

Lyn Is Essential for Fc γ Receptor III–mediated Systemic Anaphylaxis but Not for the Arthus Reaction

By Takae Yuasa,* Masao Ono,* Takeshi Watanabe,† and Toshiyuki Takai*

From the *Department of Experimental Immunology and the Core Research for Evolutional Science and Technology (CREST) Program of Japan Science and Technology, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-8575, Japan; and the †Department of Molecular Immunology, Medical Institute of Bioregulation, Fukuoka 812-8582, Japan

Abstract

The Src family kinase Lyn initiates intracellular signal transduction by associating with a variety of immune receptors such as antigen receptor on B cells and high-affinity Fc receptor (FcR) for immunoglobulin Ig(E) (Fc ϵ RI) on mast cells. Involvement of Lyn in the IgE-mediated immediate-type hypersensitivity is well documented, but the physiological significance of Lyn in IgG-dependent, type III low-affinity FcR for IgG (Fc γ RIII)-mediated responses is largely unknown. In this study, we generated a double-mutant mouse strain deficient in both type II FcR for IgG (Fc γ RIIB) and Lyn to exclude any involvement of inhibitory signaling by Fc γ RIIB, which otherwise downregulates Fc γ RIII-mediated cellular responses. Fc γ RIIB-deficient but Lyn-sufficient mice served as controls. The Lyn deficiency attenuated IgG-mediated systemic anaphylaxis in vivo, and significantly reduced calcium mobilization and degranulation responses of bone marrow-derived mast cells (BMMCs) in vitro. However, we found that either interleukin 4 or tumor necrosis factor α release by BMMCs was comparable to that from Lyn-deficient and control mice, and the reverse-passive Arthus reaction was equally induced in both mutant mice, indicating that Lyn is not involved in the onset of the IgG-mediated, Fc γ RIII-dependent late phase responses of mast cells. These findings provide us with insight into distinct signaling mechanisms in mast cells underlying the development of diverse pathologies as well as a therapeutic potential for selective treatment of allergic disorders.

Key words: Lyn • Fc receptor • hypersensitivity • mast cells • Arthus reaction

Introduction

Fc receptors (FcRs) expressed on immune effector cells are capable of initiating a variety of immune and inflammatory responses in an antibody-dependent manner. Because of the complexity stemming from overlapped FcR expression on diverse cell types, the defined role of each class of FcR had not been assigned until recent efforts to generate several strains of mice genetically deficient in FcR expression. Currently, their roles are implicated not only in host defense but also in the development of such diverse diseases as allergy (1, 2), Arthus reaction (3, 4), rheumatoid arthritis (5, 6), immune glomerulonephritis (7, 8), autoimmune vasculitis (9), systemic lupus erythematosus (10), and Goodpasture's syndrome (11). The findings obtained from these mutant mice have promoted an understanding of the

indispensable role of each FcR in a variety of immune processes (for reviews, see references 12–14).

As notable findings in association with our current study, previous studies have defined the central role of low-affinity stimulatory FcR for IgG, Fc γ RIII, on mast cells in initiating two distinct classes of immune responses, that is, the IgG-dependent anaphylaxis (15–17) and the reverse-passive Arthus reaction (3, 4, 18–20). These in vivo experimental models of inflammation are mutually distinguishable by the time of disease onset and their histological features. Anaphylaxis displays edematous lesions in skin or systemic insufficiency of blood supply (shock) immediately after antigen challenge, whereas the Arthus reaction appears as bleeding and tissue-damaged lesions and takes several hours for their development after antigen challenge. To date, pathogenesis of these distinctive models has been interpreted as a consequence of time-constrained releases of proinflammatory mediators, for example, prestored factors such as histamine and serotonin, or de novo synthesized factors such as arachi-

Address correspondence to Toshiyuki Takai, Dept. of Experimental Immunology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryō, Sendai 980-8575, Japan. Phone: 81-22-717-8501; Fax: 81-22-717-8505; E-mail: tostakai@idac.tohoku.ac.jp

donic acid metabolites and cytokines. However, an intracellular mechanism underlying the pathogenesis of these models remains unclear.

Lyn is a Src family tyrosine kinase which functions to initiate intracellular signal transduction by associating with a variety of immune receptors bearing the cytoplasmic amino acid motif termed immunoreceptor tyrosine-based activation motif (ITAM),¹ which serves as the binding sequence recognized by the Src homology 2 domain of cytoplasmic tyrosine kinase (21, 22). A recent study using Lyn-deficient mice demonstrated the essential role of Lyn in normal functions of B cells and mast cells in vivo (23–25). In fact, Lyn-deficient mice displayed markedly impaired anaphylactic response to IgE plus antigen challenge in skin, suggesting a critical role of Lyn in FcεRI-mediated signal transduction (26).

In this study, we examined whether Lyn-deficient mice could display the intact function of FcγRIII, which associates with FcRβ and FcRγ, the same ITAM-bearing subunits as FcεRI, and which serves as the main initiator of IgG-mediated immune responses in mice (for a review, see reference 12). To achieve this objective, we first prepared double-homozygous mice bearing null mutations for Lyn (24) and FcγRIIB (27). Since FcγRIIB affects FcγRIII-mediated signaling by its inhibitory nature when these two FcRs are present on a cell, as in the case of mast cells (17, 27), the removal of FcγRIIB makes it possible to assess the net effect of FcγRIII-mediated stimulation on the onset of diseases. Using these double-mutant mice, we obtained results that demonstrated the dispensable role of Lyn in cytokine production in mast cells and in the onset of reverse-passive Arthus reaction in contrast to its indispensable role in IgG-mediated anaphylactic response. Current findings provide an insight into distinct signaling mechanisms in mast cells underlying the development of diverse pathologies and suggest a way for selective treatment of hypersensitivities.

Materials and Methods

Mice. All experiments were performed using 7–12-wk-old mice housed under conditions monthly certified as being specific-pathogen free. The mice homozygous of null mutations in both Lyn and FcγRIIB (Lyn^{-/-}IIB^{-/-}) were generated by serial cross-breeding between Lyn^{-/-} (24) and FcγRIIB^{-/-} (27) strains of mice. Their littermates of Lyn^{+/-}FcγRIIB^{-/-} (IIB^{-/-}) were used as control mice.

Cells and Antibodies. Bone marrow-derived mast cells (BMMCs) were obtained from a culture of bone marrow cells in RPMI 1640 supplemented with 5 ng/ml murine IL-3 (R&D Systems), 10% FCS, nonessential amino acids (GIBCO BRL), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 μM 2-ME. To obtain the different type of cultured mast cells, BMMCs were further cultured on confluent monolayer of Swiss 3T3 fibroblasts (American Type Culture Collection) in the same medium for

2 wk. We used the BMMCs tightly adhered to monolayer cells (3T3-BMMCs) for our assays. Peritoneal resident mast cells (PMCs) were collected by peritoneal washings with Tyrode's solution supplemented with heparin (5 U/ml). Positive cells for mouse c-kit staining were analyzed as PMCs. Mouse anti-trinitrophenyl hapten (TNP) IgE (IGELa2; American Type Culture Collection) and mouse anti-TNP IgG1 (G1; reference 28) were prepared with DEAE-cellulose column chromatography from hybridoma culture supernatants. Rat anti-mouse FcγRII/III (2.4G2; BD PharMingen), anti-mouse c-kit (2B8; BD PharMingen), rabbit IgG anti-OVA (Sigma-Aldrich), F(ab')₂ fragment of goat anti-rat IgG (Immunotech), and mouse antiphosphotyrosine antibody (4G10; Upstate Biotechnology) were purchased.

Induction of Passive Systemic Anaphylaxis. Each mouse was sensitized by intravenous administration of 200 μg anti-TNP IgG or 20 μg anti-TNP IgE. At 30 min (IgG sensitization) or 24 h (IgE sensitization) after the sensitization, anaphylaxis was induced by intravenous administration of 1 mg TNP-conjugated OVA (Sigma-Aldrich) in 100 μl PBS per mouse. Control mice received PBS in place of antibody for sensitization. Rectal temperature was monitored at various time points after induction using a rectal probe coupled to a digital thermometer (Natsume Seisakusyo Co.).

Measurements of Degranulation and Cytokine Release. BMMCs (5 × 10⁵/ml) were labeled with 6.6 μCi/ml of 5-[1,2-³H]-hydroxytryptamine creatinine sulfate (American Radiolabeled Chemicals, Inc.) for 16 h, and sensitized with 5 μg/ml 2.4G2 or 1 μg/ml of mouse anti-TNP IgE for 30 min of the labeling period. After unbound antibodies were washed out, the receptor of interest was aggregated with 0.3–10 μg/ml F(ab')₂ fragment of goat anti-rat IgG or 0.03–30 ng/ml TNP-OVA for 1 h. The percentage of serotonin release (percent degranulation) was calculated as described previously. For cytokine measurements, BMMCs (5 × 10⁴ per sample) and 3T3-BMMCs (10⁵ per sample) without the labeling process were stimulated as described above, and culture supernatant was harvested at 3–12 h after induction for TNF-α or IL-4 measurement, respectively. The amount of released cytokine was measured by ELISA kit (BD PharMingen). Tissue extract samples were prepared from punched-out skin specimens (12.5 mm²) at 3 h after induction of reverse-passive Arthus reaction. Tissue specimens were homogenized in 400 μl PBS containing 2 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 2 μg/ml pepstatin. The amount of TNF-α in supernatant was measured with ELISA.

Analysis of Cytoplasmic Calcium Mobilization. BMMCs labeled with 2 μM Fura-2/acetoxymethyl ester (Molecular Probes) were sensitized with 2 μg/ml biotinylated mouse anti-TNP IgE or 5 μg/ml biotinylated 2.4G2 for 10 min at 25°C. Free antibody was washed out, after which the cells were stimulated with 10 μg of streptavidin (Wako Pure Chemicals) in 2 ml PBS containing 1 mM CaCl₂ and 1 mM MgCl₂. Cytoplasmic calcium mobilization was monitored at a 510-nm emission wavelength excited by 340 and 360 nm with a fluorescence spectrophotometer (model F4500; Hitachi Ltd.). Calibration and calculation of calcium concentration were performed as described (29).

Immunoblot Analyses. BMMCs (10⁶) sensitized with 2.4G2 or biotinylated mouse IgE were treated with 30 μg/ml F(ab')₂ fragment of goat anti-rat IgG or 5 μg/ml streptavidin for 0.5, 1, and 5 min at 37°C in 100 μl PBS supplemented with 1 mM CaCl₂ and 1 mM MgCl₂. The treatment was terminated by adding an equal volume of ice-chilled lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 137 mM NaCl, 2 mM EDTA, 50 mM NaF, 2 mM NaVO₄, 1 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml leu-

¹Abbreviations used in this paper: BMMC, bone marrow-derived mast cell; ITAM, immunoreceptor tyrosine-based activation motif; MPO, myeloperoxidase; PMC, peritoneal resident mast cell; TNP, trinitrophenyl.

peptin, 2 $\mu\text{g/ml}$ pepstatin). The cell extract cleared of nuclear fraction was used for the detection of protein phosphorylation. Immunoblotting was performed with antiphosphotyrosine monoclonal antibody (4G10).

Flow Cytometric Analysis. Expression level of Fc ϵ RI and Fc γ RIII on mast cells was measured by staining with mouse IgE plus FITC-conjugated anti-mouse IgE and R-phycoerythrin (R-PE)-conjugated anti-mouse Fc γ RII/III (2.4G2), respectively. Background levels of Fc ϵ RI and Fc γ RIII stainings for the cultured mast cells were shown by single staining of FITC-anti-IgE and R-PE-conjugated rat IgG2b isotype control antibody, respectively. In the case of PMCs, we used the PMC from Fc γ RIIB-Fc γ double deficient mice, in which cell surface expression of Fc ϵ RI and Fc γ RIII is diminished, as a negative control to determine the level of background signal. Increased expression of Fc ϵ RI on BMMCs was observed after the culture for 24 h in the presence of 1 $\mu\text{g/ml}$ of IgE. Flow cytometric analyses were performed with a FACSCalibur™ (Becton Dickinson), and c-kit-positive cells in 3T3-BMMCs and PMCs were analyzed as mast cells.

Histologic and Cytologic Examinations. Paraffin-embedded skin sections were prepared after fixation in 10% (vol/vol) neutral-buffered formalin. Peritoneal cells were placed onto slide glass plate by cytospin centrifugation and fixed as above. Specimens were stained with toluidine blue dye, pH 2.5, or hematoxylin and eosin.

Reverse Cutaneous Arthus Reaction. Mice intradermally received 20 μl of saline containing 0, 16, and 80 μg of rabbit anti-OVA in a discrete spot on the shaved back of the trunk, and immediately thereafter received 200 μl of saline containing 1 mg of OVA intravenously. Mice were killed at 8 h, and the dorsal skins were harvested. Myeloperoxidase (MPO) assay was performed as described by Bradley et al. (30). In brief, the punched-out area (12.5 mm²) of lesion of interest was homogenized in 400 μl of 50 mM phosphate buffer, pH 6.0, and further extracted by adding hexadecyltrimethylammonium bromide (HTAB; Sigma-Aldrich) to 0.5%, followed by sonication for 20 s and three cycles of freezing and thawing. The supernatant of extract obtained by centrifugation at 14,000 rpm for 15 min was used for measurement of MPO activity. 20 μl of the supernatant was mixed with 200 μl of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml *o*-dianisidine dihydrochloride (Sigma-Aldrich) and 0.0005% hydrogen peroxide (Wako Pure Chemicals), and color change was measured with light absorbance at 460 nm. 1 U of MPO activity was arbitrarily defined as the activity given by 0.25 μl of mouse whole blood.

Results

Impaired Fc γ RIII Function for Passive Systemic Anaphylaxis Induction in Lyn-deficient Mice. Previous studies have shown that Lyn-deficient mice failed to display IgE-dependent passive cutaneous anaphylaxis, suggesting a critical role of Lyn in triggering the signal downstream of Fc ϵ RI on mast cells (23). We further examined the role of Lyn in triggering the signal downstream of Fc γ RIII by inducing passive systemic anaphylaxis with IgG immune complexes. In all experiments in this report, we used a double-mutant mouse strain of Lyn and Fc γ RIIB (Lyn⁻IIB⁻) to eliminate involvement of Fc γ RIIB, which exerts an inhibitory function on Fc γ RIII-mediated responses (13, 17, 27). We induced systemic anaphylaxis in Lyn-deficient and control

mice by challenging with hapten-specific antibody (anti-TNP IgG or anti-TNP IgE) and antigen (TNP-OVA) through the tail vein. Decrease in rectal temperature was monitored as a measure of systemic anaphylactic response. Both in IgG- and IgE-based stimulations, Lyn-deficient mice displayed markedly reduced responses, whereas the control mice showed substantial responses (Fig. 1). The finding of impaired responsiveness in Lyn-deficient mice indicates that activation of Lyn in mast cells is necessary for intact anaphylactic response triggered by Fc γ RIII as well as by Fc ϵ RI.

Reduced Fc γ RIII Functions Detectable in BMMCs from Lyn-deficient Mice. Using BMMCs in culture, we examined mast cell function by measuring serotonin release, which is a consequence of mast cell degranulation, a representative of immediate-phase responses, and a critical event that leads to anaphylaxis. We induced mast cell activation with graded cross-linking of Fc γ RIII or Fc ϵ RI, and measured radiolabeled serotonin released into the culture medium (Fig. 2). Upon either Fc γ RIII- or Fc ϵ RI-mediated stimulation, degranulation responses of Lyn-deficient BMMCs were significantly low within the range of low grade stimulation, that is, 1–3 $\mu\text{g/ml}$ for anti-rat IgG or 0.3–3 ng/ml for TNP-OVA, although comparable degranulation was achieved within the range of high grade stimulation in Lyn-deficient BMMCs. These differences between Lyn-deficient and control mice were further confirmed in three experiments using two independently established mast cell cultures. We next examined intracellular defective traits in Lyn-deficient BMMCs by detecting cytoplasmic calcium mobilization and tyrosine phosphorylation of whole cellular proteins (Fig. 3, A and B). We used biotin-conjugated antibodies and streptavidin for BMMC stimulation. This

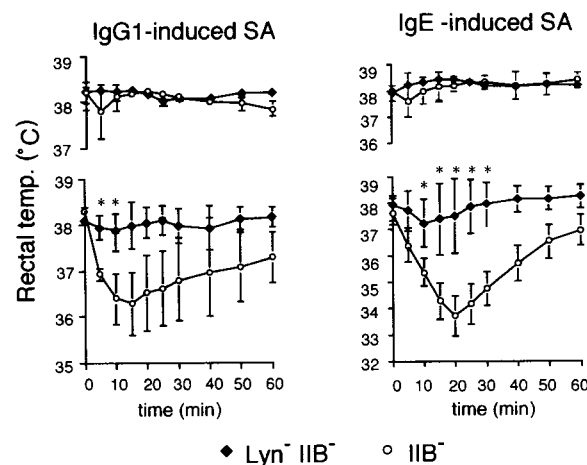


Figure 1. Passive systemic anaphylaxis (SA) in Lyn⁻IIB⁻ and control mice (IIB⁻). Anaphylaxis was induced with intravenous administration of 200 μg of anti-TNP IgG1 (left, $n = 4$) or 20 μg of anti-TNP IgE (right, $n = 6$) followed by 1 mg of TNP-OVA antigen per mouse. Control experiments were performed as well except for antibody injection (upper figures). Anaphylaxis was periodically monitored by changes in rectal temperature from the time of antigen injection. Mean \pm SD. * $P < 0.01$ (Student's t test).

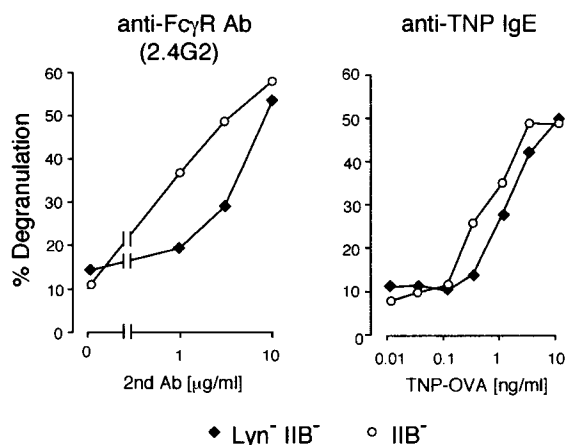


Figure 2. Degranulation responses of Lyn^{-/-}IIB^{-/-} and control (IIB^{-/-}) BMMCs. Degranulation of BMMCs was evoked by FcγRIII or FcεRI aggregation with rat anti-FcγRII/III (2.4G2) plus F(ab')₂ goat anti-rat IgG (left), or anti-TNP IgE plus TNP-OVA (right), respectively. Degranulation was evaluated 1 h after stimulation with the release of serotonin into culture medium as described in Materials and Methods. Data represent the mean percentage of duplicate samples.

mode of stimulation had been determined to be equivalent to the high grade stimulation for degranulation assay. To either FcγRIII- or FcεRI-mediated stimulation, the Lyn-deficient BMMCs displayed a reduced calcium mobilization. This response was especially affected in the early phase (<150 s) of induction, but substantially induced in the late phase (>300 s; Fig. 3 A). As long as it was observed at 5 min after stimulation, induction of tyrosine phosphorylation was markedly affected in Lyn-deficient BMMCs (Fig. 3 B).

Normal FcR Expression and Development of Mast Cells in Lyn-deficient Mice. Lyn has been shown to be involved not only in the FcR-mediated but also in c-kit-mediated signal transduction (31–33). As the c-kit-mediated growth signal is especially important for the development of mast cells in vivo, the question arises whether Lyn deficiency could affect the development of mast cells in vivo. If so, this consequence could account for the defect of mast cell-related phenotypes in Lyn-deficient mice. To address this issue, we examined mast cell phenotypes of Lyn-deficient and control mice for various mast cell preparations. In this study, we prepared c-kit-dependent mast cells by coculturing BMMCs for 2 wk with Swiss 3T3 fibroblastic cell line (3T3-BMMCs), which serve as c-kit ligand and make BMMCs differentiate towards the connective tissue-type mast cells (34). The detection of mast cells in vivo was done by histological preparation with toluidine blue staining. The results clearly revealed that Lyn deficiency in 3T3-BMMCs and PMCs as well as in BMMCs does not affect the steady expression of c-kit (data not shown), FcεRI, and FcγRIII (Fig. 4 A). We also observed normal increase of FcεRI expression on Lyn-deficient BMMC by monomeric IgE binding (Fig. 4 A). Importantly, resident mast cells in skin and PMCs from Lyn-deficient and control mice were mutually indistinguishable in cell number, cell shape, cell size,

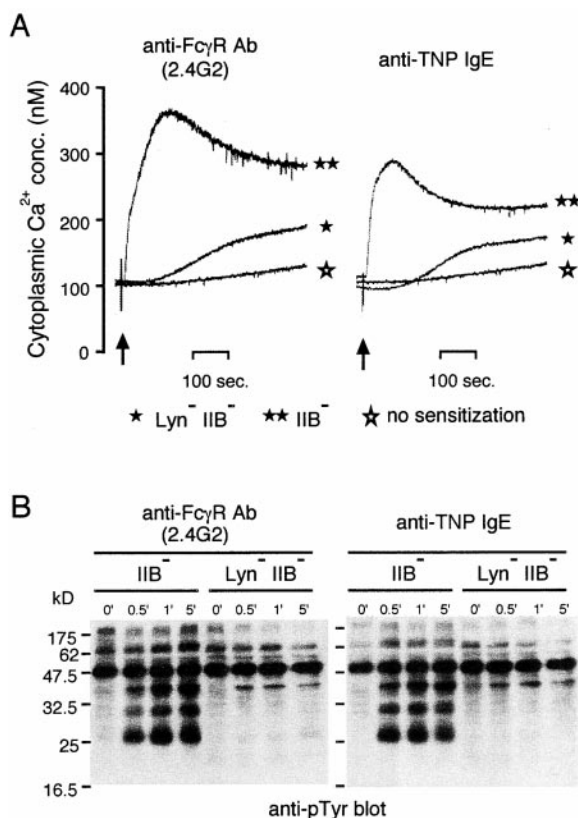


Figure 3. Cytoplasmic Ca²⁺ mobilization (A) and tyrosine phosphorylation of whole cellular protein (B) in Lyn-deficient and control BMMCs induced by FcR cross-linking. (A) Fura-2-loaded BMMCs of Lyn^{-/-}IIB^{-/-} (left) and control (IIB^{-/-}, right) mice were treated with biotinylated anti-FcγRII/III (*2.4G2) or **biotinylated IgE followed by streptavidin cross-linking (arrow). Calcium mobilization was detected as 510-nm emission with a fluorospectrophotometer. (B) BMMCs were treated as in A, and FcR-mediated stimulation was terminated by adding lysis buffer at the indicated time points. Cell lysates (2.5 × 10⁴ equivalents per lane) precluded of nuclear fractions were used for immunoblotting with antiphosphotyrosine monoclonal antibody (anti-pTyr, 4G10). Relative molecular masses are indicated.

and intracellular granule density (Fig. 4 B). These findings indicate the normal development of mast cells in Lyn-deficient mice. Accordingly, we concluded that defective phenotypes in Lyn-deficient mast cells and mice are not because of lowered expression of FcRs and developmental abnormality, and thus Lyn is central to the activation of FcR-mediated signal transduction for the immediate phase of mast cell activation.

Normal FcγRIII Function for Cytokine Production in BMMCs from Lyn-deficient Mice. It has been shown that FcR-mediated stimulation causes de novo synthesis of cytokines in mast cells (35–39). TNF-α and IL-4, well-analyzed cytokines in association with mast cell activation, are known to be released at a few and several hours, respectively, after FcR-mediated stimulation. The cytokine releases of mast cells have been shown to be important for development of mast cell-related tissue injuries (9, 40, 41) and allergic phenotypes (42). In this respect, we further examined the effect of Lyn depletion on TNF-α and IL-4 production as a

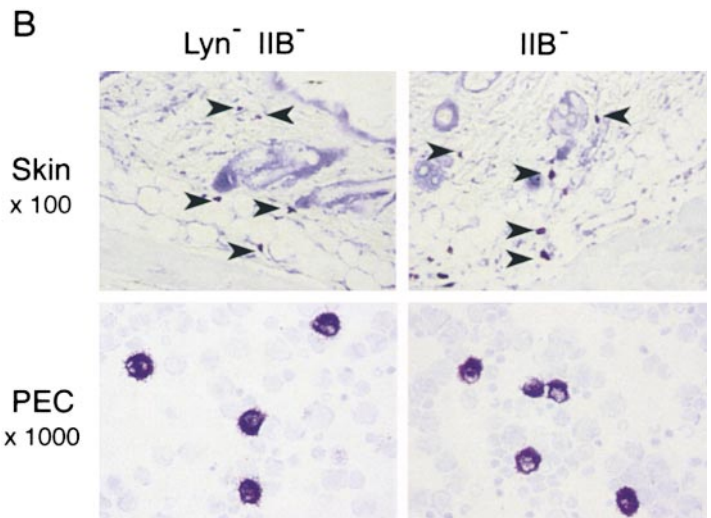
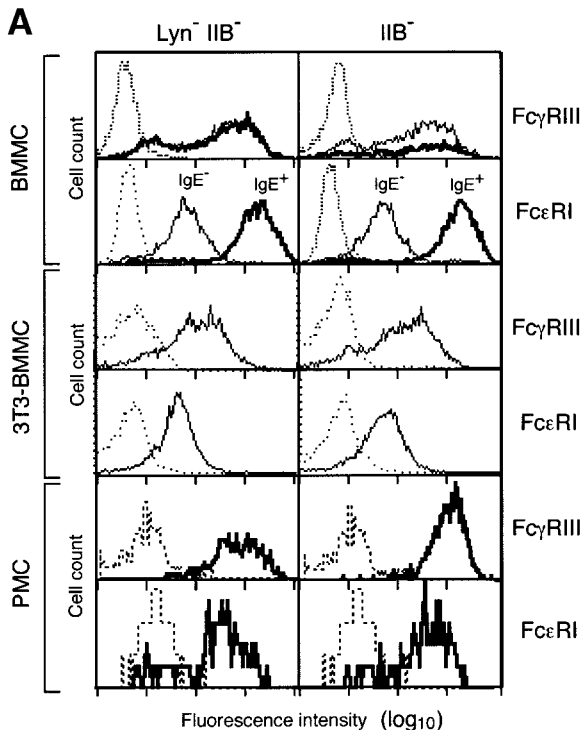


Figure 4. Normal features of Lyn-deficient mast cells. (A) Histograms for cell surface expression of FcεRI and FcγRIII on IL-3-dependent BMMCs, BMMCs cocultured with Swiss 3T3-BMMCs, and PMCs. FcεRI and FcγRIII expression was detected by mouse IgE plus FITC-conjugated anti-mouse IgE and PE-conjugated anti-mouse FcγRII/III (2.4G2), respectively (solid line). Background staining was traced with a single second antibody or isotype-matched antibody staining in the case of BMMCs and 3T3-BMMCs, or with full-stained mast cells prepared from FcγRIIB and Fcγ double deficient mice, which lack expression of all FcRs on mast cells, in the case of PMCs. Increased expression of FcεRI after IgE

incubation (1 μg/ml, 24 h) was shown with BMMCs (bold line with IgE⁺). FcγRIII expression was unchanged after this treatment (bold line). (B) Photomicrographs of mast cells resident in skin and peritoneal cavity. Mast cells are shown by metachromatic staining with toluidine blue dye (pH 2.5), and in skin sections are indicated by arrowheads. PMC preparation was made with cytopsin centrifugation. Original magnifications: ×100 for skin sections; ×1,000 for PMC preparations. PEC, peritoneal cells.

downstream event of FcR-mediated stimulation. The 3T3-BMMCs and BMMCs were stimulated for several hours in the presence of anti-FcγRII/III (2.4G2) and graded amounts of secondary antibody for FcγRIII cross-linking. We measured the amount of IL-4 and TNF-α in culture medium with ELISA at 12 and 3 h, respectively, after cross-linking. The results clearly demonstrated no defects in TNF-α and IL-4 induction in Lyn-deficient 3T3-BMMCs (Fig. 5) as well as in BMMCs (data not shown), indicating that the signaling pathway downstream of FcγRIII is linked to cytokine production independently of Lyn activation in mast cells. The paralleled experiments using 3T3-BMMCs and BMMCs with FcεRI cross-linking also gave rise to the results indicating no defect in cytokine induction (data not shown). These findings sharply contrast with the attenuated induction of immediate phase responses in Lyn-deficient mice and BMMCs as shown in Figs. 2 and 3, suggesting the distinctive signaling pathways associated with immediate and late phase responses of mast cells.

Normal FcγRIII Function for Reverse Cutaneous Arthus Reaction in Lyn-deficient Mice. The *in vitro* results for Lyn-independent cytokine induction prompted us to examine the role of Lyn in reverse-passive Arthus reaction. It has been shown that this model represents an immune complex-triggered mode of inflammation *in vivo*, and that its onset in the murine model largely depends on FcγRIII-mediated mast cell activation (3, 4, 15, 16, 19). TNF-α is known to play a pivotal role in recruitment of neutrophils

in mast cell-dependent cutaneous injury (43, 44). We examined the onset of reverse-passive Arthus reaction in Lyn-deficient mice after the treatment with intradermal injections of anti-OVA rabbit IgG followed by OVA challenge through the tail vein. Diseased reaction was evaluated by these three means: (a) histopathological feature; (b) MPO activity *in situ* at the late inflamed phase (8 h), which paral-

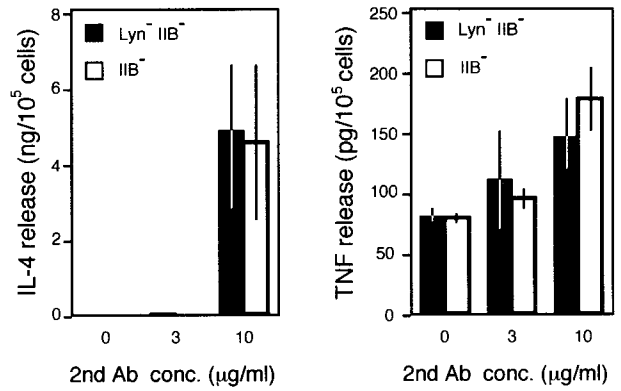


Figure 5. Cytokine release from Lyn^{-/-}IIB^{-/-} and control (IIB^{-/-}) 3T3-BMMCs (10⁵) evoked by FcγRIII aggregation. BMMCs were stimulated the same way as in the degranulation assay except for the time point for sample harvest. Culture supernatants at 12 or 3 h after stimulation were used for measurement of IL-4 (left) or TNF-α (right) release, respectively. Each column represents the mean ± SD of IL-4 (ng/10⁵ cells) and TNF-α (pg/10⁵ cells) obtained from triplicate assays.

leled the extent of PMN infiltration; and (c) TNF- α release at the initial phase (3 h). We first showed that Lyn-deficient mice as well as control mice display inflammatory tissue damage with massive neutrophil infiltration in the challenged sites (Fig. 6 A). Consistently, comparable induction of MPO activity in situ was detected in these two strains of mice (Fig. 6 B). We next examined increase of TNF- α production at the initial phase of the Arthus reaction in the challenged sites (dorsal skin and ear). Although the results from two different lesions are somewhat unparallel, there was found to be no statistic significance in the TNF- α production between Lyn-deficient and control mice (Fig. 6 C). This finding cannot affirm the intact TNF- α production in Lyn-deficient mice; however, it may mean that in vivo mechanisms for TNF- α production are not affected as much as mechanisms for the immediate phase reaction in Lyn-deficient mice. Collectively, these findings indicate

that activation of the Lyn-independent signaling pathway downstream of Fc γ RIII is sufficient for initiating the reverse-passive Arthus reaction, suggesting that Lyn is not necessary for the onset of late phase responses of mast cells.

Discussion

The Fc γ RIIIB deficiency made it possible to examine Fc γ RIII function in mast cell activation, whereas Lyn deficiency provided us with an opportunity to reevaluate the nature of signaling mechanisms leading to various types of immune responses in vivo. By introducing both deficiencies in mice, we investigated the nature of Fc γ RIII function in relation to mast cell-dependent pathogenesis. The notable points of the present findings are summarized as follows. (a) Lyn is required for intact induction of immediate phase responses as revealed in the degranulation response and systemic anaphylaxis (Figs. 1–3). Since Lyn is unlikely to be involved in mechanisms for FcR expression and maturation of mast cell in vivo (Fig. 4), the defect in immediate phase responses in Lyn-deficient mice can be interpreted as a consequence of the defect in signal transduction downstream of FcRs. (b) In contrast, Lyn is dispensable for late phase responses as revealed in cytokine induction and the reverse-passive Arthus reaction (Figs. 5 and 6). These contrasting results imply that Lyn-dependent and -independent signaling pathways downstream of Fc γ RIII underlie the onset of the anaphylactic response and the Arthus reaction, respectively. Consistent with our present results, two independent studies have demonstrated that Lyn-deficient mice do not display passive cutaneous anaphylaxis triggered by IgE plus antigen (23), while Lyn-deficient BMMCs were capable of activating such cytokine genes as IL-4, IL-5, IL-6, and TNF- α in response to Fc ϵ R1 aggregation (26). Thus, it is suggested that Fc γ RIII and Fc ϵ R1 commonly involve Lyn-dependent and -independent modes of signal transduction in triggering immediate and late phase responses of mast cells in vivo, respectively.

It has been well documented that Lyn is physically and functionally associated with cell surface receptors such as B cell receptor (22, 45, 46), Fc ϵ R1, Fc γ RIII on mast cells (47), IL-2 receptor (48), and GM-CSF receptor (49, 50). However, current findings have directed our interest towards molecular mechanisms for a Lyn-independent signaling pathway in mast cells. Previous studies have delineated signaling events required for cytokine gene activation upon Fc ϵ R1 aggregation. In those studies, inactivation of Btk (51), cyclosporin A-sensitive calcium-dependent pathway (52), phosphatidylinositol 3-kinase (53, 54), protein kinase C β (55), or c-Jun NH $_2$ -terminal kinase (JNK)-related pathway (56, 57), respectively, caused attenuation of cytokine gene activation. Accordingly, the Lyn-independent pathway may involve activation of these diverse signaling molecules in mast cells as well as the Lyn-dependent pathway. Notably, the requirement of Btk for cytokine induction suggests the presence of a tyrosine kinase other than Lyn upstream of Btk, because activation of Btk requires another tyrosine kinase such as Lyn to phosphorylate Btk

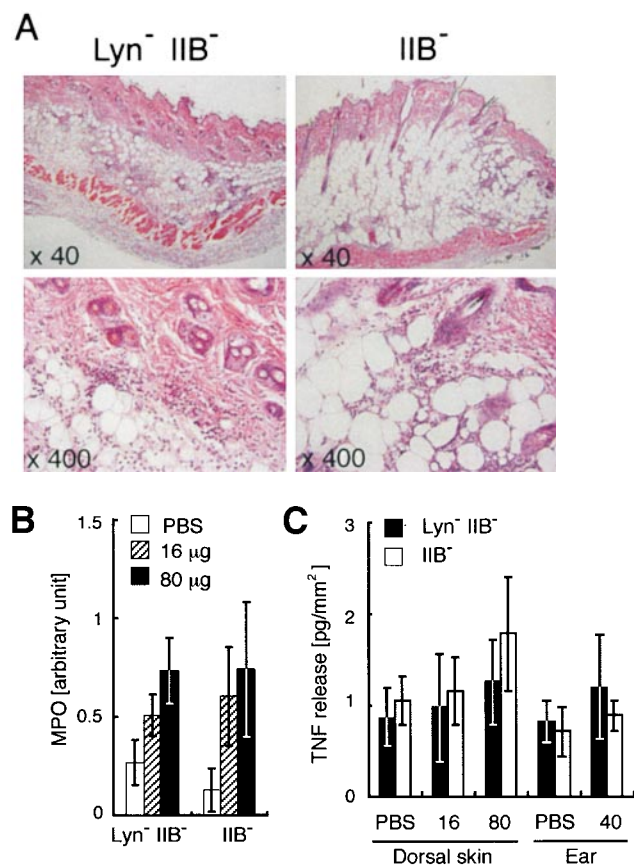


Figure 6. Reverse-passive Arthus reaction in Lyn^{-/-}IIB^{-/-} and control (IIB^{-/-}) mice evoked by IgG immune complexes. Each mouse was treated with intradermal injection of 0 (PBS), 16, or 80 μ g of rabbit anti-OVA IgG per site followed by intravenous injection of 1 mg of OVA in 0.2 ml saline. (A) Photomicrographs of a representative lesion of the Arthus reaction at 8 h after antigen challenge. Hematoxylin and eosin staining. Original magnifications: $\times 40$ or $\times 1,000$ as indicated in each photomicrograph. (B) MPO activity in a skin lesion at 8 h after antigen challenge. Mean \pm SD of five mice is indicated. One unit represents MPO activity in 0.25 μ l of whole blood sample. (C) Amount of TNF- α released in skin lesions at 3 h after antigen challenge. Each column represents the mean \pm SD of TNF- α density (pg/mm 2) in skin lesions obtained from five mice.

(58–60). In fact, Nishizumi and Yamamoto have demonstrated the activation of c-Src kinase on FcεRI aggregation in Lyn-deficient mast cells, and suggested a compensatory mechanism for cytokine induction in place of Lyn (26). However, it remains to be determined whether c-Src is functionally sufficient for cytokine induction or not. Here, we again support the presence of an undetermined tyrosine kinase as a mechanism of Lyn-independent signal transduction that sufficiently functions for cytokine induction. The mechanisms for time-constrained mast cell activation, which leads to immediate and late phase responses, may be attributed to the differential natures of Lyn and this putative tyrosine kinase.

There have been two conflicting results reported regarding IgE-dependent anaphylaxis in Lyn-deficient mice. Hibbs and Dunn have shown that Lyn-deficient mice displayed a defect in the induction of passive cutaneous anaphylaxis by IgE (21). On the other hand, Nishizumi and Yamamoto have mentioned that Lyn-deficient mice were capable of developing passive cutaneous anaphylaxis by IgE, and that Lyn-deficient BMMC indeed displayed no defect in degranulation in response to FcεRI aggregation (26). In our current results, Lyn-deficient mice failed to exhibit passive systemic anaphylaxis, whereas Lyn-deficient BMMCs were found to be responsive to high dose stimulation for FcεRI. In conclusion, the Lyn-dependent pathway serves as the immediate type of mast cell activation in vivo in response to low dose, probably biologically feasible, FcR-mediated stimulation.

Our findings indicate the presence of a Lyn-independent signaling mechanism in mast cells, and its involvement in cytokine induction and immune complex-triggered immunity represented by the Arthus reaction. As a possible mechanism for Lyn-independent signal transduction, a tyrosine kinase other than Lyn might be postulated for the signaling events. This notion may provide a novel therapeutic approach to allergy, in which targeted blocking of Lyn in mast cells can selectively suppress immediate-type hypersensitivities without affecting a common, immune complex-triggered immunity.

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