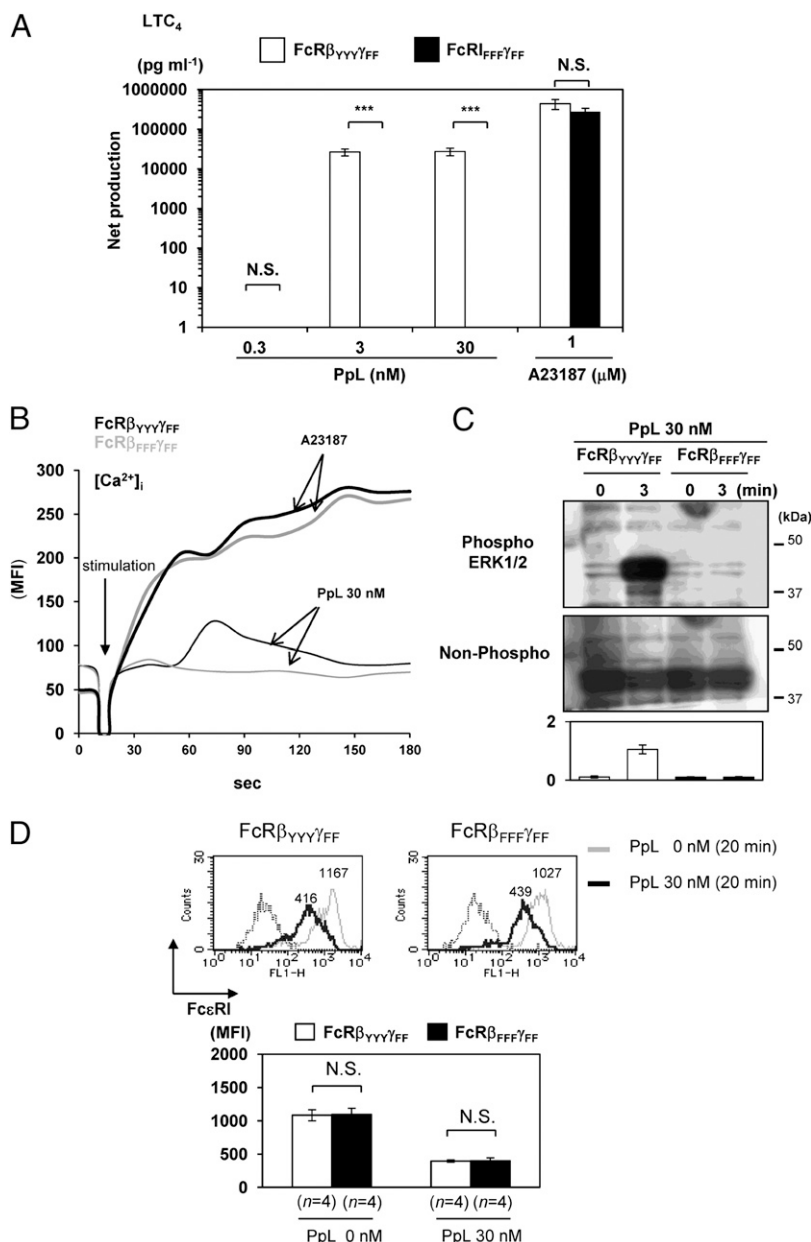


FIGURE 10. The $\text{FcR}\beta$ -ITAM regulates LTC_4 secretion in an $\text{Fc}\gamma\text{R}$ -ITAM-independent manner. $\text{FcR}\beta_{\text{YY}\gamma\text{FF}}$ and $\text{FcR}\beta_{\text{FF}\gamma\text{FF}}$ mast cells were sensitized with 15.3.2 and stimulated with or without PpL at the indicated concentrations. In **(A)** and **(B)**, A23187 (1 μM) was used as a nonimmunological positive control in place of PpL. **(A)** The net LTC_4 production. Data shown are the mean $\pm$ SD of quadruplicates. Similar results were obtained in three independent experiments. **(B)** PpL-mediated calcium mobilization was analyzed in the 15.3.2-sensitized $\text{FcR}\beta_{\text{YY}\gamma\text{FF}}$ and $\text{FcR}\beta_{\text{FF}\gamma\text{FF}}$ mast cells, as shown in Fig. 4B. The results shown are representative of three independent experiments with similar results. **(C)** The ERK1/2 phosphorylation was analyzed by immunoblotting with anti-phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴) Ab. The blot was reprobbed with anti-nonphospho-Ab. Densitometric data obtained from three independent experiments (bottom panel) are shown as mean $\pm$ SD. **(D)** The changes in cell-surface $\text{Fc}\epsilon\text{RI}$ expression in 15.3.2-sensitized $\text{FcR}\beta_{\text{YY}\gamma\text{FF}}$ and $\text{FcR}\beta_{\text{FF}\gamma\text{FF}}$ mast cells by PpL stimulation. Data obtained from three independent experiments (bottom panel) are shown as mean $\pm$ SD. The statistical analysis was performed using Student *t* test. ****p* < 0.001.

$\gamma^{-/-}$ BMMCs failed to express the FcR β and FcR γ proteins (Fig. 9A). We then prepared two cotransfectants (FcR $\beta_{YYY\gamma_{FF}}$ and FcR $\beta_{FFF\gamma_{FF}}$) that harbored mutations in the tyrosine residues of each ITAM (Fig. 9B, 9C). Because both FcR β and FcR γ are indispensable for cell-surface Fc ϵ RI expression (26, 31), we recognized the Fc ϵ RI-positive cells as mast cells with the exogenous FcR β and FcR γ genes and enriched this cell population (Fig. 9C). As shown in Fig. 10A, the 15.3.2-sensitized FcR $\beta_{FFF\gamma_{FF}}$ mast cells did not produce LTC $_4$ in response to PpL loading. Consistent with this finding, $[Ca^{2+}]_i$ mobilization and ERK1/2 activation in response to PpL were entirely abolished in the 15.3.2-sensitized FcR $\beta_{FFF\gamma_{FF}}$ mast cells (Fig. 10B, 10C). Conversely, cell-surface Fc ϵ RI was downregulated in a comparable manner in the 15.3.2-sensitized FcR $\beta_{YYY\gamma_{FF}}$ and FcR $\beta_{FFF\gamma_{FF}}$ mast cells following PpL stimulation (Fig. 10D), suggesting that Fc ϵ RI cross-linking-induced receptor internalization is unaltered by introducing mutations into the FcR β -ITAM. Fig. 11 illustrates a model of mast cell activation triggered by 15.3.2 and PpL.

Discussion

Mast cells are resident in various tissues, where they act as innate immune cells against pathogens. Previous studies demonstrated that PpL activates human mast cells by interacting with IgE κ (14, 15), suggesting that Fc ϵ RI, the high-affinity receptor for IgE, is

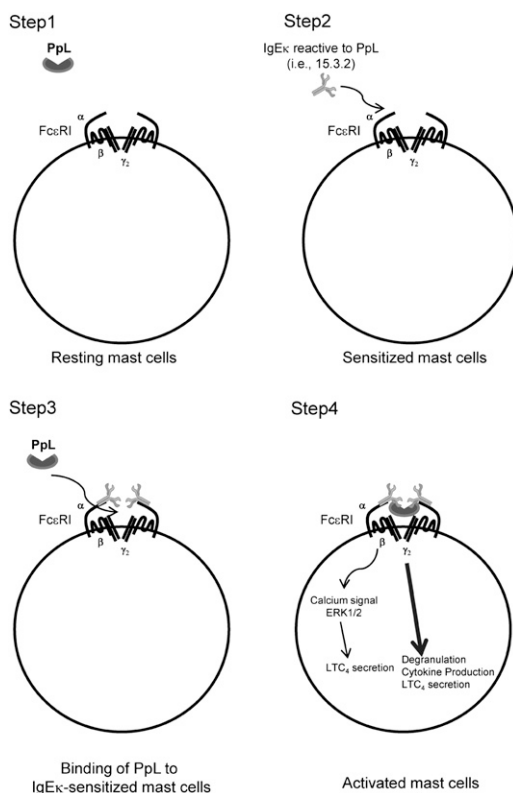


FIGURE 11. A model of mast cell activation triggered by 15.3.2 and PpL. PpL fails to activate mast cells in the absence of IgE κ (Step1). Binding of IgE κ (15.3.2) to the cell-surface Fc ϵ RI of mast cells (Step2). PpL exists as a monomer but has multiple (four or five) Ig κ binding regions. Thereby, one PpL molecule can bind to multiple IgE κ -Fc ϵ RI complexes through the Ig κ binding sites (Step3). Engagement of IgE κ -Fc ϵ RI complexes with PpL induces cytokine production, the degranulation response, and LTC $_4$ synthesis in mast cells (Step4). Additional stimulation of Fc ϵ RI with IgE κ and its specific Ag is dispensable for the PpL-mediated mast cell activation. The FcR γ subunit plays important roles in the Fc ϵ RI-dependent mast cell activation. The FcR β subunit also activates ERK1/2, calcium signals and LTC $_4$ secretion independently of the FcR γ subunit.

a critical participant in PpL-mediated mast cell activation. Consistent with these studies, we also found that sensitization of mast cells with IgE κ (15.3.2) is required for cytokine production, the degranulation response, and LTC $_4$ release in murine mast cells upon PpL loading. Like Fc ϵ RI, other Fc receptors (such as Fc γ RI, Fc γ RIII, and Fc γ RIV) also require FcR γ for their cell-surface expression. It has been reported that Fc γ RIV acts as a low-affinity receptor for mouse IgE (32, 33). However, murine mast cells do not express Fc γ RIV (33), and 15.3.2 does not bind to Fc γ RI, Fc γ RIII, or Fc γ RIV (32). In addition, we showed that FcR $\gamma^{-/-}$ and Fc ϵ RI $\alpha^{-/-}$ mast cells lack PpL-dependent cell responses. Therefore, we conclude that Fc ϵ RI definitely contributes to the activation of murine mast cells induced by IgE κ (15.3.2) and PpL.

In this study, the murine IgE κ clones that were examined, with the exception of 15.3.2, showed little or no binding to PpL, which was similar to the previous finding that out of six murine IgG κ clones, at least three could not bind to PpL (34). Murine IgE clones have been divided into two allotypes, IgE ^{a} and IgE ^{b} (35). Because 15.3.2, 27.74, and H1- ϵ -26.82 are IgE ^{a} allotypes, and C48-2 is an IgE ^{b} allotype, we believe that the specific IgE allotype does not determine the ability of IgE κ to bind to PpL. As demonstrated in the current study, the avidity between 15.3.2 and PpL is sufficient to allow Fc ϵ RI-dependent cytokine production, the degranulation response, and LTC $_4$ secretion by mast cells. However, we think that the avidity between 15.3.2 and PpL may be much lower than that between human polyclonal IgE κ and PpL for the following reasons: 1) the interaction between human polyclonal IgE κ and PpL elicits a robust degranulation response (36); 2) the interaction between 15.3.2 and PpL elicited an apparent but weak degranulation response; and 3) high-avidity interactions between IgE and multivalent ligands are required for robust degranulation (21). We speculate that the avidity between 15.3.2 and PpL may explain why stimulation of Fc ϵ RI with 15.3.2 and PpL does not induce a strong degranulation response.

In the case of stimulation of Fc ϵ RI by IgE and its specific Ag, FcR γ -ITAM is a well-known signal transducer responsible for Fc ϵ RI-dependent mast cell function. By contrast, FcR β -ITAM is a known modulator of FcR γ -ITAM-dependent signaling (31, 37, 38). Sakurai et al. (39) and Yamashita et al. (40) have independently reported that FcR γ -ITAM is indispensable for cytokine production, the degranulation response, and PGD $_2$ secretion in mast cells upon Fc ϵ RI stimulation. With respect to IL-6 production and the degranulation response, our data supported these previous studies. Interestingly, our results on LTC $_4$ secretion were not consistent with the current theory that all aspects of mast cell activation depend on the FcR γ -ITAM functions. The discrepancy in the secretion of LTC $_4$ and PGD $_2$ may be explained by a previous report, which demonstrated that BMMCs predominantly synthesize LTC $_4$, not PGD $_2$, upon Fc ϵ RI cross-linking (41), suggesting that LTC $_4$ is a more highly detected lipid mediator than PGD $_2$. Our experiments employing cotransfectants (FcR $\beta_{YYY\gamma_{FF}}$ and FcR $\beta_{FFF\gamma_{FF}}$) clearly revealed that the FcR β -ITAM contributes to FcR γ -ITAM-independent LTC $_4$ production from mast cells stimulated with 15.3.2 and PpL by activating ERK1/2 and $[Ca^{2+}]_i$ mobilization. To our knowledge, this is the first report demonstrating a novel role for the FcR β -ITAM as a functional signal transducer in Fc ϵ RI-mediated mast cell activation. Currently, it is unclear how the FcR β -ITAM itself regulates ERK1/2 and calcium signaling in the absence of a functional FcR γ -ITAM. Because the tyrosine-phosphorylated FcR β -ITAM peptide can bind to Lyn, Syk, and PLC γ 1 (31, 42, 43), these signaling molecules are likely to play pivotal roles in the FcR β -ITAM-dependent regulation of ERK1/2 and calcium signaling. Recently, it was reported that the internalization of Fc ϵ RI indepen-

dently activates calcium signaling in mast cells (44). We previously reported that the FcR β -ITAM is essential for Fc ϵ RI internalization upon the stimulation of Fc ϵ RI by highly cytokinergic IgE (20); we therefore expected that FcR β _{FF} γ _{FF} mast cells would display reduced Fc ϵ RI internalization compared with FcR β _{YY} γ _{FF} cells. However, the Fc ϵ RI internalization induced by I5.3.2 and PpL was comparable in the FcR β _{YY} γ _{FF} and FcR β _{FF} γ _{FF} mast cells (Fig. 10D). This result suggests that Fc ϵ RI internalization is not involved in the molecular mechanisms underlying the FcR β -ITAM-dependent LTC₄ secretion.

In this study, we focused on IgE κ and PpL and employed murine mast cells to investigate the underlying mechanisms of PpL-dependent mast cell activation. However, *Staphylococcus aureus* also possesses an Ig-binding protein, called protein A, that binds to VH3 domain of human IgE (15) and thereby induces human mast cell activation. Furthermore, it was reported that the murine anti-TNP IgE κ clone (IGEL b4) considerably enhances the mast cell degranulation response and LTC₄ secretion elicited by extracts of the opportunistic skin yeast *Malassezia sympodialis* (45). This finding raises the possibility that *M. sympodialis* extracts include PpL or a novel type of Ig κ -binding protein, such as MUB (46). It is suggested that mast cells can respond both to PpL and to other bacterial products to produce inflammatory mediators in an IgE-dependent manner. Mast cell-derived inflammatory mediators, including TNF- α , are essential for the recruitment of leukocytes and the initiation of adaptive immunity (47–49). Recent studies suggest that LTC₄ also plays important roles in both innate and adaptive immunity (50–52). Therefore, our novel findings that the complete secretion of LTC₄ elicited by I5.3.2 and PpL requires both the FcR β - and γ -ITAMs will provide useful information for understanding the roles of IgE and mast cells in the immune system.

Disclosures

The authors have no financial conflicts of interest.

References

- Blank, U., C. Ra, L. Miller, K. White, H. Metzger, and J. P. Kinet. 1989. Complete structure and expression in transfected cells of high affinity IgE receptor. *Nature* 337: 187–189.
- Ra, C., M. H. Jouvin, and J. P. Kinet. 1989. Complete structure of the mouse mast cell receptor for IgE (Fc epsilon RI) and surface expression of chimeric receptors (rat-mouse-human) on transfected cells. *J. Biol. Chem.* 264: 15323–15327.
- Gould, H. J., B. J. Sutton, A. J. Beavil, R. L. Beavil, N. McCloskey, H. A. Coker, D. Fear, and L. Smurthwaite. 2003. The biology of IGE and the basis of allergic disease. *Annu. Rev. Immunol.* 21: 579–628.
- Malaviya, R., E. Ross, B. A. Jakschik, and S. N. Abraham. 1994. Mast cell degranulation induced by type 1 fimbriated *Escherichia coli* in mice. *J. Clin. Invest.* 93: 1645–1653.
- Dreskin, S. C., and S. N. Abraham. 1999. Production of TNF-alpha by murine bone marrow derived mast cells activated by the bacterial fimbrial protein, FimH. *Clin. Immunol.* 90: 420–424.
- Supajatura, V., H. Ushio, A. Nakao, K. Okumura, C. Ra, and H. Ogawa. 2001. Protective roles of mast cells against enterobacterial infection are mediated by Toll-like receptor 4. *J. Immunol.* 167: 2250–2256.
- Supajatura, V., H. Ushio, A. Nakao, S. Akira, K. Okumura, C. Ra, and H. Ogawa. 2002. Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *J. Clin. Invest.* 109: 1351–1359.
- Muñoz, S., R. Hernández-Pando, S. N. Abraham, and J. A. Enciso. 2003. Mast cell activation by *Mycobacterium tuberculosis*: mediator release and role of CD48. *J. Immunol.* 170: 5590–5596.
- Gon, Y., S. Nunomura, and C. Ra. 2005. Common and distinct signalling cascades in the production of tumour necrosis factor-alpha and interleukin-13 induced by lipopolysaccharide in RBL-2H3 cells. *Clin. Exp. Allergy* 35: 635–642.
- Murdoch, D. A. 1998. Gram-positive anaerobic cocci. *Clin. Microbiol. Rev.* 11: 81–120.
- Stephens, P., I. B. Wall, M. J. Wilson, K. E. Hill, C. E. Davies, C. M. Hill, K. G. Harding, and D. W. Thomas. 2003. Anaerobic cocci populating the deep tissues of chronic wounds impair cellular wound healing responses in vitro. *Br. J. Dermatol.* 148: 456–466.
- Akerström, B., and L. Björck. 1989. Protein L: an immunoglobulin light chain-binding bacterial protein. Characterization of binding and physicochemical properties. *J. Biol. Chem.* 264: 19740–19746.
- Nilson, B. H., A. Solomon, L. Björck, and B. Akerström. 1992. Protein L from *Peptostreptococcus magnus* binds to the kappa light chain variable domain. *J. Biol. Chem.* 267: 2234–2239.
- Patella, V., V. Casolaro, L. Björck, and G. Marone. 1990. Protein L. A bacterial Ig-binding protein that activates human basophils and mast cells. *J. Immunol.* 145: 3054–3061.
- Genovese, A., J. P. Bouvet, G. Florio, B. Lamparter-Schummert, L. Björck, and G. Marone. 2000. Bacterial immunoglobulin superantigen proteins A and L activate human heart mast cells by interacting with immunoglobulin E. *Infect. Immun.* 68: 5517–5524.
- Genovese, A., G. Borgia, L. Björck, A. Petraroli, A. de Paulis, M. Piazza, and G. Marone. 2003. Immunoglobulin superantigen protein L induces IL-4 and IL-13 secretion from human Fc epsilon RI+ cells through interaction with the kappa light chains of IgE. *J. Immunol.* 170: 1854–1861.
- Kastern, W., U. Sjöbring, and L. Björck. 1992. Structure of peptostreptococcal protein L and identification of a repeated immunoglobulin light chain-binding domain. *J. Biol. Chem.* 267: 12820–12825.
- Blank, U., and J. Rivera. 2004. The ins and outs of IgE-dependent mast-cell exocytosis. *Trends Immunol.* 25: 266–273.
- Kalesnikoff, J., M. Huber, V. Lam, J. E. Damen, J. Zhang, R. P. Siraganian, and G. Krystal. 2001. Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity* 14: 801–811.
- Nunomura, S., Y. Gon, T. Yoshimaru, Y. Suzuki, H. Nishimoto, T. Kawakami, and C. Ra. 2005. Role of the Fc epsilon RI beta-chain ITAM as a signal regulator for mast cell activation with monomeric IgE. *Int. Immunol.* 17: 685–694.
- Collins, A. M., D. Theliam, and M. Basil. 1995. Antigen valency as a determinant of the responsiveness of IgE-sensitized rat basophil leukemia cells. *Int. Arch. Allergy Immunol.* 107: 547–556.
- Torigoe, C., J. K. Inman, and H. Metzger. 1998. An unusual mechanism for ligand antagonism. *Science* 281: 568–572.
- Gonzalez-Espinosa, C., S. Odom, A. Olivera, J. P. Hobson, M. E. Martinez, A. Oliveira-Dos-Santos, L. Barra, S. Spiegel, J. M. Penninger, and J. Rivera. 2003. Preferential signaling and induction of allergy-promoting lymphokines upon weak stimulation of the high affinity IgE receptor on mast cells. *J. Exp. Med.* 197: 1453–1465.
- Grodzki, A. C., K. D. Moon, E. H. Berenstein, and R. P. Siraganian. 2009. Fc epsilon RI-induced activation by low antigen concentrations results in nuclear signals in the absence of degranulation. *Mol. Immunol.* 46: 2539–2547.
- Park, S. Y., S. Ueda, H. Ohno, Y. Hamano, M. Tanaka, T. Shiratori, T. Yamazaki, H. Arase, N. Arase, A. Karasawa, et al. 1998. Resistance of Fc receptor-deficient mice to fatal glomerulonephritis. *J. Clin. Invest.* 102: 1229–1238.
- Kobayashi, M., S. Nunomura, Y. Gon, D. Endo, S. Kishiro, M. Fukunaga, Y. Kitahata, T. Terui, and C. Ra. 2010. Abrogation of high-affinity IgE receptor-mediated mast cell activation at the effector phase prevents contact hypersensitivity to oxazolone. *J. Invest. Dermatol.* 130: 725–731.
- Nunomura, S., Y. Gon, T. Yoshimaru, J. Kashiwakura, T. Kawakami, and C. Ra. 2010. Fc epsilon RI beta-chain ITAM amplifies PI3K-signaling to ensure synergistic degranulation response via Fc epsilon RI and adenosine receptors. *Eur. J. Immunol.* 40: 1205–1217.
- Kitaura, J., J. Song, M. Tsai, K. Asai, M. Maeda-Yamamoto, A. Mocsai, Y. Kawakami, F. T. Liu, C. A. Lowell, B. G. Barisas, et al. 2003. Evidence that IgE molecules mediate a spectrum of effects on mast cell survival and activation via aggregation of the Fc epsilon RI. *Proc. Natl. Acad. Sci. USA* 100: 12911–12916.
- Sly, L. M., J. Kalesnikoff, V. Lam, D. Wong, C. Song, S. Omeis, K. Chan, C. W. Lee, R. P. Siraganian, J. Rivera, and G. Krystal. 2008. IgE-induced mast cell survival requires the prolonged generation of reactive oxygen species. *J. Immunol.* 181: 3850–3860.
- Wilson, B. S., N. Kapp, R. J. Lee, J. R. Pfeiffer, A. M. Martinez, Y. Platt, F. Letourneur, and J. M. Oliver. 1995. Distinct functions of the Fc epsilon RI gamma and beta subunits in the control of Fc epsilon RI-mediated tyrosine kinase activation and signaling responses in RBL-2H3 mast cells. *J. Biol. Chem.* 270: 4013–4022.
- Furumoto, Y., S. Nunomura, T. Terada, J. Rivera, and C. Ra. 2004. The Fc epsilon RIbeta immunoreceptor tyrosine-based activation motif exerts inhibitory control on MAPK and IkappaB kinase phosphorylation and mast cell cytokine production. *J. Biol. Chem.* 279: 49177–49187.
- Mancardi, D. A., B. Iannascoli, S. Hoos, P. England, M. Daëron, and P. Bruhns. 2008. Fc gamma RIV is a mouse IgE receptor that resembles macrophage Fc epsilon RI in humans and promotes IgE-induced lung inflammation. *J. Clin. Invest.* 118: 3738–3750.
- Hirano, M., R. S. Davis, W. D. Fine, S. Nakamura, K. Shimizu, H. Yagi, K. Kato, R. P. Stephan, and M. D. Cooper. 2007. IgEb immune complexes activate macrophages through Fc gamma RIV binding. *Nat. Immunol.* 8: 762–771.
- De Chateau, M., B. H. Nilson, M. Ertell, E. Myhre, C. G. Magnusson, B. Akerström, and L. Björck. 1993. On the interaction between protein L and immunoglobulins of various mammalian species. *Scand. J. Immunol.* 37: 399–405.
- Miyajima, H., K. Abe, C. Ushiyama, K. Okumura, Z. Ovary, and T. Hirano. 1996. IgE allotypes in sera of mice with autoimmune diseases and in mice with graft-versus-host disease after transfusion or bone marrow transplantation. *Int. Arch. Allergy Immunol.* 111: 152–155.
- Kastern, W., E. Holst, E. Nielsen, U. Sjöbring, and L. Björck. 1990. Protein L, a bacterial immunoglobulin-binding protein and possible virulence determinant. *Infect. Immun.* 58: 1217–1222.
- Xiao, W., H. Nishimoto, H. Hong, J. Kitaura, S. Nunomura, M. Maeda-Yamamoto, Y. Kawakami, C. A. Lowell, C. Ra, and T. Kawakami. 2005. Positive and negative regulation of mast cell activation by Lyn via the Fc epsilon RI. *J. Immunol.* 175: 6885–6892.

38. On, M., J. M. Billingsley, M. H. Jouvin, and J. P. Kinet. 2004. Molecular dissection of the FcRbeta signaling amplifier. *J. Biol. Chem.* 279: 45782–45790.
39. Sakurai, D., S. Yamasaki, K. Arase, S. Y. Park, H. Arase, A. Konno, and T. Saito. 2004. Fc epsilon RI gamma-ITAM is differentially required for mast cell function in vivo. *J. Immunol.* 172: 2374–2381.
40. Yamashita, T., R. Suzuki, P. S. Backlund, Y. Yamashita, A. L. Yergey, and J. Rivera. 2008. Differential dephosphorylation of the FcRgamma immunoreceptor tyrosine-based activation motif tyrosines with dissimilar potential for activating Syk. *J. Biol. Chem.* 283: 28584–28594.
41. Murakami, M., K. F. Austen, C. O. Bingham, III, D. S. Friend, J. F. Penrose, and J. P. Arm. 1995. Interleukin-3 regulates development of the 5-lipoxygenase/leukotriene C4 synthase pathway in mouse mast cells. *J. Biol. Chem.* 270: 22653–22656.
42. Kimura, T., H. Kihara, S. Bhattacharyya, H. Sakamoto, E. Appella, and R. P. Siraganian. 1996. Downstream signaling molecules bind to different phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) peptides of the high affinity IgE receptor. *J. Biol. Chem.* 271: 27962–27968.
43. Soto-Cruz, I., J. M. Oliver, and E. Ortega. 2007. Analysis of proteins binding to the ITAM motif of the beta-subunit of the high-affinity receptor for IgE (FcepsilonRI). *J. Recept. Signal Transduct. Res.* 27: 67–81.
44. Ryu, S. D., H. S. Lee, H. Y. Suk, C. S. Park, and O. H. Choi. 2009. Cross-linking of FcepsilonRI causes Ca²⁺ mobilization via a sphingosine kinase pathway in a clathrin-dependent manner. *Cell Calcium* 45: 99–108.
45. Selander, C., C. Engblom, G. Nilsson, A. Scheynius, and C. L. Andersson. 2009. TLR2/MyD88-dependent and -independent activation of mast cell IgE responses by the skin commensal yeast *Malassezia sympodialis*. *J. Immunol.* 182: 4208–4216.
46. MacKenzie, D. A., L. E. Tailford, A. M. Hemmings, and N. Juge. 2009. Crystal structure of a mucus-binding protein repeat reveals an unexpected functional immunoglobulin binding activity. *J. Biol. Chem.* 284: 32444–32453.
47. Malaviya, R., T. Ikeda, E. Ross, and S. N. Abraham. 1996. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature* 381: 77–80.
48. McLachlan, J. B., J. P. Hart, S. V. Pizzo, C. P. Shelburne, H. F. Staats, M. D. Gunn, and S. N. Abraham. 2003. Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection. *Nat. Immunol.* 4: 1199–1205.
49. Kunder, C. A., A. L. St John, G. Li, K. W. Leong, B. Berwin, H. F. Staats, and S. N. Abraham. 2009. Mast cell-derived particles deliver peripheral signals to remote lymph nodes. *J. Exp. Med.* 206: 2455–2467.
50. Barrett, N. A., O. M. Rahman, J. M. Fernandez, M. W. Parsons, W. Xing, K. F. Austen, and Y. Kanaoka. 2011. Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes. *J. Exp. Med.* 208: 593–604.
51. Medeiros, A. I., A. Sá-Nunes, W. M. Turato, A. Secatto, F. G. Frantz, C. A. Sorgi, C. H. Serezani, G. S. Deepe, Jr., and L. H. Faccioli. 2008. Leukotrienes are potent adjuvant during fungal infection: effects on memory T cells. *J. Immunol.* 181: 8544–8551.
52. Kanaoka, Y., A. Maekawa, J. F. Penrose, K. F. Austen, and B. K. Lam. 2001. Attenuated zymosan-induced peritoneal vascular permeability and IgE-dependent passive cutaneous anaphylaxis in mice lacking leukotriene C4 synthase. *J. Biol. Chem.* 276: 22608–22613.