The quantity and duration of FcRγ signals determine mast cell degranulation and survival

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Immunoglobulin E (IgE) bound to multivalent antigen (Ag) elicits mast cell degranulation but not survival; on the contrary, IgE in the absence of Ag (IgE(-Ag)) induces survival only but not degranulation. Although these distinct responses are mediated through the same receptor, FcεRI, the molecular mechanism generating the divergence is largely unknown. We recently showed that the signals through FcεRI chain are essential for IgE(-Ag)–induced mast cell survival as well as IgE(+Ag)–induced degranulation.

To determine whether the cellular output is regulated by the quantity of FcεRI signal, we expressed CD8/FcεRI chimeras (CD8/γ) in bone marrow–derived mast cells (BMMCs) from FcεRI−/− mice to manipulate the strength of FcεRI signals by anti-CD8 cross-linking. Cross-linking of CD8/γ induced mast cell survival and degranulation. Survival was induced by weaker stimulation than needed for degranulation in terms of anti-CD8 concentration and the valency of chimera. However, sustained extracellular signal-regulated kinase (Erk) activation seems to regulate survival even when the activation signal was strong enough to elicit degranulation. Generation of sustained Erk activation by active mitogen-activated protein kinase kinase (MEK)–induced BMMC survival. These results suggest that the duration and the magnitude of FcεRI signals may determine mast cell survival and degranulation, respectively.

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Introduction

Immunoglobulin E (IgE) triggers antiparasitic immunity or allergic responses via binding to FcεRI on the surface of mast cells and basophils. Rodent FcεRI is expressed as a tetramer composed of specific α, β (FcεRIα, FcεRIβ), and common γ (FcεRIγ) homodimers shared with FcγRI or FcγRIII. The α chain mediates binding to IgE. The β and γ chains possess immunoreceptor tyrosine-based activation motifs (ITAMs) within their cytoplasmic domains. Cross-linking of FcεRI with IgE and multivalent antigen (IgE(+Ag)) initiates an activation signal cascade via tyrosine phosphorylation of these ITAMs by Lyn. Syk is then recruited to the phospho-ITAMs of FcεRIγ, where it is activated to phosphorylate various substrates in the downstream cascade, which leads to degranulation or cytokine production. It is recently reported that IgE in the absence of Ag (IgE(-Ag)) actively promotes mast cell survival in addition to passive sensitization. IgE(+Ag) can evoke degranulation but not survival, whereas IgE(-Ag) induces survival but not degranulation. Although both these responses are mediated through FcεRI, the molecular mechanism underlying it remains to be fully elucidated. We have recently demonstrated that ITAM in the FcεRIγ chain is essential for IgE(-Ag)–induced mast cell survival as well as degranulation and cytokine production evoked by IgE(+Ag). This suggests that distinct cellular responses like mediator release and survival are triggered by a similar signaling mechanism, in an FcγRI ITAM-dependent manner. One possible hypothesis for the distinct responses mediated by the same receptor is that the strength of FcεRI signals determines the type of response similar to the situation observed during immature T- or B-cell development. Extracellular signal-regulated kinase (Erk), a pleiotropic mitogen-activated protein (MAP) kinase, is one of the possible molecules that transmit different nature of ligand-receptor interaction into appropriate cellular output. Sustained Erk activation is reported to induce cell survival in many types of cells. In mast cells, it has been reported that IgE(-Ag) elicits more prolonged Erk activation than IgE(+Ag). IgE(-Ag)–induced Erk activation is abrogated by the disruption of the lipid raft structure. Lyn is exclusively localized in these rafts. Although FcεRI is virtually absent in rafts of resting cells, it translocates into rafts upon IgE(+Ag) stimulation. In T cells, constitutive localization of pre–T-cell receptor (pre-TCR) within the raft is suggested to confer survival signals during early thymic development. It is therefore possible that IgE(-Ag) may also induce the prolonged association of FcεRI with rafts to deliver survival signals.

In this report, the correlation between FcεRI signal strength or duration and mast cell responses was investigated using FcεRI chimera with CD8, a coexpressed expressed in T cell, in bone marrow–derived mast cells (BMMCs).

Materials and methods

Mice

C57BL/6 mice were obtained from Japan SLC (Hamamatsu, Japan). The establishment and characterization of FcεRI−/− mice have been described previously. Lyn−/− mice were kindly provided by T. Yamamoto (University of Tokyo). All mice were maintained within a filter-air laminar flow enclosure and provided with standard laboratory food and water ad libitum.
All animal experiments were performed in accordance with Helsinki protocol and Chiba University guidelines.

**Antibodies**

Mouse antidinitrophenol (anti-DNP) IgE (H1 DNP-c-26) was kindly provided by F. Liu (La Jolla Institute, Ontario, CA). H1 DNP-c-26 was centrifuged at 100,000 g for 10 minutes before use to remove possible aggregated complex contaminants. 53.6.7, anti-CD8α, was purchased from eBioscience (San Diego, CA). Anti-CD8α Fab was prepared by papain digestion. Briefly, 200 μg anti-CD8 was digested with 0.02 mg/mL papain in 1 mL digestion buffer (0.02 M EDTA [ethylenediaminetetraacetic acid] and 0.02 M t-cysteine) at 37 °C for 4 hours. After the reaction was stopped with 3 mM iodoacetamide, digested Fc portion or undigested whole antibody (Ab) was removed with protein A–Sepharose (30 μL × 3) and protein G–Sepharose (30 μL × 1). After dialysis against phosphate-buffered saline (PBS), the purity and titer of anti-CD8α Fab were confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (more than 95%) and cell surface staining, respectively. Polyclonal rabbit antimonochrome CD8α was kindly provided by R. Zamoyska (National Institute for Medical Research [NIMR], London, England) for immunoblotting. Fluorescein isothiocyanate (FITC)–conjugated antimonochrome IgE monoclonal antibody (mAb) (R35-72) and phycoerythrin (PE)–conjugated antimonochrome c–kit mAb (2B8) were purchased from BD Pharmingen (San Diego, CA) and eBioscience, respectively. Anti–phospho-Erk, anti–phospho-Jun N-terminal kinase (anti–phospho-JNK), and anti–phospho-p38 Abs were purchased from Promega (Madison, WI). Anti–phospho-Akt (Ser473) was from Cell Signaling Technology (Beverly, MA). Anti–FcRy Ab was from UBI (Lake Placid, NY). Anti–Lyn was kindly provided by T. Yamamoto (University of Tokyo, Tokyo, Japan). Goat antirat Ig was purchased from Cappel (West Chester, PA).

**Construction**

To construct the CD8/y chimera, the cytoplasmic domain of murine FcRy (amino acids [aa] 45 to 86) was fused to the extracellular and transmembrane domain of murine CD8α (aa 1 to 217) by polymerase chain reaction (PCR). For mutant CD8(α)y, C151(TGC) and C166(TGT) of CD8 were mutated to S(TCC) and S(TCT), respectively. For the LAT/y chimera, the cytoplasmic domain of FcRy (aa 45 to 86) was fused to the truncated LAT (aa 1 to 36). Fragments encoding these chimeras were cloned into the retroviral vector, pMX or pMX-ires-GFP (kindly provided by T. Kitamura, University of Tokyo). MEK.WT and MEK.ΔSESE were kindly provided by E. Nishida (Kyoto University, Kyoto, Japan). 19 MEK.WT and MEK.ΔSESE were kindly provided by R. Zamoyska (National Institute for Medical Research [NIMR], London, England) for immunoblotting.

**Retroviral infection and BMMC induction**

Retrovirus transfections were performed as previously described.20 BM cells were obtained from femurs and tibias of 10– to 15-week-old FcRy−/−, wild-type, or Lyn−/− mice, and 5 × 10^6 cells were suspended in RPMI supplemented with 10% fetal calf serum (FCS) and 10% supernatant of X-63-interleukin-3 (X-63-IL-3) cells as a source of IL-3 (kindly provided by H. Karasuyama, Tokyo Medical and Dental University, Tokyo, Japan). For retrovirus infection, 10-fold concentrated virus supernatant was added to cell cultures at days 0, 1, and 2. After 6 days, CD8+ cells were sorted by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in the same medium. Adherent cells were removed every 3 to 5 days. After 4 weeks of culture, more than 95% of the cells were c-kit+ and CD8+.

**Degranulation assays**

For antigen cross-linking, mast cells were sensitized with 1 μg/mL mouse anti-DNP IgE at 37 °C for 4 hours and then with the indicated amount of dinitrophenol–human serum albumin (DNP-HSA). Degranulation assays were performed as previously described.21 Briefly, 5 × 10^6 cells per 100 μL mast cells were treated with various amounts of anti-CD8 or IgE for 30 minutes in Tyrode buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], and 0.1% bovine serum albumin [BSA], pH 7.4) in 96-well plates (Corning Costar, Cambridge, MA). Supernatants and cell lysates were assayed for β-hexosaminidase activity. The percentage of specific β-hexosaminidase release was calculated as [supernatant activity/ (supernatant activity + cell lysate activity)] × 100. Prostaglandin D2 (PGD2) in supernatant was measured by enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI). For long-term degranulation, mast cells were cultured in RPMI containing 10% FCS. At indicated times, supernatant was harvested and histamine concentration was measured by Histamine enzyme-linked immunosorbent assay (ELISA) kit (MBL, Nagoya, Japan).

**Survival assays**

A total of 5 × 10^4 mast cells were cultured in 200 μL IL-3–free medium with various treatments on flat-bottom 96-well plates (Corning Costar). At indicated days, cells were stained with propidium iodide (PI), and live cells (PI-negative cells) were counted by FACScalibur (BD Bioscience, San Jose, CA).

**Internalization assay**

CD8 chimera–expressing BMMCs were treated with anti-CD8 at 37 °C for indicated periods. For secondary cross-linking, cells were incubated with anti-CD8 on ice for 30 minutes, washed twice, and treated with goat antirat Ig at 37 °C. Surface expression was assessed by staining with PE-conjugated antirat Ig x light chain (MRK-1; BD Pharmingen). Data were expressed by percentage of surface expression when mean fluorescence intensity (MFI) of 0 minutes was set at 100%.

**Stimulation with immobilized IgE(+/Ag)**

A 24-well plate (FALCON, Lincoln Park, NJ) was coated with 30 μg/mL DNP-HSA at 37 °C for 2 hours. After washing 3 times with PBS, 10 μg/mL IgE was added and incubated at 37 °C for 1 hour. After washing 3 times with PBS, BMMCs were incubated on the coated well at a concentration of 1 × 10^6/mL and centrifuged at 400g (1500 rpm) for 2 minutes, followed by incubation at 37 °C for indicated periods.

**Ca2+ mobilization**

BMMCs (1 × 10^7/mL) were incubated with 10 μM indo-1-AM (Molecular Probes, Eugene, OR) in the presence of Fl27 and 0.2% FCS at 37 °C for 30 minutes. Cells were washed twice and resuspended in 500 μL Hanks balanced salt solution (HBSS) containing 1% FCS and stimulated with anti-CD8. For antigen stimulation, normal BMMCs were sensitized with 1 μg/mL IgE overnight before antigen stimulation. Ca2+ mobilization was analyzed in a BD-LSR (BD Bioscience) equipped with UV lasers. Ratio metric analysis of Ca2+-bound indo-1 (FL5)/Ca2+-free indo-1 (FL4) was performed using CellQuest (BD Bioscience) and FlowJo (Tree Star, San Carlos, CA) software programs.

**Western blot analysis**

For phospho-Erk analysis, BMMCs were cultured in the absence of IL-3 for 2 hours followed by stimulation with indicated conditions and lysed in 1% Nonidet P-40 (NP-40) lysis buffer as previously described.22 For the precipitation of Ab-bound chimera, cell lysates were incubated with protein G–Sepharose at 4 °C for 1 hour.

**Results**

CD8/FcRy chimera–induced degranulation, cytokine production, and cell survival in mast cells

Initially, we investigated whether the strength of FcRy signals determined the outcome of mast cell responses. For this purpose, we established CD8/FcRy chimera (CD8/y)–expressing BMMCs so that the strength of FcRy signaling could be manipulated by cross-linking with various concentrations of anti-CD8. To avoid the
possible involvement of endogenous FcRγ chain, FcRγγ−/− BM cells were used for retrovirus-mediated gene transfer. Similar chimeras have been used in the analysis of cell lines but not in normal mast cells.24-26 When CD8-expressing cells were sorted and cultured for 4 weeks in the presence of IL-3, approximately 95% of the cells expressed CD8 and c-kit, but not FcεRI on the cell surface, as expected (Figure 1Ai; and data not shown). The expression of the entire chimeric molecule was confirmed by immunoblotting with anti-CD8 and anti-FcRγ.

Next, we examined whether CD8/γ engagement induced BMMC activation. Upon cross-linking CD8/γ with soluble anti-CD8, β-hexosaminidase (β-hex) secretion as a representative of degranulation, (Figure 1B, left), PGD2 secretion as a representative of prostaglandin (PG) synthesis (Figure 1B, right), and cytokine production such as IL-6 and tumor necrosis factor (TNF) (Figure 1C) were measured. The activation levels of CD8/γ-stimulated mast cells, as assessed using these criteria, were equivalent to those exhibited by wild-type BMMCs stimulated with IgE and Ag (IgE(+Ag)) (data not shown; and Sakurai et al).50 We also investigated whether stimulation with anti-CD8 induced BMMC survival in the absence of any exogenous growth factors such as IL-3. Anti-CD8 treatment clearly enhanced survival of FcRγγ−/− BMMCs expressing CD8/γ (Figure 1D). Because even low concentrations of soluble anti-CD8 mAb induced mast cell survival, we examined whether cross-linking with monovalent mAb is sufficient for survival. We compared survival induced by monovalent versus divalent anti-CD8. As shown in Figure 1E, anti-CD8 Fab did not evoke mast cell survival, suggesting that cross-linking of the chimera by a divalent Ab is required to induce survival. Degranulation and survival by CD8/γ was abrogated in both Lyn−/− BMMCs (Figure 1F) and in wild-type BMMCs expressing truncated CD8 lacking the cytoplasmic region containing the ITAM (data not shown). This suggested that phosphorylation of ITAM within the cytoplasmic domain of CD8/γ triggered survival and degranulation. These results confirm that cross-linking of chimeric FcRγ is sufficient for induction of degranulation, PG synthesis, and cytokine production in BMMCs lacking endogenous FcRγ. Furthermore, our data demonstrate for the first time that FcRγ engagement is sufficient for the induction of BMMC survival.

**Distinct signal thresholds for the induction of mast cell survival and degranulation**

Next, we examined the correlation between signal strength through CD8/γ and various cellular responses, particularly degranulation and cell survival. CD8/γ-expressing BMMCs were stimulated by cross-linking with various concentrations of soluble anti-CD8. Concentrations as low as 0.01 μg/mL anti-CD8 mAb induced a significant level of mast cell survival in CD8/γ-expressing BMMCs. The dose of anti-CD8 inducing half maximum response (EC50) for survival was 0.036 μg/mL (Figure 2A).

The concentration of anti-CD8 mAb required for degranulation as measured by β-hex release was much higher (EC50 = 0.26 μg/mL) than that needed for cell survival. Degranulation was only induced by concentrations higher than 0.1 μg/mL anti-CD8. This was confirmed by analyzing histamine release, another known indicator of degranulation. The high stimulation threshold required for degranulation was not simply due to the short stimulation period because anti-CD8–induced histamine release after 24 hours of stimulation also shows similar dose responses (data not shown). Figure 2B (inset) shows the correlation between the input concentration of anti-CD8 and surface-bound anti-CD8 as assessed by flow cytometry. These results confirm that cross-linking of chimeric FcRγ chain, FcRγγ−/− BM cells were used for retrovirus-mediated gene transfer. Similar chimeras have been used in the analysis of cell lines but not in normal mast cells.24-26 When CD8-expressing cells were sorted and cultured for 4 weeks in the presence of IL-3, approximately 95% of the cells expressed CD8 and c-kit, but not FcεRI on the cell surface, as expected (Figure 1Ai; and data not shown). The expression of the entire chimeric molecule was confirmed by immunoblotting with anti-CD8 and anti-FcRγ.

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mean fluorescence intensity (MFI) using antirat Ig staining. When each cellular response was plotted as the percentage of the engaged CD8γ/δ, the difference in the signaling thresholds required for mast cell survival and degranulation became even more prominent (Figure 2B).

Sustained Erk activation correlates with mast cell survival

To investigate differences in cellular signaling events associated with survival and degranulation, active forms of MAP kinases (MAPKs) were analyzed using antiphospho-MAPK mAbs. CD8γ/δ-expressing cells were stimulated with either 0.03 μg/mL anti-CD8 mAb, which increased survival but did not induce degranulation, or with 3 μg/mL anti-CD8 that induced both survival and degranulation. The amount of anti-CD8 mAb bound to CD8γ/δ reached a plateau within 2 minutes after the treatment with 3 μg/mL anti-CD8 (Figure 3A, lower panel, lanes 7-10). In contrast, CD8γ/δ could hardly be detected by 0.03 μg/mL anti-CD8 (Figure 3A, lower panel, lanes 2-5). Nevertheless, comparable levels of sustained Erk activation were observed with both 0.03 μg/mL and 3 μg/mL mAbs. Activation of prosurvival kinase Akt, assessed by phosphorylation at Ser473, was also induced by both concentrations of anti-CD8. In contrast, the phosphorylation levels of JNK and p38 upon stimulation with 0.03 μg/mL anti-CD8 mAb were much lower compared with the levels when 3 μg/mL was used (Figure 3A). Erk activation was sustained for days in survival-inducing condition (Figure 3B).

Our data demonstrating sustained Erk activation is associated with survival are consistent with a previous report. To test whether sustained Erk activation is sufficient for the survival, the active form of MAPK kinase (MEK), a direct activator of Erk, was introduced into BMMCs. Wild-type MEK (MEK WT) showed no enhancement of survival over the vector alone (Mock). In contrast, the active form of MEK (MEK.ASESE) significantly, albeit moderately, enhanced survival (Figure 3C). This suggests that constitutive activation of Erk is capable of inducing survival in normal BMMCs. To confirm the correlation between sustained Erk activation and survival, BMMC survival was analyzed during transient Erk activation by further cross-linking of anti-CD8 mAb with a secondary Ab. Erk phosphorylation was induced more strongly by secondary cross-linking than anti-CD8 alone. The level reached a peak at 2 minutes and rapidly decreased, whereas anti-CD8 alone induced a sustained level of Erk phosphorylation (Figure 4A). Secondary cross-linking also induced rapid internalization of CD8γ/δ from the surface (Figure 4B), with similar kinetics to Erk phosphorylation. In terms of survival, viability of BMMCs cross-linked with anti-CD8 plus second Ab was significantly lower compared with those treated with anti-CD8 alone (Figure 4C). These results suggest that sustained but not transient Erk activation correlates with cell survival.

Although strong cross-linking with IgE (Ag) does not significantly induce survival, high anti-CD8 concentrations could still induce substantial BMMC survival (Figure 2A). These results raise the possibility that failure to induce BMMC survival by IgE (Ag) was due to the limited duration of signaling rather than excessive...
with anti-CD8 only or anti-CD8 plus anti-(C) Survival of CD8+/H9253 -bearing BMMCs by secondary cross-linking. Cells were treated with anti-CD8/ H9253, FcR/ H9280 immobilized IgE/(H9253/H11001 Ag) in normal BMMCs. IL-3 similarly to Figure 1D. (D) Kinetics of Erk phosphorylation after stimulation with 0.01 μg/mL mAb. Ca2+ mobilization was hardly detected (Figure 5A), but substantial cell survival was induced (Figure 2). Similarly, when FcR/IgE Ag, was stimulated, IgE(-Ag) did not induce Ca2+ mobilization in our assay, whereas IgE(+Ag) did (Figure 5B). These results suggest that the transient increase in [Ca2+]i seems to be highly correlated with degranulation rather than survival signals.

FcRγ localization to lipid rafts is not sufficient to promote survival

A recent report suggested that disruption of lipid rafts abrogates IgE(-Ag)-mediated signal transduction.6 To investigate whether constitutive localization of FcRγ chain to lipid rafts triggers downstream survival signals, the intracellular domain of the FcRγ chain was fused to the N-terminal 36 amino acids of linker of activated T cells (LAT) containing palmitoylation sites that target proteins to lipid rafts (Figure 6A). Density gradient centrifugation of FcRγ/-/- BMMCs expressing chimeric LAT/γ showed that LAT/γ was predominantly localized in the lipid raft fraction as expected (Figure 6B). The expression of each chimera, LAT/γ and LAT(CA)/γ, was comparable with that of C27/30A in the N-terminal of LAT that abolishes raft localization,30,31 was also constructed (Figure 6B). The expression of each chimera, LAT/γ and LAT(CA)/γ, was comparable with that of CD8/γ and higher than endogenous FcRγ in WT BMMCs as determined by an anti-FcRγ blot (data not shown). In addition, FcRγ/-/- BMMCs infected with pMX-IRES-GFP vector only (Mock), LAT/γ, and LAT(CA)/γ all developed normally in terms of c-kit expression (data not shown). The survival advantage of chimera-expressing population was compared by culturing bulk BMMCs infected with vector only, LAT/γ, and LAT(CA)/γ in the absence of IL-3 and examining the percentage of viable cells at indicated days by chasing the bicistronic expression of GFP. As shown in Figure 6C, there was no difference in the survival rates of GFP-positive (infected) and GFP-negative (uninfected internal control) populations in each line. Taken together, this suggests that raft localization of FcRγ is not sufficient to augment survival and that further cross-linking within the raft may be required to trigger survival signals.

Degranulation required higher valency of cross-linking than cell survival

We adopted another mutagenesis approach to investigate the effect of valency of the chimera on the induction of mast cell survival and degranulation. Because CD8α dimerizes via a disulfide linkage between extracellular domains,32 each CD8/γ chimera is presumably expressed as a homodimer on the cell surface (Figure 7A). signaling. To test this, we utilized an approach that enabled signal duration to be manipulated. IgE(+Ag) induces FcR/IgE internalization, which may terminate prolonged activation,27 whereas IgE(+immobilized Ag) does not cause receptor internalization.28,29 This implies that immobilized Ag can transduce both strong and prolonged signals simultaneously. Indeed, the plate-coated DNP-HSA plus anti-DNP IgE induced strong and sustained Erk activation (Figure 4D). Furthermore, Figure 4E shows that
Indeed, most of CD8/γ was detected as a dimer in nonreducing SDS-PAGE and a monomer in reducing conditions (Figure 7B, lanes 2 and 5). To compare the signaling capacity of monomeric versus dimeric CD8/γ, 2 cysteine residues involved in the interchain disulfide linkage were mutated to serine (CD8(CS)/γ). CD8(CS)/γ was expressed as a monomer even under a nonreducing condition (Figure 7B, lane 3). This was also confirmed by an anti-CD8 blot (data not shown). Surface staining with anti-CD8 mAb showed no difference in the fluorescence intensity between CD8/γ- and CD8(CS)/γ-expressing cells (data not shown), indicating that this mutation impaired only valency of the chimera without changing surface expression or anti-CD8 affinity.

We then compared the mast cell function upon anti-CD8 stimulation of CD8/γ- and CD8(CS)/γ-expressing cells. Mast cell survival in CD8(CS)/γ-expressing BMMCs was comparable to CD8/γ-expressing BMMCs when anti-CD8 was added at concentrations of 0.1 µg/mL and 1 µg/mL. In contrast, CD8/γ-expressing BMMCs, but not CD8(CS)/γ-expressing BMMCs, induced substantial levels of degranulation (Figure 7D) in response to anti-CD8 stimulation. Again, this result suggests that the signaling threshold for mast cell survival is lower than that required for degranulation.

**Discussion**

We have recently demonstrated that BMMCs from FcRγ−/− mice reconstituted with ITAM-mutated FcRγ fail to induce survival, cytokine production, and degranulation.50 This indicates that FcRγ plays a central role in both IgE(-Ag)− and IgE(+Ag)− dependent responses. How these distinct cellular responses are regulated through the same receptor subunit in the same cellular context is not clear. We hypothesized that the strength of the FcRγ signals determined the outcome of the response. To verify it we established CD8/FcRγ-expressing BMMCs to stimulate FcRγ directly in a qualitative manner. Cross-linking of FcRγ at a low valency was sufficient to induce...
mast cell survival, whereas strong cross-linking was required for
degranulation. Cross-linking was required for survival because
anti-CD8 Fab could not induce survival. This is consistent with
the model that cell surface aggregation of FcεRI is required for
IgE(-Ag)-induced survival although the nature of IgE cross-
linking in the absence of Ag is not yet clear.35 Requirements of
Lyn (Figure 1F) and the cytoplasmic region of FcγRI (Figure 7D)
for the CD8γ chimera to function suggested that phosphoryla-
tion of the ITAMs was important. Notably, IgE(+Ag)-mediated
degranulation is not impaired in Lyn-/- BMMCs.34 The differen-
tial requirement of Lyn in degranulation through FcεRI and
CD8γ may be explained by the distinct contribution of FcεRIβ,
which is phosphorylated by Lyn and binds SH2 domain-
containing 5′ inositol phosphatase (SHIP) in vitro.35 These
results demonstrate that the FcγRIγ signal is necessary and
sufficient for the induction of various mast cell responses and
also suggest that the quantity of the signals can control the types
of responses induced. Notably, the types of responses induced
cannot be attributed to different sensitivities of the assay system
utilized because EC50 values were essentially constant regard-
less of the sensitivity of the assay. Our data also confirm that
survival signals were triggered by FcεRI subunits rather than
other IgE-binding molecules such as galectin-3, consistent with
the previous report.5

Mast cell survival induced by IgE(-Ag) is associated with
simultaneous FcεRI up-regulation,26 but whether this up-regulation
is required for mast cell survival has not been investigated. FcεRI
up-regulation is FcγRI-ITAM independent37 but FcεRIα depen-
dent.37,38 Consistently, we showed surface expression of CD8γ
was not significantly up-regulated upon anti-CD8 treatment despite
the fact that mast cell survival was enhanced (Figure 4B). Thus,
mast cell survival can occur in the absence of receptor up-regulation.

The involvement of lipid rafts in the IgE(+Ag) response via
FcεRI is well documented.16 Kalesnikoff et al reported that
disruption of raft structure also abrogated IgE(-Ag)-induced Erk
activation.5 In T cells, constitutive localization of pre-TCR
within rafts is thought to trigger survival signals in early thymocyte
development.37 It is possible that IgE(-Ag)-induced FcεRI associa-
tion with lipid raft confers survival signals without aggregation.
However, we found forced expression of FcγRI within lipid rafts
did not promote BMMC survival by itself, suggesting that a certain
type of receptor cross-linking is required to promote mast cell
survival after the recruitment of FcγRI to the lipid raft.39

At first sight it seems contradictory that we found high
concentrations of anti-CD8 mAb still induced mast cell survival as
degranulation because previous work shows that massive
cross-linking of FcεRI by IgE(+Ag) does not trigger survival
signals.4 This may reflect the difference in systems between
the present CD8γ system and the physiologic IgE-FcεRI system. The
strength and duration of FcεRI signals are mutually exclusive in
physiological conditions because IgE plus multivalent Ag induces
strong activation signals as well as receptor internalization simulta-
neously, and the latter leads to rapid desensitization to Ag.27
However, our in vitro studies using CD8 chimeras indicated that
sustained FcγRI signals regulated mast cell survival even when the
activation signal was strong enough to elicit degranulation (Figure
4). Although the difference of the dose-response curve between
survival and degranulation was significant, it was smaller than
expected. This indicates that distinct responses can be induced by
slight alterations of the FcγRI signal. Under physiological con-
ditions, the full signaling capacity of FcεRI can be achieved by
cooperation between the β and γ chains. Although the role of β
chain as an amplifier of FcεRI signal is well characterized,40-42 it
also associates with SHIP in vitro,43 which is known to function as
a negative regulator. The presence of the β chain may confer the
FcεRI complex with the additional ability to draw a strict boundary
between survival and degranulation. Indeed, because substantial
degranulation was observed upon IgE(-Ag) stimulation in SHIP-
deficient BMMCs, β chain–associated SHIP may play a role
in avoiding unnecessary (if any harmful) degranulation upon IgE(-
Ag) stimulation.53

We and others have shown that antigen receptor signal strength
or duration or both determines the fate of immature T and B
cells.4,42 Recently, Gonzalez-Espinosa et al reported that strength of
FcεRI signals determines the cytokine profile produced by mast
cells.44 Thus, it could be that a general property of Ag receptors via
downstream signal transduction machinery is to convert quantita-
tive or durational input into qualitative output. Consistently,
Torigoe et al showed that various FcεRI ligand avidity strengths
could elicit distinct responses by recruiting different downstream
signaling molecules,45 just like agonistic or partial agonistic
peptides during T-cell receptor (TCR) signaling.46 Currently,
intracellular molecular mechanisms that convert “strength” and “duration” into distinct responses are not known. The “kinetic proofreading model” is widely accepted for several Ag receptors including FcεRI.45 In this model, downstream signals depend not only on the concentration but also on the lifetime of the “activated” receptor. However, it is difficult to explain the distinct outcome by IgE-(Ag) and IgE(+Ag) stimulation using this model.47 Our observation that even lower Ag concentrations were unable to induce survival (data not shown) suggests that “a sustained signal” cannot be simply proofread by weak transient signals. The precise molecular mechanism that senses “sustained” activation is unknown. One possible explanation is that such signals are translated into sustained Erk signals that can induce cell survival in many types of cells.13,14 Two antiapoptotic molecules belonging to the bcl-2 family, bcl-xL and Bcl2, are involved in mast cell survival.6,48 Activation of prosurvival kinase Akt was also observed in survival-inducing condition (Figure 3A), suggesting the Akt-induced bcl-xL activation pathway may contribute to the survival. The correlation between sustained Erk activation and the expression of bcl-2 family proteins needs to be clarified. Although forced prolonged Erk activation could bypass partial survival (Figure 3C), whether such sustained signals directly induce survival or not is still unclear. Recent reports have shown that weak FcεRI signals favor the production of certain cytokines or chemokines, which favor one of the candidates responsible for the survival described here.6,44,47

The involvement of Ca2+ signals in degradation is well documented.1 Consistently, our data show that Ca2+ mobilization correlates well with degradation. On the other hand, survival appeared to be independent of transient Ca2+ mobilization. However, we cannot exclude the possibility that undetectable Ca2+ levels were triggered in IgE-(Ag)-stimulated mast cells, because Huber et al reported that anti-DNP IgE could induce slight Ca2+ influx even in a wild-type BMMC.33 Although the precise molecular mechanism is still unclear, our system would provide an efficient tool for analyzing downstream effector molecules that direct these divergent responses. The conclusion that prolonged cross-linking of FcεRI is sufficient to induce mast cell survival may support the implication that the high-affinity Fc receptor for IgG associated with FcεRI can also regulate the survival of FcγR-expressing cells such as phagocytes.49 Furthermore, the present study could lead to the development of therapeutic strategies that specifically inhibit certain allergic responses mediated by FcεRI on mast cells.

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References


30. Huber M, Helgason CD, Damen JE, Liu L, Humphries RK, Krystal G. The src homology 2-containing inositol phosphatase (SHIP) is the


The quantity and duration of FcRγ signals determine mast cell degranulation and survival

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