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Monomer hapten and hapten-specific IgG inhibit mast cell activation evoked by multivalent hapten with different mechanisms

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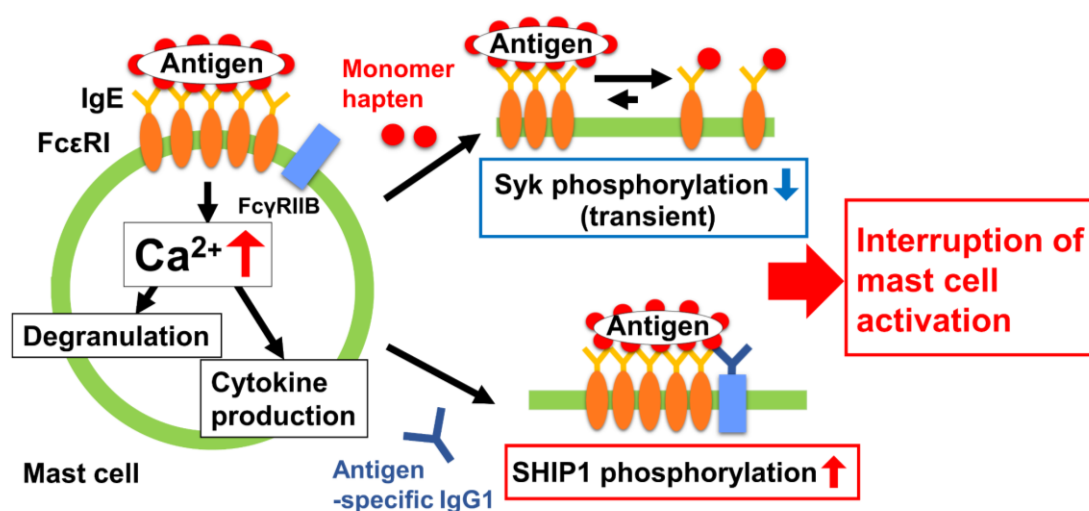
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(ABSTRACT)

Aggregation of IgE bound to high affinity IgE receptor (FcεRI) by multivalent antigen induces mast cell activation. Reportedly, disaggregation of aggregated FcεRI immediately terminated degranulation, and formation of co-ligated FcεRI and low affinity IgG receptor FcγRIIB blocked degranulation by inhibitory signal via SH2-containing inositol 5'-phosphatase 1 (SHIP1) phosphorylation. However, their molecular mechanisms to inhibit mast cell activation have been unclear in detail. Herein, we found that addition of excess monomeric hapten (TNP-alanine) to multivalent antigen (TNP-OVA)-activated rat basophilic leukemia cells and mouse bone marrow-derived mast cells induced immediate and transient Syk dephosphorylation, which was previously phosphorylated by TNP-OVA addition. Syk dephosphorylation correlated to rapidly decreased intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$), terminated degranulation, and suppressed cytokine production through inhibition of Akt and ERK phosphorylation. Addition of hapten-specific IgG monoclonal antibody (anti-TNP IgG1) to activated mast cells induced translocation of SHIP1 to the plasma membrane and its phosphorylation, indicating that co-ligation of FcεRI and FcγRIIB after FcεRI aggregation can lead to SHIP1 activation. SHIP1 phosphorylation led to gradually decreased $[\text{Ca}^{2+}]_i$, weak inhibition of degranulation, and strong inhibition of cytokine production. Our findings clearly show the inhibitory mechanism of cell function in activated mast cells by operating Fc receptor crosslinking.

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Dissociation of aggregated FcεRI by monomer hapten induces transient dephosphorylation of phosphorylated Syk. Coligation of aggregated FcεRI with FcγRIIB by hapten-specific IgG leads to SHIP1 phosphorylation. Those changes in formation of aggregated FcεRI trigger interruption of mast cell activation.



Introduction

Interaction between multivalent antigen and high affinity receptor for IgE (FcεRI)-bound IgE on the plasma membrane is an essential step for mast cell activation. FcεRI is expressed as a tetrameric receptor composed of the IgE-binding α chain, tetra-spanning β chain, and disulfide-linked homodimeric γ chains [1]. FcεRI crosslinking by multivalent antigen initiates activation of Src family tyrosine kinase cascades, including Lyn and Fyn, that cause degranulation and cytokine production to induce allergic reactions [2, 3]. FcεRI associates with Lyn, which phosphorylates tyrosine residues in immunoreceptor tyrosine-based activation motifs (ITAMs) in β and γ chains upon FcεRI crosslinking. Further, phosphorylated Lyn induces phosphorylation of Syk tyrosine kinase, which subsequently activates several adaptor proteins, e.g., linker of activated T cells (LAT) 1 and 2. Phosphorylated LAT1 leads to association and phosphorylation of phospholipase Cγ (PLCγ), which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) to form inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). Then, IP₃ binds to its receptor (IP₃R) on the endoplasmic reticulum (ER) membrane and triggers Ca²⁺ release from the ER, and the decreased Ca²⁺ concentration in the ER subsequently induces an extracellular Ca²⁺ influx into the cytoplasm via a STIM–Orai interaction. DAG is involved in activating protein kinase C (PKC), which is essential in mast cell activation by cooperation with increased intracellular Ca²⁺ concentration ([Ca²⁺]_i). In addition to Lyn, Fyn is a positive regulator of mast cell activation and activates a complementary cascade to Lyn. Phosphorylated Fyn activates PI3K through the adaptor protein Gab2. Phosphorylated PI3K induces Akt activation and PIP₂ phosphorylation to produce phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃). These cytosolic signaling cascades finally activate NF-κB, NFAT, and ERK that translocate to the nucleus from the cytoplasm. Consequently, NF-κB, NFAT, and ERK-dependent transcription factor Fos increase the transcriptional activity of genes of inflammatory cytokines (tumor necrosis factor (TNF)-α and interleukin (IL)-4) in the nucleus.

Hapten-conjugated proteins have generally been used as multivalent antigens to aggregate monoclonal IgE bound to FcεRI on mast cells. Reportedly, larger oligomers of IgE are effective in mast cell activation [4, 5], whereas multivalent antigen results in bell-shaped dose-response curves for mast cell activation [6, 7]. Multivalent antigen induces β and γ chain phosphorylation in FcεRI, IP₃ generation, and histamine secretion. The subsequent addition of

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excess monovalent hapten, which leads to dissociation of aggregated FcεRI, returns FcεRI phosphorylation and IP₃ generation to basal levels and arrests histamine secretion in activated mast cells [8-11]. Thus, aggregated FcεRI is intimately involved in intracellular signal transduction in mast cells.

Several negative regulators modulate intracellular signaling in mast cells. When FcεRI is co-ligated with low affinity IgG receptor FcγRIIB, Lyn-mediated phosphorylation of tyrosine residues in immunoreceptor tyrosine-based inhibition motifs (ITIMs) in FcγRIIB occurs [12], followed by SHIP1 recruitment by phosphorylated FcγRIIB. Then, activated SHIP1 dephosphorylates PI(3,4,5)P₃ generated by PI3K to produce PI(3,4)P₂ and dampens intracellular activation signaling in mast cells. The immune complex composed of hapten-specific IgG and hapten-conjugated proteins inhibits sustained [Ca²⁺]_i increase and degranulation by SHIP1 phosphorylation for mast cells sensitized with hapten-specific IgE [13, 14]. Further, bone marrow-derived mast cells (BMMCs) from SHIP1 knockout mice show enhanced extracellular Ca²⁺ entry and degranulation after multivalent antigen stimulation compared with those from wild type mice, indicating involvement of SHIP1 in moderating mast cell activation mediated by FcεRI aggregation [15]. Studies investigating inhibitory signaling through FcγRIIB have been conducted in both mast and B cells. Co-ligation of B cell receptor (BCR) with FcγRIIB was reported to repress cellular activation through tyrosine phosphorylation of FcγRIIB ITIM and SHIP1 recruitment to phosphorylated ITIM [16-18]. However, it remains unclear how the co-ligation between antigen receptors and FcγRIIB affects activation in cells already stimulated with multivalent antigens.

In this study, we re-evaluated the inhibitory effects of monomeric hapten and hapten-specific IgG on FcεRI-mediated mast cell activation such as degranulation and cytokine production and compared their suppression mechanisms in intracellular signaling. Our finding will be a useful clue to develop strategy to relieve symptoms of allergic diseases.

Results

Elimination of sustained increase in $[Ca^{2+}]_i$ by monomer hapten and hapten-specific IgG

Aggregation of FcεRI by hapten-conjugated proteins induced sustained increase of $[Ca^{2+}]_i$ to mast cells. To investigate how FcεRI disaggregation and inhibitory signaling from co-ligation of FcεRI and FcγRIIB affected $[Ca^{2+}]_i$, we measured $[Ca^{2+}]_i$ after the addition of monomeric hapten (trinitrophenyl (TNP)-alanine) or hapten-specific IgG (anti-TNP IgG1) to rat basophilic leukemia (RBL-2H3) cells and BMMCs stimulated with hapten-conjugated proteins (TNP-ovalbumin (OVA)). It is well known that multivalent antigen shows a dose-dependent bell-shaped curve for mast cell activation [6, 7]. Recently, co-ligation of FcεRI and FcγRIIB expressed on the plasma membrane is found to be involved in negative regulation of mast cell activation via increased SHIP1 phosphorylation by supra-optimal antigen stimulation [19]. To avoid the inhibitory effect at supra-optimal concentration, we first examined an optimal concentration of TNP-OVA to stimulate RBL-2H3 cells and BMMCs. Because the optimal concentration of TNP-OVA was determined as 1 and 0.1 μg/mL in RBL-2H3 cells and BMMCs (data not shown), respectively, the cells were stimulated with the optimal concentration of TNP-OVA in following experiments.

In RBL-2H3 cells, addition of TNP-OVA induced sustained increase of $[Ca^{2+}]_i$, which peaked at approximately 50 s after stimulation. The addition of immune complex composed of TNP-OVA and anti-TNP IgG1 led to a smaller and temporal increase of $[Ca^{2+}]_i$ (Fig 1A). When TNP-alanine or anti-TNP IgG1 was added 50 s after TNP-OVA stimulation, $[Ca^{2+}]_i$ rapidly or gradually decreased, respectively, and thereby returned to basal levels. Area under the curve (AUC) derived from the time course of $[Ca^{2+}]_i$ movement was significantly smaller upon subsequent addition of TNP-alanine or anti-TNP IgG1 and immune complex stimulation compared with TNP-OVA stimulation (Fig 1B). Notably, using non-specific IgG instead of anti-TNP IgG1, neither immune complex stimulation nor subsequent IgG addition inhibited the $[Ca^{2+}]_i$ increase, suggesting that addition of TNP-OVA followed by anti-TNP IgG1 formed the co-crosslinking of FcεRI and FcγRIIB to transduce the inhibitory signal. In BMMCs, the stimulation with TNP-OVA induced the sustained $[Ca^{2+}]_i$ increase and the subsequent addition

of TNP-alanine or anti-TNP IgG1 significantly decreased the $[Ca^{2+}]_i$ similar to RBL-2H3 cells (Fig 1C and 1D).

These results indicate that FcεRI disaggregation and co-ligation of FcεRI and FcγRIIB reduced $[Ca^{2+}]_i$ increase even after FcεRI aggregated in mast cells.

Effect of monomeric hapten and hapten-specific IgG on β-hexosaminidase release

We investigated the effect of the subsequent addition of TNP-alanine and anti-TNP IgG1 on β-hexosaminidase release mediated by TNP-OVA in RBL-2H3 cells. The amount of secreted β-hexosaminidase gradually increased 30 min after TNP-OVA stimulation. As previously reported [8, 9], addition of TNP-alanine at 50 s, 5 min, and 15 min after TNP-OVA stimulation completely discontinued β-hexosaminidase release (Fig 2A). Stimulation with the immune complex of TNP-OVA and anti-TNP IgG1 did not elicit β-hexosaminidase release, as reported previously [14]. However, unlike TNP-alanine, the subsequent addition of anti-TNP IgG1 induced partial suppression of β-hexosaminidase release (Fig 2B). These results suggest that disaggregation of FcεRI, but not co-ligation of FcεRI and FcγRIIB, induced an immediate complete blockade of degranulation mediated by FcεRI aggregation.

Difference between monomeric hapten and hapten-specific IgG in Syk and SHIP1 phosphorylation

Syk phosphorylation is a key event in mast cell activation mediated by FcεRI aggregation. Additionally, co-ligation of FcεRI and FcγRIIB by immune complex induced SHIP1 phosphorylation and inhibited mast cell degranulation [13]. Next, we investigated how the subsequent addition of TNP-alanine and anti-TNP IgG1 to mast cells activated with TNP-OVA affects the phosphorylated states of Syk and SHIP1.

Syk possesses a number of tyrosine residues phosphorylated according to FcεRI aggregation and each phosphorylated residue has specific function in mast cell signal

transduction [20, 21]. Here we investigated that the phosphorylation of Y519/Y520 (Y525/Y526 of human Syk) and Y317 (Y323 of human Syk), which exist in the activation loop of kinase domain [22] and the binding site for a negative signaling regulator Cbl [23, 24], respectively, because the phosphorylation of their tyrosine residues is known to be induced by FcεRI aggregation.

In RBL-2H3 cells, Syk phosphorylation (Y317) increased within 50 s and maintained for 5 min after TNP-OVA addition (Fig 3A). Interestingly, TNP-alanine addition at 50 s after TNP-OVA stimulation immediately dephosphorylated Syk. This dephosphorylation was transient, with phosphorylation levels recovered at 5 min. The anti-TNP IgG1 addition at 50 s after TNP-OVA stimulation, similar to the immune complex of TNP-OVA and anti-TNP IgG1, did not induce Syk dephosphorylation and showed a similar pattern as TNP-OVA. In BMMCs, Syk phosphorylation (Y519/Y520) also increased within 50 s and maintained for 5 min after TNP-OVA stimulation (Fig 3B). Additionally, TNP-alanine addition at 50 s after TNP-OVA stimulation presented a similar transient dephosphorylation pattern to Syk in RBL-2H3 cells.

SHIP1 also has tyrosine residues phosphorylated according to co-ligation of FcεRI and FcγRIIB. The phosphorylated Y1020 of mouse and rat SHIP1 is involved in interaction with adaptor proteins and is used as a marker of SHIP activity [25]. When we measured the phosphorylated Y1020 of SHIP1, the phosphorylation after immune complex addition was expectedly higher than TNP-OVA addition in RBL-2H3 cells (Fig 4A). The immune complex increased SHIP1 phosphorylation at 5 min, which was maintained for 15 min. Anti-TNP IgG1 addition at 50 s after TNP-OVA stimulation also induced SHIP1 phosphorylation, but not TNP-alanine addition. The increased SHIP1 phosphorylation by subsequent anti-TNP IgG1 addition after TNP-OVA stimulation was expectedly found in BMMCs (Fig. 4B).

Next, we observed the intracellular SHIP1 distribution in RBL-2H3 cells because SHIP1 is reportedly recruited to the plasma membrane where it is phosphorylated according to the co-ligation of BCR and FcγRIIB in B cells [18]. We found that SHIP1 was distributed in the cytoplasm of resting RBL-2H3 cells while it accumulated on the plasma membrane at 5 min after the immune complex and subsequent anti-TNP IgG1 addition, but not the TNP-OVA and subsequent TNP-alanine addition (Fig 5), suggesting that co-ligation of FcεRI and FcγRIIB led to plasma membrane recruitment and SHIP1 phosphorylation.

Effect of monomeric hapten and hapten-specific IgG on cytokine production

Because TNP-alanine and anti-TNP IgG1 suppressed degranulation in RBL-2H3 cells activated with TNP-OVA, we investigated the effect of the subsequent addition of TNP-alanine and anti-TNP IgG1 on cytokine production mediated by TNP-OVA. While the amount of produced TNF- α and IL-4 significantly increased via TNP-OVA stimulation for 3 h, immune complex addition induced neither TNF- α nor IL-4 production (Fig 6). Further, TNP-alanine or anti-TNP IgG1 addition at 50 s after TNP-OVA stimulation inhibited TNF- α and IL-4 production.

Further, we measured phosphorylation of Akt and ERK that are involved in cytokine production [26, 27]. TNP-OVA induced their phosphorylation, whereas the subsequent TNP-alanine and anti-TNP IgG1 addition at 50 s after TNP-OVA stimulation significantly decreased the phosphorylation levels of Akt and ERK, and similar findings were observed regarding the immune complex (Fig 7), suggesting that inhibition of cytokine production by subsequent TNP-alanine or anti-TNP IgG1 addition is due to Akt and ERK dephosphorylation.

In all experiments described above, the excess amount of TNP-OVA that does not bind to IgE is present in extracellular solution during stimulation. Here, to examine if anti-TNP IgG1 could lead to the inhibitory effect of cell activation in the absence of free TNP-OVA, we measured cytokine production according to anti-TNP IgG1 addition after washing out free TNP-OVA. When free TNP-OVA was washed out at 50 s after addition, we confirmed that the subsequent anti-TNP IgG1 decreased the both TNF- α (93.9 ± 5.0 % inhibition, $n = 3$, $P = 0.041$) and IL-4 (89.1 ± 6.7 % inhibition in IL-4, $n = 3$, $P = 0.016$) production for 3 h. This result suggested that anti-TNP IgG1 alone could bind to the Fc ϵ RI-IgE-TNP-OVA complex on the plasma membrane and suppress the Fc ϵ RI-mediated signal transduction.

Discussion

For over three decades, the relationship of FcεRI aggregation with mast cell activation has been investigated using hapten-conjugated proteins and hapten-specific IgE monoclonal antibody. The tyrosine phosphorylation of β and γ chains of FcεRI by Src family tyrosine kinases upon FcεRI aggregation was discovered in the early 1990s [10, 28, 29]. Subsequent monomeric hapten addition rapidly induces dephosphorylation of not only β and γ chains of FcεRI but also Lyn, Syk, and LAT phosphorylated by FcεRI aggregation [30, 31]. Recently, there has been an increase in knowledge regarding the intracellular signal transduction network from FcεRI aggregation in mast cells [2, 3, 32]. Co-ligation of FcεRI and FcγRIIB expressed on the plasma membrane of mast cells previously played a negative role in cellular activation through SHIP1 [12]. Similar to mast cells, B cells expressing BCR and FcγRIIB possess the Syk-dependent activation and SHIP1-mediated inhibition pathways by BCR aggregation and co-ligation of BCR and FcγRIIB, respectively [25, 33, 34]. SHIP functions to not only inhibit activation signals in immune cells but also regulate hyperactivity and resulting autoimmunity [25].

Herein, we compared the suppressive mechanism of FcεRI disaggregation and co-ligation of FcεRI and FcγRIIB on mast cell activation originally mediated by FcεRI aggregation following the addition of TNP-OVA at the optimal concentration. We showed that the disaggregation of FcεRI by excess TNP-alanine immediately decreased $[Ca^{2+}]_i$ (Fig 1) and rapidly terminated degranulation (Fig 2) through transient Syk dephosphorylation (Fig 3) in mast cells activated by TNP-OVA. The transient Syk dephosphorylation elicited Akt and ERK dephosphorylation (Fig 7) and thereby significantly inhibited TNF-α and IL-4 production (Fig 6). Our data suggest that Syk dephosphorylation, even if transient, in mast cell activation causes significant inhibition of cellular function.

In addition, co-ligation of FcεRI and FcγRIIB by anti-TNP IgG1 gradually decreased $[Ca^{2+}]_i$ (Fig 1) and partially suppressed degranulation (Fig 2) through SHIP1 recruitment to the plasma membrane and its phosphorylation (Figs 4 and 5) in mast cells activated with TNP-OVA. SHIP1 plasma membrane recruitment and phosphorylation also elicited Akt and ERK dephosphorylation (Fig 7) and thereby induced complete inhibition of TNF-α and IL-4 production (Fig 6). In previous studies, an immune complex of hapten-conjugated proteins and hapten-specific IgG was used to co-ligate FcεRI and FcγRIIB for mast cells sensitized with

hapten-specific IgE. In the data presented here, even in the case wherein FcεRI was aggregated by hapten-conjugated proteins, the subsequent hapten-specific IgG addition reduced $[Ca^{2+}]_i$ (Fig 1) and induced complete inhibition of cytokine production (Fig 6) in mast cells, probably due to the co-crosslinking of FcεRI and FcγRIIB via the binding of IgG to FcεRI-IgE-antigen complex on the plasma membrane.

A novel mechanism of inhibition of mast cell responses was proposed as trans-inhibition [36]. In trans-inhibition, receptors recruiting sufficient amounts of SHIP1 can inhibit FcεRI-mediated mast cell activation, whereas co-ligation of FcεRI and FcγRIIB blocks mast cell activation in cis-inhibition, as well-known previously. Our results suggest that FcεRI dissociation and co-ligation of FcεRI and FcγRIIB following FcεRI aggregation completely blocked degranulation and cytokine production, which are major cellular outputs in the early and late phases, respectively, in immediate allergy elicited by antigen-activated mast cells. The difference is possibly due to the responsiveness in the dissociation of preformed FcεRI aggregates and new formation of FcεRI and FcγRIIB co-ligation. Because the formation of FcεRI and FcγRIIB co-ligation may gradually occur with low reversibility and trigger a novel signaling pathway, the plasma membrane recruitment and phosphorylation of SHIP1 are considered to be maintained. This inhibitory process is considered to be classified as trans-inhibition. The lasting SHIP1 activation probably affects the inhibition of cytokine production in the late phase rather than the degranulation in the early phase. However, we presume that the dissociation of aggregated FcεRI via excess hapten monomers rapidly occurs with high reversibility and influences the former activating signaling pathways with high sensitivity. When the equilibrium status of FcεRI aggregation on the plasma membrane is shifted toward dissociation, the intracellular signaling proteins anchored to the plasma membrane possibly sense FcεRI disaggregation and terminate signal transduction. Syk dephosphorylation is rapidly induced, with Syk phosphorylation levels recovering after several minutes. Transient Syk dephosphorylation in the activation process probably leads to immediate inhibition of $[Ca^{2+}]_i$ and degranulation in the early phase rather than cytokine production in the late phase.

We herein propose that the temporal dephosphorylation of Syk and continuous phosphorylation of SHIP1 are primary inhibitory events due to dissociation of FcεRI and co-ligation of FcεRI and FcγRIIB, respectively, in mast cells activated by FcεRI aggregation (Fig 8). We investigated the inhibitory effect of cellular function and its molecular mechanism

in mast cells. FcγRIIB has been used as target to inhibit allergic reaction in vivo. Several kinds of fusion proteins to induce co-ligation of FcεRI and FcγRIIB on resting mast cells actually succeeded to suppress cutaneous anaphylaxis reaction in experimental animals [37-39]. If FcεRI disaggregation can similarly inhibit degranulation in mast cells activated with multivalent allergens in the body, the development of reagents that dissociate aggregated IgE by strongly binding to allergen-specific IgE may be useful in alleviating acute allergic symptoms. Additionally, the allergen-specific IgG monoclonal antibody may be a prime candidate in relieving allergic symptoms, particularly in chronic inflammatory diseases. Therefore, understanding inhibition of mast cell activation due to regulation of Fc receptor crosslinking may provide novel insight in the development of viable options to relieve allergies.

Materials and methods

Reagents

Fluo 4-AM and TNP-alanine were obtained from Dojindo (Kumamoto, Japan) and Research Organics (Cleveland, OH, USA), respectively. Mouse hybridoma cells to produce anti-DNP IgE monoclonal antibody (IgE-53-569), which cross-reacts to the TNP group, were provided by Prof. T. Kishimoto (Osaka University, Suita, Japan). We obtained ascites including abundant anti-DNP IgE monoclonal antibody from mice administrated the hybridoma cells in abdominal cavity and used the diluted ascites to sensitize mast cells after verifying the optimal dilution rate to evoke cell response ($[Ca^{2+}]_i$ increase and degranulation) against antigen. TNP-OVA and mouse anti-TNP IgG1 monoclonal antibody were prepared as previously described [14]. In this study, an average of 21 TNP groups was conjugated to a single OVA molecule. Rabbit anti-phospho-Syk(Y323) antibody, rabbit anti-phospho-Syk(Y525/Y526) antibody, rabbit anti-phospho-SHIP1(Y1020) antibody, rabbit anti-phospho-Akt(T308) antibody, rabbit anti-phospho-ERK1/2(T202/Y204) antibody, horse radish peroxidase (HRP)-labeled goat anti-rabbit IgG antibody, and HRP-labeled goat anti-mouse IgG antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Further, rabbit

anti-SHIP1 antibody was purchased from Proteintech Group (Chicago, IL, USA), Mouse anti- β -actin antibody and Alexa Fluor 555-labeled goat anti-rabbit IgG antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Molecular Probes (Cambridge Cambridgeshire, UK), respectively. Rat TNF- α and rat IL-4 immunoassay systems were obtained from R&D Systems (Minneapolis, MN, USA).

Cell culture

RBL-2H3 cells were cultured in an atmosphere of 5% CO₂ at 37°C in minimum essential medium (MEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (Roche, Mannheim, Germany). BMMCs were obtained by culturing bone marrow cells from 8 week-old BALB/c mice (Japan SLC, Shizuoka, Japan) in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FCS, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 ng/mL recombinant murine IL-3 (Peprotech, London, UK), and 2 ng/ml recombinant murine stem cell factor (Peprotech) [40]. After 4 weeks of culture, >95% of the viable cells were confirmed to be mast cells, as assessed by staining with acidic toluidine blue. BALB/c mice were housed under standard conditions and in compliance with Nagoya City University and Aichi Gakuin University guidelines for the use of experimental animals, and all animal experiments were performed with approval by the Animal Care and Use Committee of Nagoya City University and Aichi Gakuin University.

Measurement of [Ca²⁺]_i

RBL-2H3 cells (1×10^6 cells) were loaded with anti-DNP IgE (mouse ascites; 1:50000) and Fluo 4-AM (1 μ M) for 20 min at 37°C and then washed twice with Hepes buffer (10 mM Hepes [pH 7.2], 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1 mM CaCl₂, 0.1% glucose, 0.1% bovine serum albumin, and 0.01% sulfinpyrazone). BMMCs (5×10^5 cells) were loaded with anti-DNP IgE (mouse ascites; 1:2500) for 30 min and Fluo 4-AM (1 μ M) for 30 min at 37°C

and then washed twice with Hepes buffer. Fluorescence intensity of Fluo 4 was measured by a spectrofluorometer (RF-5300PC; Shimadzu, Kyoto, Japan) with excitation and emission wavelengths of 488 and 525 nm, respectively, at 37°C. Data (F/F_0) were obtained by dividing fluorescence intensity (F) by (F_0) at resting level ($t = 0$) [41].

β-Hexosaminidase secretion

Degranulation of RBL-2H3 cells was monitored by measuring activity of the granule-stored enzyme β-hexosaminidase secreted into cell supernatants. The cells (5×10^4 cells/well) were plated in 24-well plates. The following day, the cells were sensitized with anti-DNP IgE (1:50000) for 10 min at 37°C, monolayers were washed with Hepes buffer, and incubated with stimulants at 37°C. Following incubation, supernatant aliquots were transferred to 96-well plates (20 μL/well) and incubated with 20 μL substrate solution (2 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide [Sigma-Aldrich] in 100 mM citrate buffer [pH 4.5]) for 1 h at 37°C. After terminating the reaction using 167 mM Na₂CO₃-NaHCO₃ buffer (160 μL), absorbance at 405 nm was measured using a microplate reader (model 680, Bio-Rad, Hercules, CA, USA). Release activity relative to total cellular β-hexosaminidase content was determined by disrupting cells with 0.1% Triton X-100 [41, 42]. Each assay was performed in triplicate.

Western blot analysis

Western blotting analysis was performed using previously described procedures [42, 43]. RBL-2H3 cells (5×10^5 cells/dish) were plated in 60-mm dishes. The following day, the cells were sensitized with anti-DNP IgE (1:50000) for 10 min at 37°C, monolayers were washed with Hepes buffer, and incubated with stimulants at 37°C. BMMCs (5×10^5 cells) were sensitized with anti-DNP IgE (1:2500) for 30 min at 37°C and washed with Hepes buffer, and incubated with stimulants at 37°C. To prepare whole cell lysates, the cells were collected using a rubber policeman, centrifuged at 1,000 rpm for 3 min, and suspended in cold lysis buffer (20

mM Hepes [pH 7.9], 0.1% NP-40, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Na_3VO_4 , 10 $\mu\text{g/mL}$ phenylmethylsulfonyl fluoride, 10 $\mu\text{g/mL}$ leupeptin, and 10% glycerol). Then, the lysates were maintained on ice for 30 min, followed by centrifugation at 15,000 rpm for 20 min at 0°C. The resulting supernatants were solubilized by treatment with Laemmli buffer for 3 min at 100°C. Subsequently, proteins were separated using 8% SDS-PAGE. Further, the separated proteins were transferred to a PVDF membrane with an electroblotter (Bio-Rad). After blocking with Blocking One (Nakalai, Tokyo, Japan), membranes were probed with rabbit anti-phospho-Syk(Y323) antibody (1:2000), rabbit anti-phospho-Syk(Y525/Y526) antibody (1:2000), rabbit anti-phospho-SHIP1(Y1020) antibody (1:2000), rabbit anti-phospho-Akt(T308) antibody (1:2000), rabbit anti-phospho-ERK1/2(T202/Y204) antibody (1:2000), and anti- β -actin antibody (1:40000) and treated with HRP-labeled goat anti-rabbit IgG antibody (1:2000) or HRP-labeled goat anti-mouse IgG antibody (1:4000). Lastly, immunoreactivity was detected by ECL (GE Healthcare, Buckinghamshire, UK) with a LAS-3000 mini (Fuji Film, Tokyo, Japan) and analyzed by Image Gauge (Fuji Film).

Confocal laser scanning microscopy

RBL-2H3 cells (1×10^4 cells/chamber) were plated on an observation chamber (Elecon, Chiba, Japan). The following day, the cells were sensitized with anti-DNP IgE (1:50000) for 10 min at 37°C, monolayers were washed with Hepes buffer, and incubated with stimulants at 37°C. The cells were washed thrice with PBS, immediately fixed with ice-cold 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 for 10 min. Then, the cells were incubated with rabbit anti-SHIP1 antibody (1:100) overnight at 4°C. Subsequently, treatment with Alexa Fluor 555-labeled goat anti-rabbit IgG antibody (1:400) was performed. Distribution of SHIP1 was analyzed using a confocal laser scanning microscope (LSM800; Zeiss, Oberkochen, Germany).

Cytokine production

RBL-2H3 cells (1×10^5 cells) were plated in 24-well plates. The following day, the cells were sensitized with anti-DNP IgE (1:50000) for 10 min at 37°C, monolayers washed with Hepes buffer, and incubated with stimulants for 3 h at 37°C. Supernatants were collected, and absolute amounts of TNF- α and IL-4 released by the cells were determined using rat TNF- α and rat IL-4 immunoassay systems, respectively, according to the manufacturer's protocol [40, 44].

Statistical analysis

One-way analysis of variance (ANOVA) and Steel–Dwass test were used to compare group differences. Results were considered statistically significant at $P < 0.05$.

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Conflict of interest statement

The authors have no financial conflicts of interest.

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(Figure legends)

Figure 1. $[Ca^{2+}]_i$ movement in mast cells. (A) Representative time courses of $[Ca^{2+}]_i$ movement after addition of TNP-OVA (1 $\mu\text{g/mL}$), TNP-OVA (1 $\mu\text{g/mL}$) + anti-TNP IgG1 (3 $\mu\text{g/mL}$; 50 s later), TNP-OVA (1 $\mu\text{g/mL}$) + TNP-alanine (12 $\mu\text{g/mL}$; 50 s later), and immune complex composed of TNP-OVA (1 $\mu\text{g/mL}$) and anti-TNP IgG1 (3 $\mu\text{g/mL}$) in RBL-2H3 cells. Fluorescence intensity of Fluo 4 was measured by a spectrofluorometer with excitation and emission wavelengths of 488 and 525 nm, respectively, at 37°C. Data (F/F_0) were shown by dividing fluorescence intensity (F) by (F_0) at resting level ($t = 0$). (B) Comparison of AUC calculated from time courses of $[Ca^{2+}]_i$ movement (0–450 s) stimulated with TNP-OVA (1 $\mu\text{g/mL}$), TNP-OVA (1 $\mu\text{g/mL}$) + anti-TNP IgG1 (3 $\mu\text{g/mL}$; 50 s later), and TNP-OVA (1 $\mu\text{g/mL}$) + TNP-alanine (12 $\mu\text{g/mL}$; 50 s later), immune complex composed of TNP-OVA (1 $\mu\text{g/mL}$) and anti-TNP IgG1 (3 $\mu\text{g/mL}$), TNP-OVA (1 $\mu\text{g/mL}$) + non-specific IgG (3 $\mu\text{g/mL}$; 50 s later), and immune complex composed of TNP-OVA (1 $\mu\text{g/mL}$) and non-specific IgG (3 $\mu\text{g/mL}$) in RBL-2H3 cells. (C) Representative time courses of $[Ca^{2+}]_i$ movement after addition of TNP-OVA (0.1 $\mu\text{g/mL}$), TNP-OVA (0.1 $\mu\text{g/mL}$) + anti-TNP IgG1 (3 $\mu\text{g/mL}$; 50 s later), and TNP-OVA (0.1 $\mu\text{g/mL}$) + TNP-alanine (12 $\mu\text{g/mL}$; 50 s later) in BMMCs. (D) Comparison of AUC calculated from time courses of $[Ca^{2+}]_i$ movement (0–450 s) stimulated with TNP-OVA (0.1 $\mu\text{g/mL}$), TNP-OVA (0.1 $\mu\text{g/mL}$) + anti-TNP IgG1 (3 $\mu\text{g/mL}$; 50 s later), and TNP-OVA (0.1 $\mu\text{g/mL}$) + TNP-alanine (12 $\mu\text{g/mL}$; 50 s later) in BMMCs. Arrows indicate the time of TNP-alanine and anti-TNP IgG1 addition. Results are expressed as mean $\pm$ SE from ≥ 3 independent experiments. *** $P < 0.001$ compared with TNP-OVA by ANOVA.

Figure 1

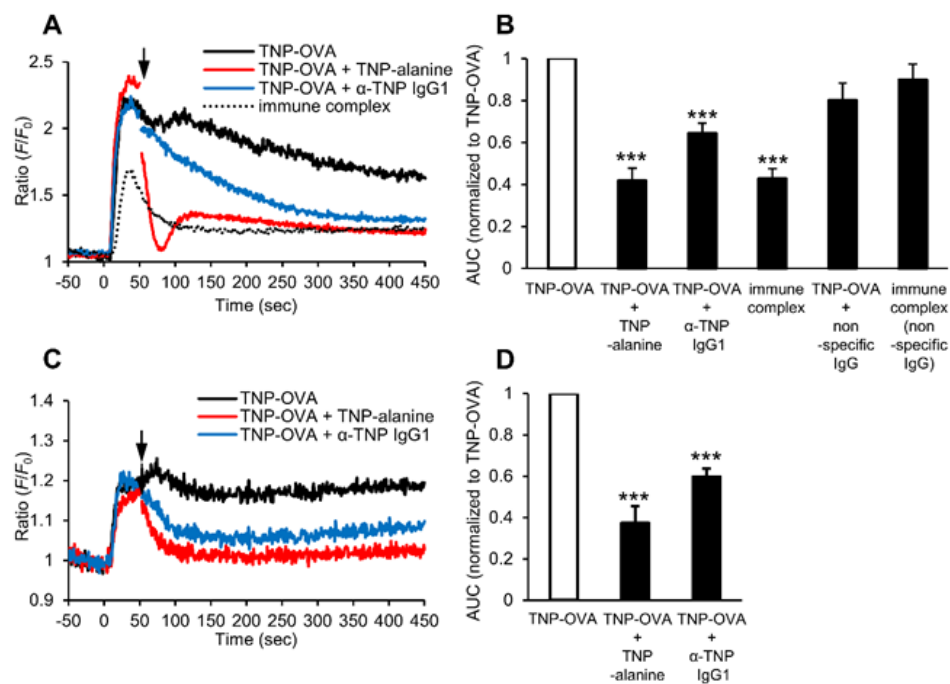


Figure 2. Time course of β -hexosaminidase release from RBL-2H3 cells. (A) Amount of released β -hexosaminidase for 30 min when TNP-alanine (12 $\mu\text{g/mL}$) was added at 50 s, 5 min, and 15 min after TNP-OVA stimulation (1 $\mu\text{g/mL}$; $\bullet$). (B) Amount of released β -hexosaminidase for 30 min when anti-TNP IgG1 (3 $\mu\text{g/mL}$) was added at 50 s, 5 min, and 15 min after TNP-OVA stimulation (1 $\mu\text{g/mL}$; $\blacktriangle$). Immune complex composed of TNP-OVA (1 $\mu\text{g/mL}$) and anti-TNP IgG1 (3 $\mu\text{g/mL}$) was also used ($\blacksquare$). Arrows indicate the time of TNP-alanine and anti-TNP IgG1 addition. Results are expressed as mean $\pm$ SE from ≥ 5 independent experiments. *** and * $P < 0.001$ and 0.05, respectively, compared with TNP-OVA by ANOVA.

Figure 2

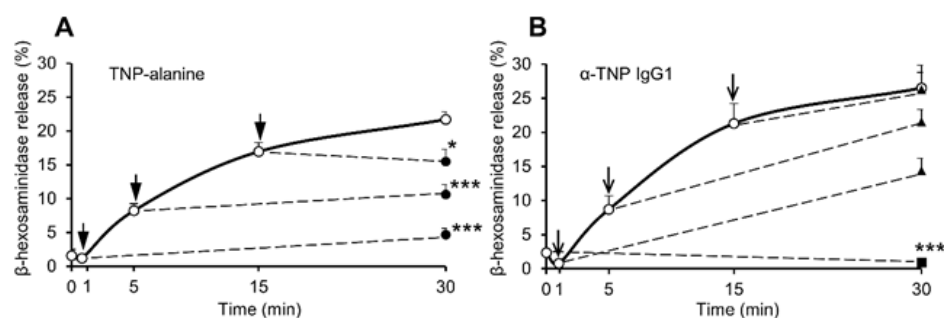


Figure 3. Time-course of Syk phosphorylation in mast cells. Phosphorylation levels of Syk were measured by Western blotting with anti-phospho-Syk(Y323) antibody, and anti-phospho-Syk(Y525/Y526) antibody. (A) RBL-2H3 cells were stimulated with TNP-OVA (1 μ g/mL), TNP-OVA (1 μ g/mL) + TNP-alanine (12 μ g/mL; 50 s later), TNP-OVA (1 μ g/mL) + anti-TNP IgG1 (3 μ g/mL; 50 s later), and immune complex composed of TNP-OVA (1 μ g/mL) and anti-TNP IgG1 (3 μ g/mL). (B) BMMCs were stimulated with TNP-OVA (0.1 μ g/mL), TNP-OVA (0.1 μ g/mL) + TNP-alanine (12 μ g/mL; 50 s later), and TNP-OVA (0.1 μ g/mL) + anti-TNP IgG1 (3 μ g/mL; 50 s later). Relative phosphorylation levels were normalized to resting cells after controlling for protein loading. β -actin served as the control. Results are expressed as mean $\pm$ SE from ≥ 6 independent experiments. ***, **, and * P < 0.001, 0.01, and 0.05, respectively, compared with TNP-OVA by Steel–Dwass test.

Figure 3

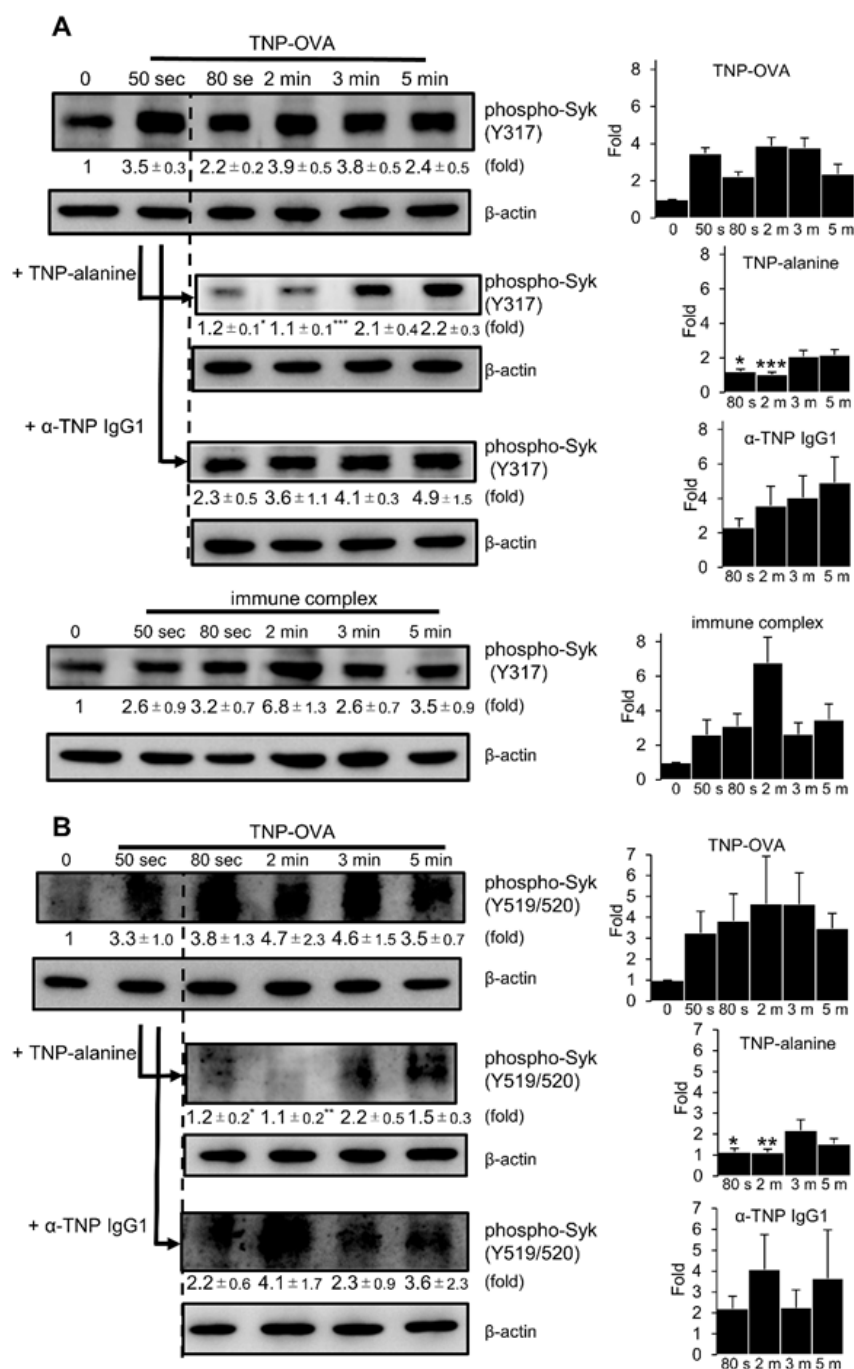


Figure 4. Time-course of SHIP1 phosphorylation in RBL-2H3 cells. Phosphorylation levels of SHIP1 were measured by Western blotting with anti-phospho-SHIP1(Y1020) antibody. (A) RBL-2H3 cells were stimulated with TNP-OVA (1 $\mu\text{g/mL}$), TNP-OVA (1 $\mu\text{g/mL}$) + TNP-alanine (12 $\mu\text{g/mL}$; 50 s later), TNP-OVA (1 $\mu\text{g/mL}$) + anti-TNP IgG1 (3 $\mu\text{g/mL}$; 50 s later), and immune complex composed of TNP-OVA (1 $\mu\text{g/mL}$) and anti-TNP IgG1 (3 $\mu\text{g/mL}$). (B) BMMCs were stimulated with TNP-OVA (0.1 $\mu\text{g/mL}$), TNP-OVA (0.1 $\mu\text{g/mL}$) + TNP-alanine (12 $\mu\text{g/mL}$; 50 s later), and TNP-OVA (0.1 $\mu\text{g/mL}$) + anti-TNP IgG1 (3 $\mu\text{g/mL}$; 50 s later). Relative phosphorylation levels were normalized to resting cells after controlling for protein loading. β -actin served as the control. Results are expressed as mean $\pm$ SE from ≥ 7 independent experiments. ***, **, and * $P < 0.001$, 0.01, and 0.05, respectively, compared with TNP-OVA by Steel–Dwass test.

Figure 4

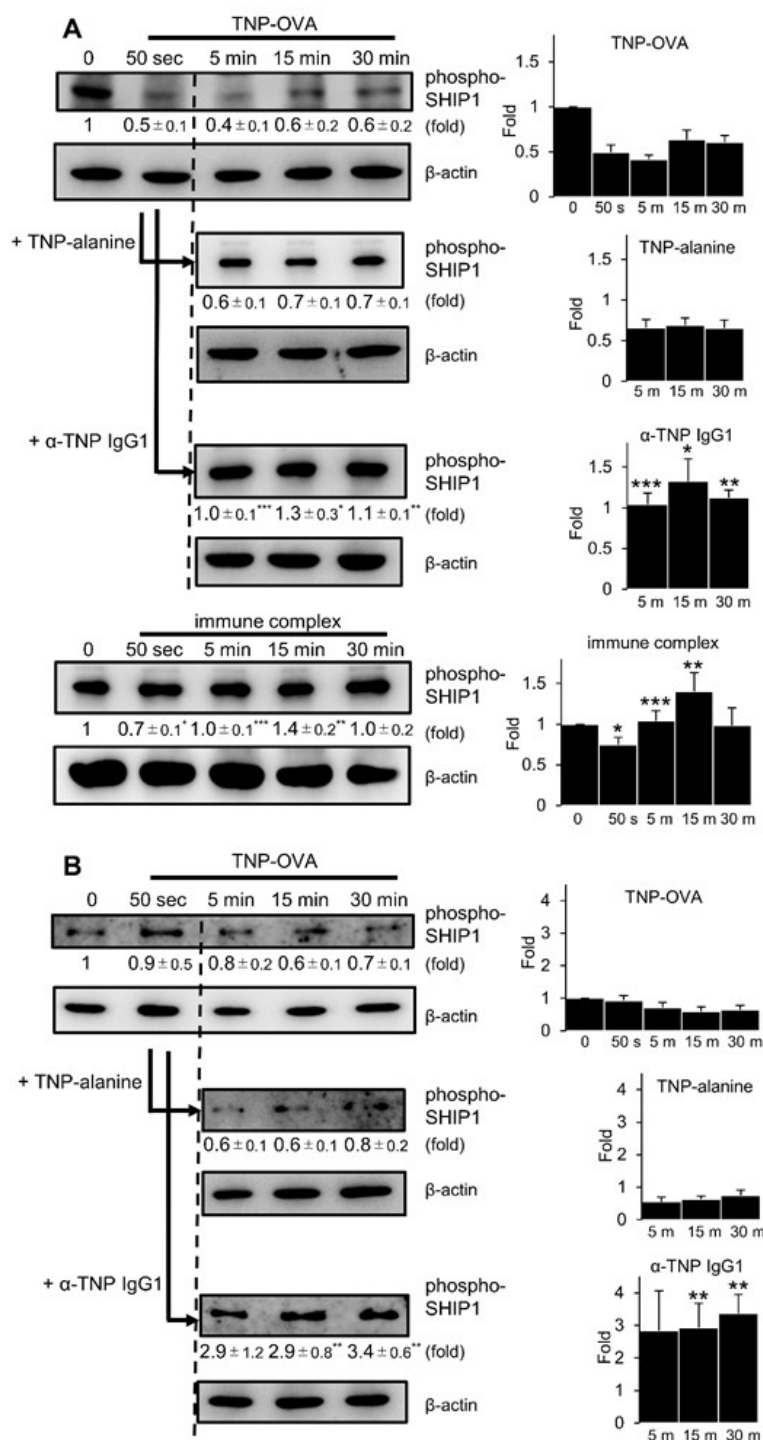


Figure 5. Intracellular distribution of SHIP1 in RBL-2H3 cells. Cells were treated with anti-SHIP1 antibody and Alexa Fluor 555-labeled anti-rabbit IgG antibody as a secondary antibody. Distribution of SHIP1 was analyzed using a confocal laser scanning microscope (LSM800; Zeiss).

Localization of SHIP1 in RBL-2H3 cells after 5 min of adding TNP-OVA (1 $\mu\text{g/mL}$), TNP-OVA (1 $\mu\text{g/mL}$) + TNP-alanine (12 $\mu\text{g/mL}$; 50 s later), TNP-OVA (1 $\mu\text{g/mL}$) + anti-TNP IgG1 (3 $\mu\text{g/mL}$; 50 s later), and immune complex composed of TNP-OVA (1 $\mu\text{g/mL}$) and anti-TNP IgG1 (3 $\mu\text{g/mL}$). Scale bars = 5 μm .

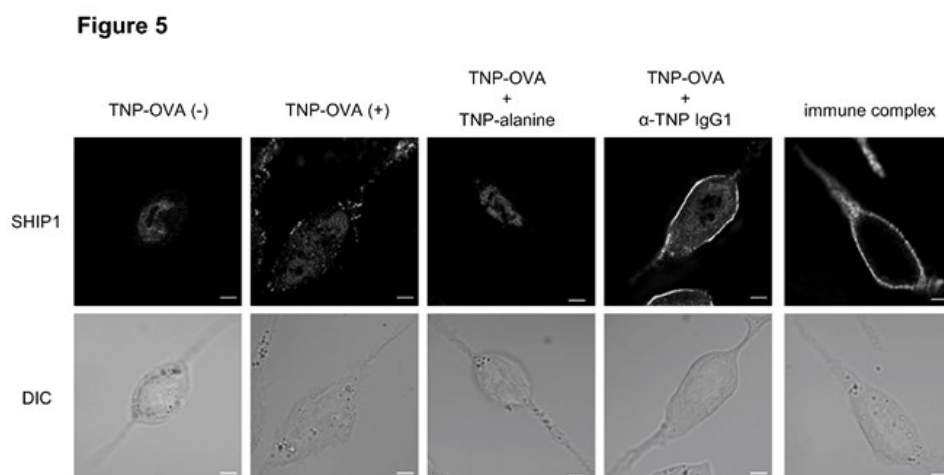


Figure 6. Cytokine production in RBL-2H3 cells determined by ELISA. Concentration of (A) TNF- α and (B) IL-4 secreted in medium after 3 h of adding TNP-OVA (1 $\mu\text{g/mL}$), TNP-OVA (1 $\mu\text{g/mL}$) + TNP-alanine (12 $\mu\text{g/mL}$; 50 s later), TNP-OVA (1 $\mu\text{g/mL}$) + anti-TNP IgG1 (3 $\mu\text{g/mL}$; 50 s later), and immune complex composed of TNP-OVA (1 $\mu\text{g/mL}$) and anti-TNP IgG1 (3 $\mu\text{g/mL}$). Results are expressed as mean $\pm$ SE from ≥ 5 independent experiments. *** P < 0.001 compared with TNP-OVA by ANOVA.

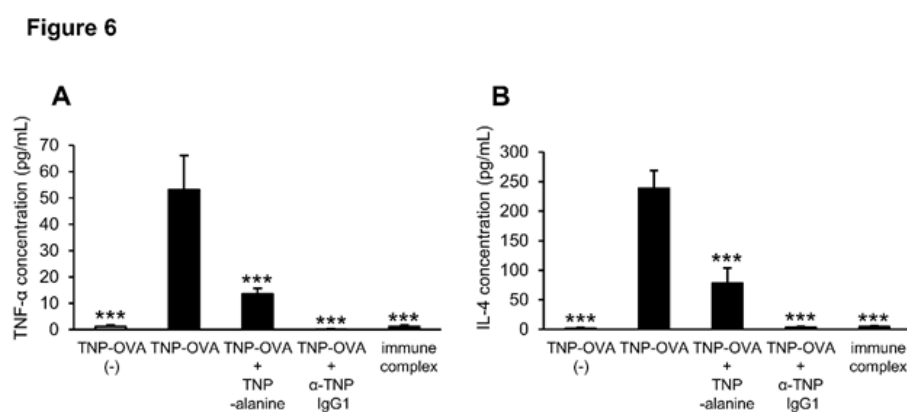


Figure 7. Time course of phosphorylation of Akt and ERKs in RBL-2H3 cells.

Phosphorylation levels of (A) Akt and (B) ERKs were measured by Western blotting with anti-phospho-Akt(T308) antibody and anti-phospho-ERK1/2(T202/Y204) antibody. Cells were stimulated with TNP-OVA (1 $\mu\text{g/mL}$), TNP-OVA (1 $\mu\text{g/mL}$) + TNP-alanine (12 $\mu\text{g/mL}$; 50 s later), TNP-OVA (1 $\mu\text{g/mL}$) + anti-TNP IgG1 (3 $\mu\text{g/mL}$; 50 s later), and immune complex composed of TNP-OVA (1 $\mu\text{g/mL}$) and anti-TNP IgG1 (3 $\mu\text{g/mL}$). Relative phosphorylation levels were normalized to resting cells after controlling for protein loading. β -actin served as the control. Data are representative of ≥ 8 independent experiments. Results are expressed as mean $\pm$ SE. *** and ** $P < 0.001$ and 0.01, respectively, compared with TNP-OVA by Steel–Dwass test.

Figure 7

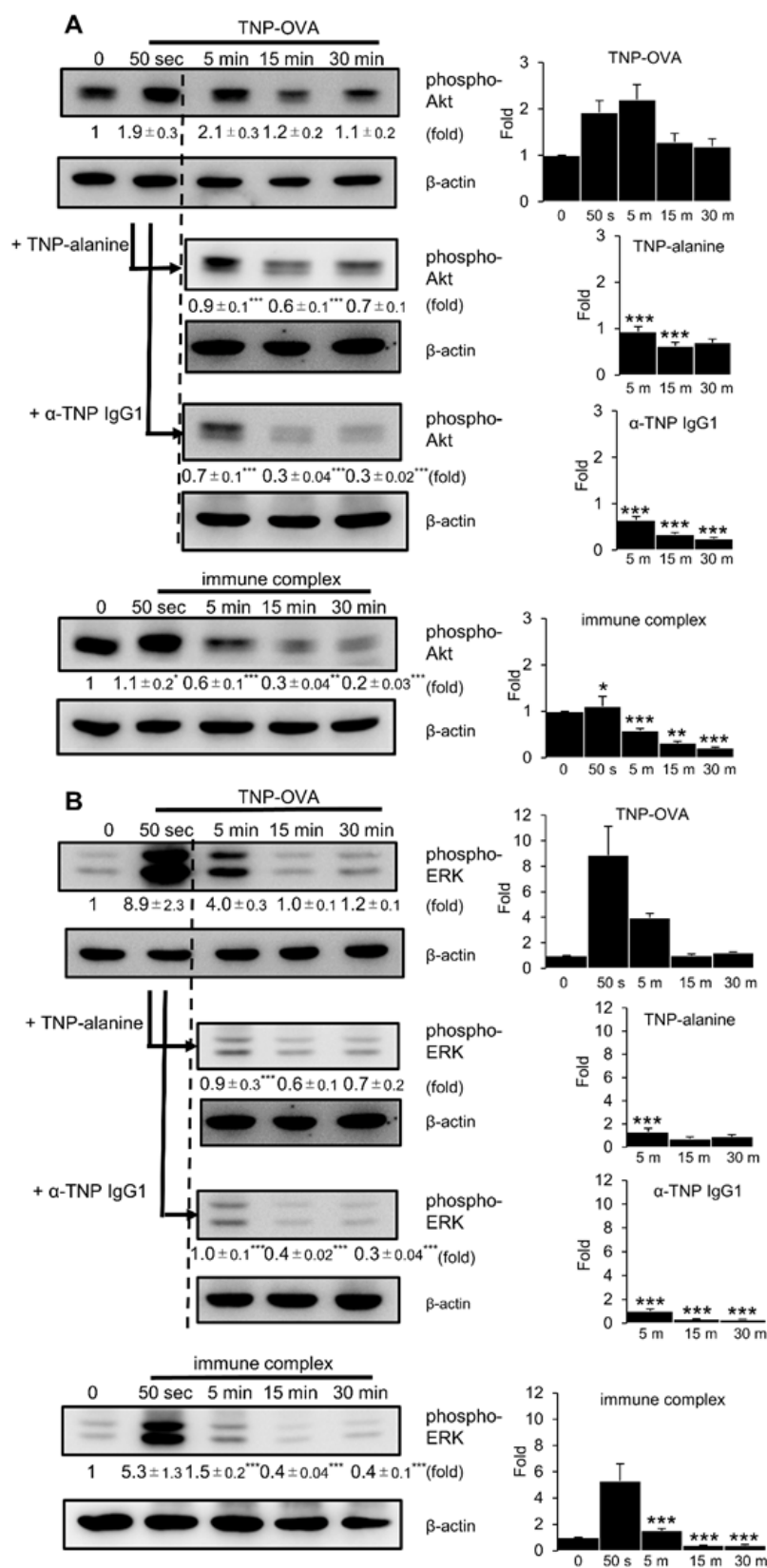


Figure 8. Schematic representation of the inhibition mechanism by monomer hapten and hapten-specific IgG in mast cell activation evoked by multivalent antigen. (left panel) Crosslinking of FcεRI by multivalent antigen (TNP-OVA) activates signaling cascades including phosphorylation of Syk and PLCγ followed by sustained $[Ca^{2+}]_i$ increase, resulting in degranulation and cytokine production. (right panel) Addition of excess monomeric hapten (TNP-alanine) causes immediate and transient dephosphorylation of Syk and rapid decrease in $[Ca^{2+}]_i$, resulting in the arrest of degranulation and impaired cytokine production. However, addition of hapten-specific IgG (α -TNP IgG1) causes phosphorylation and activation of SHIP1, resulting in gradual decline of $[Ca^{2+}]_i$ and inhibition of cytokine production.

Figure 8

