

IgE signaling suppresses Fc ϵ RI β expression

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ABSTRACT

Activation of the high-affinity receptor for IgE, Fc ϵ RI, is known to elicit its rapid down-regulation through internalization and degradation. In keeping with this, expression of all three Fc ϵ RI subunits is decreased at the protein level after cross-linkage of IgE with antigen. However, we find that the Fc ϵ RI β -subunit is also selectively suppressed at the mRNA level, through a pathway primarily involving Fyn, Syk, PI3K, and NF- κ B. IgG or calcium ionophore, stimuli known to mimic portions of the IgE signaling cascade, similarly suppressed β -subunit expression. LPS, a NF- κ B-activating TLR ligand, did not alter β -subunit expression. As IgE increases Fc ϵ RI expression, we examined the coordinated regulation of Fc ϵ RI subunits during culture with IgE, followed by cross-linkage with antigen. IgE increased the expression of all three Fc ϵ RI subunits and strikingly induced expression of the antagonistic β_T . The ratio of β : β_T protein expression decreased significantly during culture with IgE and was reset to starting levels by antigen cross-linkage. These changes in protein levels were matched by similar fluctuations in β and β_T mRNAs. Fc ϵ RI β is a key regulator of IgER expression and function, a gene in which polymorphisms correlate with allergic disease prevalence. The ability of IgE and Fc ϵ RI signaling to coordinate expression of the β and β_T subunits may comprise a homeostatic feedback loop—one that could promote chronic inflammation and allergic disease if dysregulated. *J. Leukoc. Biol.* **86**: 1351–1358; 2009.

Abbreviations: ActD=actinomycin D, BMMC=bone marrow-derived mast cell, β_T =truncated β -subunit, BTK=Bruton's tyrosine kinase, cRPMI=complete RPMI, C_t=comparative threshold, Gab-2=Grb-2-associated binding protein 2, HSA=human serum albumin, KO=knockout, LAT=linker for activation of T cells, MFI=mean fluorescent intensities, NIAHMS/NIH=National Institute of Arthritis and Musculoskeletal Skin/National Institutes of Health, PLC- γ =phospholipase C- γ , PP2=protein phosphatase 2, qRT-PCR=quantitative RT=PCR, RPA=RNase protection assay, SCF=stem cell factor, STI=signal transduction inhibitor, WT=wild-type

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Mast cells are granular hematopoietic cells commonly associated with allergic responses. Their location in tissues exposed to the external environment, such as the skin, gut, and respiratory surfaces, allows them to serve as early sentinels of infection. These cells are generally responsible for protection from parasites and bacterial infections, but in pathological conditions, they are involved in allergic diseases, asthma, and systemic anaphylaxis [1].

Mast cell activation in allergic disease occurs through the high-affinity IgER, Fc ϵ RI, which is triggered by receptor-bound IgE engaged by antigen. On mast cells, the receptor is a tetramer composed of an IgE-binding α -subunit, a signal amplifying β -subunit, and a signaling dimer of disulfide-linked γ -subunits [2]. The α -subunit is composed of a large extracellular domain responsible for IgE binding, a transmembrane domain, and a short cytoplasmic tail lacking an ITAM region [3, 4]. The β -subunit has four transmembrane domains and an ITAM-bearing cytoplasmic domain [5]. The γ -subunit is a member of the $\gamma/\zeta/\eta$ family, a related set of proteins used by many different antigen receptors including the TCR [6]. Subunits in this family are known to consist of a transmembrane domain and a cytoplasmic tail bearing ITAMs. When a multivalent antigen binds receptor-bound IgE, the receptors aggregate to begin a cascade of intracellular signaling leading to the release of inflammatory factors, including histamine, lipid-derived mediators, cytokines, and chemokines [2].

Fc ϵ RI signal transduction is thought to be mediated by at least two major signaling pathways (reviewed in ref. [7]). In the primary pathway, preassociated Lyn tyrosine kinase is activated by receptor aggregation and mediates ITAM phosphorylation of the β - and γ -subunits. This promotes Syk tyrosine kinase recruitment to the phosphorylated γ -chains. Activated Syk phosphorylates the membrane-bound adaptor proteins LAT and the non-T cell activation linker. LAT phosphorylation leads to the activation of PLC- γ , a calcium regulator and activator of protein kinase C, which is involved in mast cell degranulation. LAT also recruits exchange factors responsible for

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activation of the small GTPase Ras and the subsequent activation of the MAPK pathway, which leads to the transcription of target genes important in the late-phase mast cell response, including cytokines.

A secondary pathway begins with Fyn tyrosine kinase, likely associated with the IgER. Fyn recruits and phosphorylates the adaptor protein Gab-2. PI3K is recruited by Gab-2 and phosphorylates phosphatidylinositol 4,5-bisphosphate to make phosphatidylinositol 3,4,5-trisphosphate, which recruits Tec family kinases, including BTK, to the membrane. BTK induces PLC- γ activation, leading to calcium flux and degranulation through inositol 1,4,5-trisphosphate. These pathways also elicit transcription of inflammatory cytokine genes by activating transcription factors including NFAT, AP-1, NF- κ B, and STAT family members. Several groups [8–11] have reviewed these signaling pathways recently.

Of the three Fc ϵ RI subunits, the β -chain may offer the most opportunity for receptor regulation. The β -subunit has been well-documented to enhance Fc ϵ RI expression and the strength of signaling [12, 13]. Importantly, β -chain polymorphisms are associated with an increased incidence of allergic disease [14, 15]. In addition to its amplifying role in IgE signaling, the β -subunit may offer homeostatic feedback signals. Through alternative splicing, β_T is produced. β_T acts in opposition to canonical β , by inhibiting processing and assembly of the Fc ϵ RI complex in the endoplasmic reticulum [16]. Therefore, the ratio of β : β_T may serve as one of several regulatory points that maintains homeostatic balance in IgE signaling.

While investigating feedback regulation of the mast cell response, we have focused on the β -subunit. Rapid Fc ϵ RI down-regulation by receptor internalization has been well documented [17, 18]. However, we have uncovered selective regulation of the β -subunit and its antagonistic splice mutant β_T . Our data provide evidence of mast cell homeostasis regulated partly by feedback signals from the IgER to the genes controlling its own expression.

MATERIALS AND METHODS

Mouse BMMC cultures

BMMC were cultured from BM harvested from mouse femurs and tibias of the following strains: 129, C57BL/6, BALB/c, BL/6x129, and Stat6-deficient (BALB/c background; Jackson Laboratories, Bar Harbor, ME, USA); Stat5-flox and MX1-cre $\times$ Stat5-flox (C57BL/6NCR background; kind gift of Lothar Hennighausen, NIDDK, NIH, Bethesda, MD, USA); Lyn kinase-deficient (129 background) and Fyn kinase-deficient (B6x129 background; kind gift of Juan Rivera, NIAMS/NIH, Bethesda, MD, USA). BMMC cultures were maintained in cRPMI supplemented with conditioned media containing IL-3 (1 ng/ml) and SCF (10 ng/ml) at $\sim 5 \times 10^5$ cells/ml. After 3–4 weeks in culture, these populations were >99% mast cells, as judged by morphology and flow cytometry staining for expression of Fc ϵ RI, CD13, Kit, Fc γ RII/Fc γ RIII, and T1/ST2 (data not shown). The resulting populations were generally used between Weeks 4 and 12 of culture. To obtain Stat5-deficient BMMC, MX1-cre $\times$ Stat5-flox and control Stat5-flox BMMC were cultured in the presence of 1000 U/ml IFN- β for 4 days, after which, the IFN- β was removed. Western blotting of total cell lysates from the cultures was used to ascertain selective Stat5 deletion from the MX1-cre $\times$ Stat5-flox BMMC population, which approached 90% (Supplemental Fig. 1).

Cytokines and reagents

Murine IL-3 and SCF were purchased from Peprotech (Rocky Hill, NJ, USA). Rat anti-mouse Fc γ RII/RIII clone 2.4G2, mouse IgE, and FITC-conjugated rat anti-mouse c-Kit were purchased from BD PharMingen (San Diego, CA, USA). Rat anti-mouse IgE was purchased from Southern Biotechnology Associates (Birmingham, AL, USA). Fluorescein (FITC)-conjugated rabbit anti-rat IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). FITC-conjugated rat anti-mouse T1/ST2 was purchased from Morwell Diagnostics (Switzerland). Propidium iodide, DNP-HSA, ionomycin, cycloheximide, LFM A13, and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO, USA). U0126, PP2, cell-permeable NFAT inhibitor peptide (11R-VIVIT; Catalog #480401), cell-permeable NF- κ B inhibitor [6-amino-4-(4-phenoxyphenylethylamino)quinazoline; Catalog #481406], and Syk inhibitor II [2-(2-aminoethylamino)-4-(3-trifluoromethylamino)-pyrimidine-5-carboxamide, dihydrochloride, dehydrate; Catalog #574712] were purchased from Calbiochem (San Diego, CA, USA). Tanshinone was purchased from Biomol (Plymouth Meeting, PA, USA). Concentrations for inhibitors were chosen based on recommendations from the manufacturer and were typically tenfold higher than the IC₅₀ value. These included the NFAT inhibitor at 1 μ M, NF- κ B inhibitor at 110 nM, tanshinone at 2 mM, LY294002 at 20 μ M, U0126 at 10 μ M, PP2 at 10 μ M, LFM A13 at 100 μ M, and Syk inhibitor II at 2 μ M.

Flow cytometric analysis

To detect Fc ϵ RI surface expression, BMMC were washed twice with FACS buffer (PBS/3% FCS/0.1% sodium azide) in 96-well "V" bottom plates and resuspended in unlabeled IgE (10 μ g/ml) for 45 min at 4°C in the presence of 2.4G2 antibody to block nonspecific Fc γ R interactions. Cells were then washed twice with FACS buffer and incubated for 30 min at 4°C in FITC-coupled rat anti-IgE (10 μ g/ml) for 30 min. Cells were then washed twice with FACS buffer and analyzed by FACScan flow cytometry (Becton Dickinson Immunocytometry, Baintree, MA, USA) in the presence of propidium iodide to exclude dead cells. In experiments measuring changes in Fc ϵ RI after cross-linkage with IgE + DNP-HSA, cells were stained as above after the completion of cross-linkage with antigen. By reincubating cells with IgE prior to staining, newly recycled Fc ϵ RI receptors should be detected. To detect intracellular and surface Fc ϵ RI α -chain, cells were fixed and permeabilized prior to staining with IgE plus PE-anti-IgE using a fixation/permeabilization kit from eBioscience (San Diego, CA, USA). The percent inhibition of surface Fc ϵ RI expression was calculated by comparing MFI of populations cultured in IL-3 alone with the MFI of activated cells.

Mast cell activation

BMMC (typically 3×10^6 /sample) were resuspended in cRPMI to a concentration of 6×10^6 cells/ml and incubated with IgE (10 μ g/ml) for 45 min at 4°C to prevent early receptor activation. Cells were then washed twice with RPMI and resuspended at 3×10^5 cells/ml in cRPMI. Cells were given DNP-HSA (50 ng/ml), LPS (1 μ g/ml), or ionomycin (3 μ M). Samples were incubated for 5 h at 37°C. RNA was harvested and subjected to RPA or qRT-PCR analysis. A variation of this protocol was used for cultures that were activated for periods longer than 5 h. To prevent cell death, BMMC were resuspended in cRPMI with IL-3 + SCF at 30 ng/ml each and then activated with IgE plus antigen as above. When inhibitors were used, they were added at the indicated concentrations, and cultures were incubated for 30 min prior to adding DNP-HSA. DMSO was used as a vehicle control for studies using inhibitors.

RNA isolation and RPA

Samples were harvested with 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA), using the manufacturer's protocol. The harvested RNA was subjected to RPA analysis using the RiboQuant system (BD PharMingen). Pixel intensity was determined using a Typhoon Phosphorimager 445Si system, equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). A normalized pixel intensity was determined for each mRNA of in-

terest by dividing the pixel intensity of the mRNA band of interest by the sum of the pixel intensities for L32 + GAPDH in that sample set. This normalized pixel intensity was used to compare bands from IgE-activated and unactivated samples to determine a percent change in mRNA expression.

RT and qPCR analysis

Cells were harvested, and total RNA was extracted as described above. cDNA was synthesized using the Verso cDNA kit (Thermo Scientific, Waltham, MA, USA), following the manufacturer's protocol using oligo dT primers provided in the kit. cDNA was quantified using the Thermo Scientific NanoDrop™ 1000 UV-Vis spectrophotometer, according to the manufacturer's recommended protocol. cDNA was analyzed immediately or stored at -20°C until use. qPCR analysis was performed with the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR® Green detection using a relative standard curve method. Each reaction was performed according to the manufacturer's protocol using 100 ng sample cDNA, 12.5 μl 2× Absolute QPCR SYBR® Green fluoroscein mix (Thermo Scientific), and mouse FcεRIβ, FcεRIβ_T, or GAPDH (housekeeping gene) primers in a final reaction volume of 50 μl. The primers and conditions used for this study are listed in Table 1. Following the addition of all sample DNA or standard DNA and reagents, the plates were sealed with a MicroAmp optical adhesive film (Applied Biosystems) and centrifuged at 3000 rpm for ~20 s. Amplification conditions for all reactions consisted of a heat-activation step at 95°C for 15 min followed by 40 cycles of 95°C for 15 s, 53°C for 30 s, and 72°C for 34 s. Fluorescence data were collected during the extension step of the reaction. The instrument was set to run in 9600 emulation mode with auto ramping. Resulting data were analyzed with Applied Biosystem's SDS v1.2 software package using a manual C_t of 0.20 and the auto-baseline setting.

For each gene amplified, a standard curve from serial dilutions of a known concentration of cDNA was achieved. The standard curve consisted of eight twofold dilutions of a 400-ng/reaction cDNA sample taken from unstimulated B6x129 WT cells. The standard curve dynamic range used in this study was selected, such that a reliable standard curve encompassing most unknown C_t values was generated. For each gene being analyzed, the input (concentration) of each unknown sample was calculated by comparing its C_t value with the standard curve using the equation: $10 \times [(C_t - b)/m]$, where b is the y-intercept of the standard curve, and m is the slope of the standard curve. The FcεRIβ or FcεRIβ_T relative expression values were normalized by dividing by the amount of GAPDH and are expressed herein as percent of control.

Western blot analysis

FcεRI β- and γ-subunits were detected with mouse anti-FcεRIβ (JRK antisera, kind gift of Juan Rivera, NIAMS/NIH) or rabbit anti-mouse FcεRIγ (Upstate Biotechnology, Lake Placid, NY, USA). Western blotting was performed using 50 μg total cellular protein/sample. Protein was loaded and separated over an 8–16% (β-chain) or a 4–20% gradient (γ-chain) polyacrylamide gel (Bio-Rad, Hercules, CA, USA). Proteins were transferred to nitrocellulose (Pall Corp., Ann Arbor, MI, USA) and blocked for 60 min in Blotto B (Rockland, Inc., Gilbertsville, PA, USA) plus 0.1% Tween-20. Blots were incubated in 5% BSA in a solution of TBST, with a 1:1000 dilution of

anti-FcεRIβ, a 1:1000 dilution of anti-FcεRIγ, or a 1:2000 dilution of β-actin overnight at 4°C with gentle rocking. Blots were washed six times for 10 min each in TBST, followed by incubation in Blotto B containing a 1:5000 dilution of HRP-linked anti-IgG (matched to the appropriate species, from Cell Signaling, Danvers, MA, USA). Size estimates for proteins were obtained using molecular weight standards from Bio-Rad.

RESULTS

IgE-mediated cross-linkage selectively down-regulates FcεRI β-chain mRNA

Antigen-mediated cross-linkage of occupied IgERs is known to elicit rapid internalization and degradation, suppressing the level of receptors for several hours [17, 18]. We used this as a starting point to investigate feedback responses controlling FcεRI. As shown in Figure 1, A and B, all three FcεRI subunits were decreased at the protein level for at least 5 h following mast cell activation with IgE + antigen. The average decrease for each subunit was ~50% compared with unstimulated cells. An assessment of changes in FcεRI subunit mRNA expression revealed that the β-subunit was selectively suppressed, with no consistent change in α or γ mRNAs (Fig. 1, C and D). These results were consistent in more than 20 experiments. Time-course analysis using RPA revealed the kinetics of changes in β mRNA expression. Shortly after antigen-mediated cross-linkage, there was an increase in β mRNA, peaking at ~90 min. This dropped rapidly to a nadir at 5 h after activation, with a 40% reduction from starting mRNA levels (Fig. 1E). The mRNA levels then increased gradually, returning to starting levels ~24 h after mast cell activation. Thus, of the IgER subunits, only FcεRIβ appears to be controlled at the protein and mRNA levels during IgE signaling.

The reduction in β-subunit expression after IgE cross-linkage did not appear to be a result of changes in RNA stability. Three hours after activation with IgE + antigen, the transcriptional inhibitor ActD was added to BMMC cultures. RNA was harvested at the time of ActD addition and after an additional 1.5 and 3 h. The rate at which β-subunit mRNA expression decreased from the point at which ActD was added was used to evaluate mRNA stability. In fact, the rate of change in β-subunit mRNA was not different when comparing cells activated by IgE + antigen versus unactivated BMMC (Fig. 1F). Therefore, changes in RNA stability did not appear to explain the decrease in β-chain expression during IgE signaling.

β-Chain mRNA regulation by FcεRI signaling pathways

To identify the signaling pathways regulating β-chain mRNA, we used gene-deficient BMMC and STIs, beginning at the apical end of the FcεRI signaling cascade (Fig. 2A). BMMC were activated with IgE + antigen for 5 h, and expression of the β-subunit was compared with matched control-unactivated cells by RPA analysis. Lyn kinase deficiency had no effect on β-chain regulation, as Lyn-deficient BMMC showed a reduction in β mRNA similar to WT BMMC following IgE cross-linkage. In contrast, Fyn-deficient (KO) BMMC had a significantly different response than WT BMMC ($P < 0.005$), showing no loss of β mRNA following activation. The Src family inhibitor

TABLE 1. Sequences of Primers Used for qPCR

Name	Primer Sequence
FcεRIβ	Forward 5'-GGAGCAAACATTGTTCAGTAGC-3' Reverse 5'-AAAGCAGCCGTCGTCTTC-3'
FcεRIβ _T	Forward 5'-GGAGCAAACATTGTTCAGTAGC-3' Reverse 5'-CTTAAGACAGGACCCAGAGAG-3'
GAPDH	Forward 5'-CTGGAGAAACCTGCCAAGTA-3' Reverse 5'-TGTGCTGTAGCCGTATTCA-3'

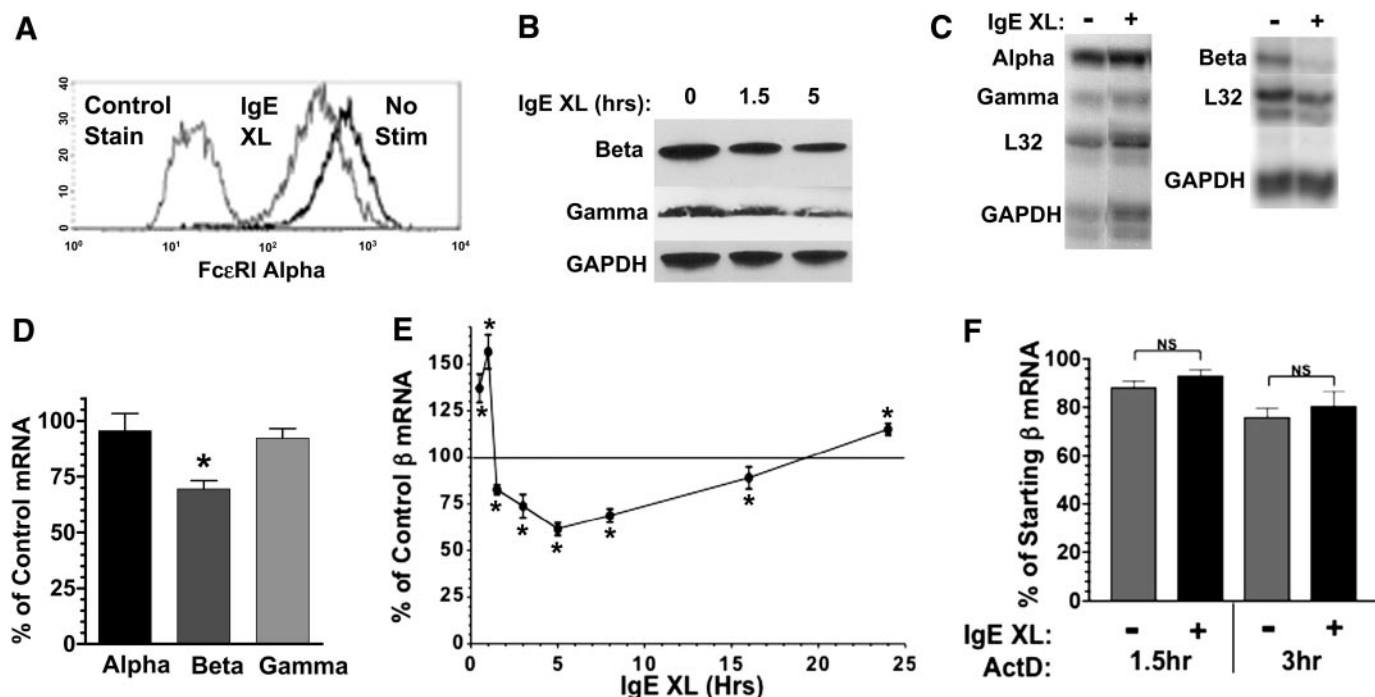


Figure 1. Mouse BMMC (B6x129 strain) were activated with IgE + DNP-HSA or left unstimulated for 1.5 h (A) or 1.5 and 5 h (B). Total expression of the α -subunit was determined by intracellular staining in A. β - and γ -Protein expression was detected by Western blotting in B. Data shown are representative of three to six samples/point. (C) BMMC were treated as in A, and mRNA expression of FcεRI subunits was analyzed 5 h after activation by RPA, as described in Materials and Methods. Data shown are representative of more than 20 experiments. (D) Summary of changes in expression in FcεRI subunit mRNAs as determined by RPA 5 h after IgE cross-linkage (XL). Samples were normalized to loading controls (L32+GAPDH) and compared with unstimulated control cells. Data shown are means and SE of 12–15 samples/point. (E) Mouse BMMC were activated as described in A for the indicated times, and β -chain mRNA expression was determined by RPA analysis. Data shown are mean $\pm$ SE change in β mRNA expression as compared with unstimulated control BMMC. Results are from three to nine samples/time-point. (F) BMMC were unstimulated or activated with IgE + DNP-HSA for 3 h, and RNA samples were harvested to serve as reference controls. ActD was added to cultures, and RNA was harvested after an additional 1.5 or 3 h. The change in β -subunit mRNA was determined by comparing expression in ActD-treated samples with starting reference samples via RPA. Data shown are mean $\pm$ SE of four to five samples/point. NS, No significant change. *, $P < .05$, compared with unstimulated cells.

PP2 corroborated these data, preventing β mRNA inhibition to an extent that matched Fyn deficiency (data not shown). In keeping with the critical role for Syk in FcεRI signal transduction, the Syk kinase inhibitor II also prevented β suppression completely. An additional Syk inhibitor, piceatanol, similarly prevented the decrease in β mRNA (data not shown). These data support the theory that Syk and Fyn kinases contribute to FcεRI β mRNA suppression, and Lyn expression is dispensable.

Several common signaling pathways lie downstream of the apical kinases Fyn and Syk. The importance of these pathways was examined using specific STIs (Fig. 2B). Blocking BTK with LFM A13 had no significant effect on β -chain mRNA levels when compared with vehicle alone (DMSO). MEK kinase blockade with U0126 also yielded no significant difference in β -chain mRNA regulation by IgE + antigen (Fig. 2B). However, blocking PI3K with LY294002 abrogated β -chain down-regulation significantly ($P=0.048$). These data support the theory that PI3K, but not BTK or MEK, plays a major role in regulating FcεRI β -chain mRNA following IgE cross-linkage.

There are several transcription factors involved in FcεRI signaling, including NFAT, NF- κ B, AP-1 [9], and the Stat family mem-

bers Stat5 and Stat6 [19, 20]. To determine which are involved in β mRNA regulation, we inhibited each separately using STIs or gene-deficient BMMC (Fig. 2C). As before, these BMMC were activated for 5 h with IgE + antigen, and β -subunit mRNA expression was compared with matched control-unactivated cells. BMMC lacking Stat5 or Stat6 expression demonstrated β mRNA suppression after IgE cross-linkage equal to their matched WT populations. BMMC activated in the presence of a cell-permeable NFAT inhibitor also showed a reduction in β mRNA that was similar to cells treated with vehicle (DMSO). In contrast, AP-1 blockade partially reversed the suppression of β mRNA compared with vehicle-treated cells. Finally, NF- κ B inhibition provided the most overt change, completely preventing the drop in β mRNA. These data support an important role for AP-1 and NF- κ B in suppressing β -chain mRNA, acting downstream of Fyn, Syk, and PI3Ks.

FcεRI β suppression by IgG-mediated signals and calcium ionophore

The pathways involved in FcεRI signaling are shared with many receptors, making it plausible that β -chain suppression

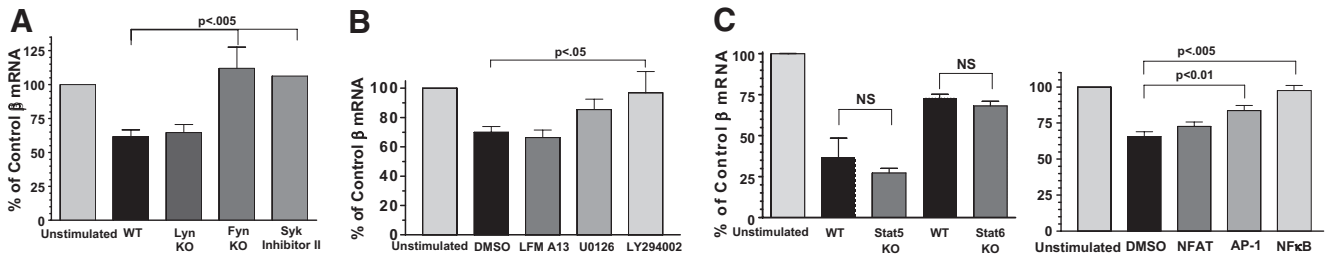


Figure 2. (A) BMMC cultured from WT (B6x129 or 129, which gave similar results), Lyn kinase-deficient (KO), and Fyn KO cells were unstimulated or activated with IgE + DNP-HSA for 5 h. WT cells were also activated in the presence of Syk Inhibitor II (2 μ M) or vehicle (DMSO; shown as control). RNA was harvested and subjected to RPA analysis. Data shown are mean $\pm$ SE for three to six samples/point, depicted as the change in β -chain mRNA expression relative to unstimulated control cells. (B) Mouse BMMC were activated as in A in the presence or absence of DMSO, the BTK inhibitor LFM A13 (100 μ M), the MEK inhibitor U0126 (10 μ M), or the PI3K inhibitor LY294002 (20 μ M). Change in β -chain mRNA expression was measured as in A, using unstimulated BMMC as control samples. Data are mean $\pm$ SE from three to six samples. (C) BMMC were activated as in A in the presence or absence of DMSO, a cell-permeable NFAT inhibitor (1 μ M), the AP-1 inhibitor tanshinone (2 mM), or a cell-permeable NF- κ B inhibitor (110 nM). Change in β -chain mRNA expression was measured as in A, using unstimulated BMMC as control samples. Data are mean $\pm$ SE from three to six samples. For inhibitor studies, BMMC were isolated from B6x129 strain mice.

could occur in response to other forms of mast cell activation. In particular, IgG-mediated signals conveyed by Fc γ RIII use the same β - and γ -subunits. Calcium flux is common to many stimuli and is critical to IgE signaling events. This pathway can be activated by Fyn or Syk via the PI3K pathway [8, 10]. In keeping with these commonalities, IgG cross-linkage suppressed β -subunit mRNA as well as Fc ϵ RI surface expression (Fig. 3). Similarly, the calcium ionophore ionomycin also decreased Fc ϵ RI β mRNA and surface IgER levels. In contrast, the TLR ligand LPS, which does not induce a calcium flux [21], had no effect on β mRNA or surface Fc ϵ RI expression.

Regulation of Fc ϵ RI protein expression by IgE cross-linkage

Our assessments of Fc ϵ RI β regulation have used cells cultured in media lacking IgE until shortly prior to antigen stimulation. Although this limits the variables affecting gene control, the natural state of the mast cell is one in which Fc ϵ RI is nearly 100% occupied. We found recently that mouse mast cells examined ex vivo had fully occupied IgERs by the time of animal maturity [22]. The effects of prior IgE exposure could be important. IgE greatly increases Fc ϵ RI expression [23], which can enhance the strength of signaling. This activity has direct relevance to atopic patients with elevated serum IgE levels.

We determined if Fc ϵ RI signaling can reverse the up-regulating effects of IgE and how this can alter expression of Fc ϵ RI subunits. Mouse BMMC were cultured with or without IgE for 3 days, washed, and activated with antigen. We first used flow cytometry to compare changes in Fc ϵ RI surface expression. As expected, IgE enhanced Fc ϵ RI surface expression dramatically. This effect was stable even after IgE was removed from the cultures. The addition of a cross-linking antigen suppressed Fc ϵ RI surface expression profoundly (Fig. 4A). Using cells that received no IgE at the start of the assay as a control group, we performed kinetic studies of this response. These experiments showed that cells preincubated with IgE started with more than tenfold the number of IgERs present on cells cultured without IgE. Following antigen addition, Fc ϵ RI surface expres-

sion dropped quickly during the following 24 h and returned to the pre-IgE baseline level within 72 h (Fig. 4B).

Flow cytometric analysis of surface Fc ϵ RI is a measure of surface α -chain, the IgE-binding subunit, whose half-life is extended by IgE binding [24]. Using fixed and permeabilized cells, we obtained data essentially identical to surface staining, indicating that total α -chain expression mirrored surface α (not shown).

We next assessed expression of the β - and γ -subunits during the IgE-up-regulating and antigen-activating phases of this study (Fig. 4C). Unlike α , γ -subunit expression was only increased slightly by culture in IgE or by subsequent cross-linkage with antigen, with values that approached but did not reach statistical significance (Fig. 4, C and D). In contrast to γ , the β -subunit revealed dynamic changes in expression (Fig. 4, C and D). β -Protein levels increased approximately threefold during culture with IgE, reaching significantly different values at 72 h. β Expression dropped subsequently after cross-linkage, returning to baseline 24–48 h after antigen addition.

Perhaps the most striking outcome of IgE addition to these cultures was the induction of the β -subunit antagonist β_T , which was difficult to detect with Western blotting prior to culture with IgE but increased approximately eightfold during the 48–72 h after IgE addition (Fig. 4, C and D). Although β_T expression never approached the levels of the canonical β -subunit, the difference in induction levels shifted the β : β_T ratio, from approximately fivefold prior to IgE to twofold after IgE addition (Fig. 4E). After antigen-mediated cross-linking, β_T diminished rapidly to undetectable levels, restoring the β : β_T ratio to its starting point within 48 h of antigen addition (Fig. 4, C–E). These data demonstrate that IgE not only induced expression of the proinflammatory Fc ϵ RI subunits but also induced a natural antagonist, β_T . Subsequent cross-linking with antigen appears to reset expression of all four subunits to an established baseline.

We used a qRT-PCR approach to measure changes in total β and β_T mRNAs during culture with IgE and subsequent antigen cross-linkage (Fig. 4F). These data revealed mRNA regula-

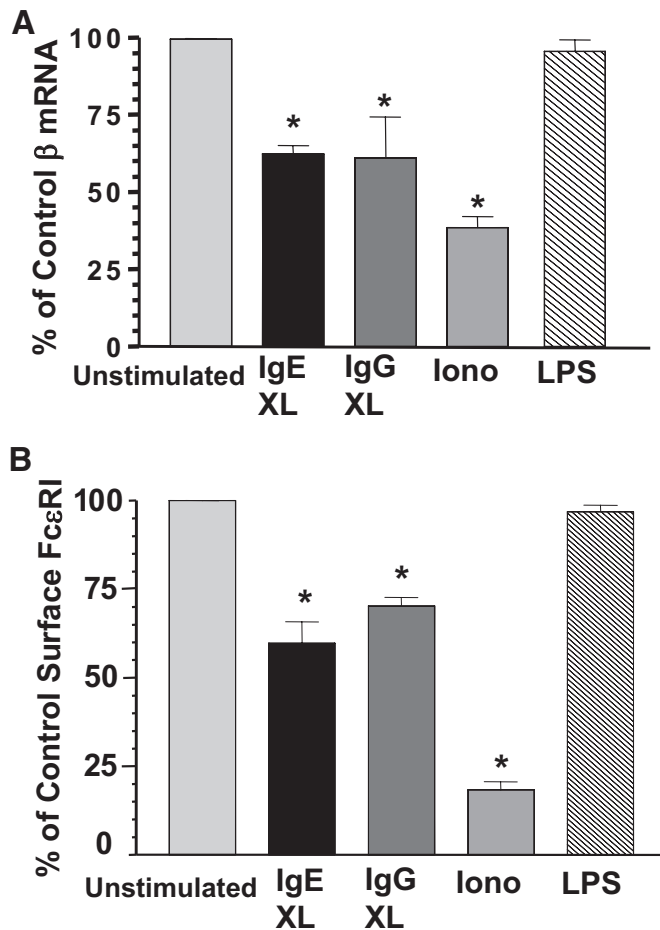


Figure 3. Mouse BMMC were activated via IgE cross-linkage, IgG cross-linkage, ionomycin (Iono), or LPS for 5 h and subjected to RPA analysis (A) or for 24 h and assessed for changes in surface Fc ϵ RI by flow cytometry (B). Data shown are mean $\pm$ SE change in β mRNA expression relative to unstimulated control cells for three to nine samples/stimulus (A) or five to 12 samples/stimulus (B). *, $P < .001$, by Student's *t*-test, when comparing samples with unstimulated control BMMC.

tion that generally matched the changes in protein expression. IgE increased expression of total β and β_T mRNAs, with a two-fold increase over baseline levels after 72 h. Following the addition of antigen, total β and β_T were diminished rapidly to $<20\%$ of the starting levels. Although total β mRNA returned to baseline levels after 24 h, β_T generally remained at a lower level, with a statistically significant difference 48 h after antigen addition compared with unstimulated control cells. Thus, the changes in β -subunit mRNAs showed a trend that was similar to changes in β and β_T protein levels, and IgE and antigen offered antagonistic control.

DISCUSSION

A common theme among organ systems is homeostasis, the ability of the body to resist destabilizing influences brought on by internal or external events. Certainly, the immune system is

also subject to this balancing effort. However, immune homeostasis and mast cell homeostasis in particular remain poorly understood. This study addresses how expression of the IgER, the best-studied and perhaps most clinically relevant stimulus for mast cell activation, is controlled. Fc ϵ RI can be expressed as a trimer of an α -subunit associated with two γ -subunits. However, this form appears to be designed for antigen presentation [25], and the β -subunit is coexpressed with α and γ on mast cells and basophils. On these cells, β is a critical component, serving as a chaperone to promote assembly and expression and a strong amplifier of IgE-mediated signals [12, 13].

Prior studies demonstrated internalization and degradation of Fc ϵ RI subunits following IgE cross-linkage [17, 18, 26]. We were surprised to find that the β -subunit was also targeted selectively for suppression at the mRNA level. The signaling pathways involved are consistent with the two major activating cascades downstream of the IgER: Syk and Fyn. Although Lyn kinase can suppress Fc ϵ RI signaling [22], its expression was not required to decrease β mRNA. Thus β suppression appears to be linked to the proinflammatory mediators of IgE signaling, perhaps serving as a means of dampening their function. Syk, Fyn, and their shared downstream signaling partners PI3K, AP-1, and NF- κ B appear to be crucial for β mRNA suppression. As LPS, a potent inducer of NF- κ B, was unable to suppress β -chain expression, it would appear that NF- κ B activation is necessary but not sufficient for β -chain regulation. In contrast, calcium mobilization, through stimulation with ionomycin, was sufficient to decrease β mRNA and surface Fc ϵ RI.

Although β suppression was fairly rapid, reaching its nadir in 5 h, we postulate that the effects of NF- κ B are indirect, as we have been unable to find a canonical NF- κ B-binding site in the first 2.3 kB of the murine β -chain promoter region or first intron (National Library of Medicine Accession Number AB033617). Given observations by our lab and others that Stat5 and Stat6 control much of the gene expression elicited by Fc ϵ RI [19, 27], we were interested in finding potential Stat5 and Stat6 binding sequences in the mouse Fc ϵ RI β sequence (nucleotides 2546–2554 and 826–835 in AB033617, respectively). However, deletion of Stat5 or Stat6 had no effect on IgE-mediated β -chain suppression. Thus, although we have narrowed the search greatly for how Fc ϵ RI controls expression of its own β -subunit, further study is needed to define the DNA-level interactions.

An important aspect of this study is the demonstration that IgE, in the absence of antigen, increases expression of not only the α - and β -subunits but also the antagonistic β_T as well. Donnadieu et al. [16] first described β_T in 2003 as an alternatively spliced form of the canonical β -subunit. Consistent with the tenets of homeostasis, β_T acts in direct opposition to the chaperone functions of canonical β . By suppressing assembly and transport of the Fc ϵ RI complex, β_T antagonizes IgER expression levels effectively and offers more evidence that the β -subunit may be the critical limiting factor controlling Fc ϵ RI expression and function. The elevation of β_T levels during culture with IgE, followed by its rapid loss after antigen cross-linking, shifts the β : β_T ratio significantly. These changes are a

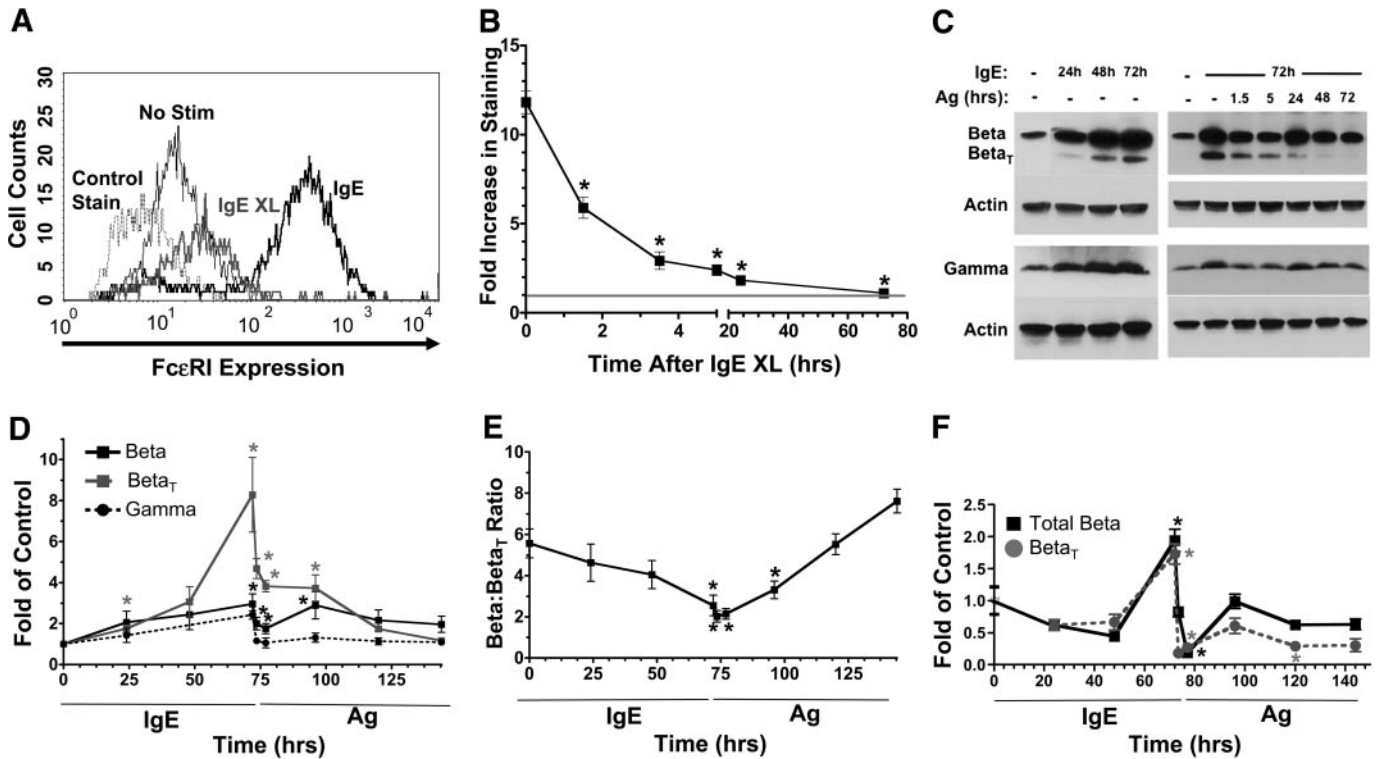


Figure 4. (A) BMMC (B6x129 strain) were cultured for 72 h with or without IgE and then washed and recultured for 24 h with or without DNP-HSA (50 ng/ml). Cells were then analyzed by flow cytometry for Fc ϵ RI surface expression. Data shown are representative of at least five experiments. (B) Mouse BMMC were cultured for 72 h in media $\pm$ IgE, followed by IgE cross-linkage for the indicated times and analyzed by flow cytometry for Fc ϵ RI surface expression. Data shown are the fold change in Fc ϵ RI expression, compared with cells cultured without IgE. Each point is the mean $\pm$ SE of four to five samples/point. *, $P < .001$, compared with control (0 h). (C) BMMC (C57BL/6 strain) were cultured with IgE (1 μ g/ml) for 72 h and then activated with DNP-HSA for an additional 72 h. Left panel shows β , β_T , and γ -subunit expression, as measured by Western blotting. Note that time-points for antigen cross-linkage are in addition to 72 h of culture with IgE. Membranes were stripped and reprobed for actin to determine protein loading. (D) Summary of changes in subunit expression during culture with IgE and subsequent cross-linkage with antigen (Ag). Samples were normalized to actin using densitometry and compared with matched control BMMC cultured without IgE to determine the fold change in expression. Data shown are mean $\pm$ SE of at least three samples/point. (E) BMMC (C57BL/6 strain) were activated as described in C, and the β : β_T ratio was determined for all samples by comparing bands from Western blots using densitometry. (F) BMMC (C57BL/6 strain) were activated as described in C, and RNA was subjected to qRT-PCR to detect expression of total β and β_T . Samples were normalized to GAPDH and compared with matched control-unactivated BMMC to determine the fold change in expression. Data shown are mean $\pm$ SE of three to 12 samples/point. (D–F) *, $P < .05$, compared with cells without IgE (0 h).

plausible support for homeostasis in the mast cell response. As IgE causes mast cells to accumulate surface Fc ϵ RI, β_T induction may serve as a brake, limiting the extent to which the cell is primed to elicit potentially harmful inflammation. However, once antigen-induced internalization has elicited a rapid return to the starting baseline Fc ϵ RI levels, β_T would no longer be needed and could even impede restoration of this baseline. Thus, β_T loss after cross-linkage and its absence in cells not cultured with IgE conform to a homeostatic function.

Our qRT-PCR studies revealed changes in total β and β_T mRNAs, which generally matched the changes in protein expression. However, β_T mRNA increased at a rate that matched total β , suggesting that the more dramatic (eightfold) increase in β_T protein may be a result of changes in mRNA and perhaps protein stability. Two limitations of our study should be stated. First, β_T is present at low levels, making measurements of its changes in expression susceptible to statistical variation.

That being said, IgE induced β_T expression consistently, from nearly undetectable to easily observed levels. Second, our qRT-PCR approach used primer sets with a matched 5' starting point and a 3' primer that was embedded outside of intron 5 (detecting total β) or inside of intron 5 (detecting β_T). Although it is intuitive that the level of canonical β could be determined by subtracting β_T from total β , this is not the case. qPCR is highly sensitive to changes in primer efficiency, and our primer sets yielded varying standard curves. Hence, our data are limited to comparing total β and β_T and do not offer a view of canonical β alone. The same is true for our RPA assays, which used a probe that detects both mRNAs. Despite this limitation, our data reveal control of β_T by IgE interacting with Fc ϵ RI and its subsequent loss after antigen cross-linkage. There has been little published about β_T since its discovery. Uncovering its regulation and its relationship to allergy—for example, knowing if β_T ex-

pression is deficient in atopic patients—has important implications for allergic disease.

These results further the dogma that the β -chain provides much of the balance for mast cell inflammatory responses to an extent that responses similar to IgE, such as IgG or calcium ionophores, also regulate β expression. Polymorphisms in the β gene correlate with allergic disease, although the physiological effects of these DNA alterations remain unclear [28]. These studies illustrate the need to fully understand how Fc ϵ RI is normally controlled and how this balance may differ in chronic inflammatory diseases such as asthma. Within the context of IgE-mediated inflammatory responses, β -chain expression is critical for acquiring a necessary threshold of receptor expression and signaling strength. Evidence that β is selectively controlled at the RNA level and that β_T appears to complement this regulatory circuit is a novel and logical addition to understanding mast cell homeostasis.

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KEY WORDS:

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