

Chapter 15

FcεRI Expression and Dynamics on Mast Cells

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Abstract

Mast cells are key effector and immunoregulatory cells in IgE-associated immune responses, including allergic disorders. IgE antibodies bind to the high-affinity IgE receptor, FcεRI, expressed on the surface of mast cells; antigen-induced cross-linking of FcεRI-bound IgE molecules activates the mast cell to release an array of proinflammatory and immunomodulatory mediators. Because mast cells often respond to very low levels of antigen in vivo, the level of FcεRI expressed on the surface of these cells is an important factor in determining the responsiveness of these cells to antigen. FcεRI surface expression is regulated by a number of processes, including FcεRI stabilization, FcεRI recycling, and antigen-induced internalization. Although members of the Rab family of small GTPases and the ubiquitin ligase, Cbl, have recently emerged as major regulators of many of the membrane trafficking events that govern FcεRI expression levels, the mechanisms and intracellular pathways that regulate FcεRI trafficking remain poorly defined. This chapter outlines a number of flow cytometry-based assays that can be used to investigate cell surface FcεRI expression and dynamics (stabilization, recycling, and internalization) on bone marrow-derived mast cells (BMMCs), the most commonly used model system for studying mast cells in vitro. Given the importance of FcεRI levels to mast cell responsiveness and function, the characterization of FcεRI expression and dynamics on different mast cell populations is critical when trying to compare IgE-dependent processes between different mast cell populations.

Key words Mast cells, BMMCs, BMMCs, Allergy, IgE, FcεRI, Receptor internalization, Ubiquitin, Membrane trafficking, Receptor recycling, Receptor stabilization

1 Introduction

Although mast cells have important effector and immunoregulatory roles in a variety of innate and adaptive immune responses that are thought to be independent of immunoglobulin E (IgE), these cells are best known for the critical roles they play in IgE-associated immediate hypersensitivity reactions and other allergic disorders. IgE primes mast cells to undergo antigen-dependent activation by binding to the high-affinity IgE receptor, FcεRI. In both humans and rodents, FcεRI is expressed on the surface of mast cells as a heterotetrameric complex composed of an IgE-binding α subunit, a four-transmembrane spanning β subunit (which serves as an

important amplifier of IgE plus antigen-induced signaling events), and two identical disulfide-linked γ subunits (which are important for initiating signaling events downstream of this receptor because they each contain one immunoreceptor tyrosine-based activation motif [ITAM]) [1, 2]. IgE binds to Fc ϵ RI at a very high affinity and this interaction has a slow rate of dissociation [1]; thus, Fc ϵ RI binds IgE and retains it for long periods setting the stage for an immediate allergic or inflammatory response upon exposure to environmental antigen. Aggregation of two or more Fc ϵ RI-bound IgE molecules by bivalent or multivalent antigens triggers the rapid release of mediators stored as preformed in mast cell granules (degranulation), the de novo synthesis of lipid mediators, and the synthesis and release of cytokines, chemokines, and growth factors, as well as mast cell adhesion and migration [2–5].

The expression of Fc ϵ RI on the surface of mast cells and the processes that regulate its expression (including receptor stabilization, recycling, and ligand-induced internalization) are critical for mast cells to mount optimal responses to antigens. Receptor regulation is particularly important in responding to low concentration of ligands, as mast cells are able to do. The membrane trafficking events that regulate Fc ϵ RI expression levels remain poorly defined; however, members of the Rab family of small GTPases [6–8] and the ubiquitin ligase, Cbl [9], have recently emerged as major regulators of many of Fc ϵ RI membrane trafficking events.

The stability of Fc ϵ RI on the mast cell surface is a chief determinant of Fc ϵ RI expression levels. Fc ϵ RI stability can be influenced by both intracellular molecules, such as RabGEF1 [10] and Rabaptin-5 [11], and external factors, such as IgE binding [2, 12, 13]. Unoccupied Fc ϵ RI complexes are not very stable on the surface of mast cells; unoccupied Fc ϵ RI is lost from the cell surface via endocytosis (not receptor shedding, as reported for the low affinity IgE receptor, Fc ϵ R2/CD23 [14]) and partially recycled back to the cell surface [11, 15, 16]. Recycling of unoccupied Fc ϵ RI back to the surface increases the chances of capturing circulating IgE molecules, which have the lowest serum concentration of all immunoglobulins. IgE binding stabilizes the Fc ϵ RI complex on the surface of mast cells [2, 12, 13]; there is a tight correlation between serum IgE levels and the density of Fc ϵ RI on the surface of mast cells in vivo [2, 13, 17–20]. IgE-mediated stabilization of Fc ϵ RI may have important implications in allergic individuals with elevated IgE levels; for example, mast cells with higher Fc ϵ RI expression levels on their surface will mount stronger responses to a given amount of antigen and will respond to lower levels of antigen [2, 11, 18, 20].

Cross-linking of Fc ϵ RI-bound IgE molecules induces the activation of Lyn, which phosphorylates Fc ϵ RI ITAMs, and other kinases and signaling molecules (both positive and negative signaling molecules) downstream of this receptor [5, 21]. Aggregation

of this receptor also triggers the ligand-induced internalization (or endocytosis) of FcεRI to remove activated receptors from the cell surface (and turn downstream signaling events “off”). While many studies suggest that rapid and efficient antigen-dependent FcεRI internalization occurs via clathrin-mediated endocytosis [22–24], the accumulation of engaged FcεRI subunits into lipid rafts (specialized regions of the plasma membrane enriched in cholesterol and glycosphingolipid) has recently been shown to be required for full activation and ubiquitination of FcεRI [25–27]. Ubiquitination is a posttranslational modification required for efficient receptor internalization and endosomal sorting [28]; thus, lipids raft appear to play an important role in antigen-dependent FcεRI internalization. Early endosomes receive cargo destined to be recycled back to the plasma membrane or to be sorted along the endocytic machinery and delivered to a lysosomal compartment for degradation. The fate of the internalized receptor (recycling versus degradation) depends, at least in part, on signals presented by the internalized receptor; for example, ubiquitination of FcεRI is required for efficient receptor internalization, sorting, and degradation by lysosomes [26, 27].

Given the importance of FcεRI levels to mast cell responsiveness and functional activation, it is very important to assess baseline levels of FcεRI expression on the surface of any mast cell populations you want to study (e.g., mast cells generated from knockout mice lacking a particular gene of interest, mast cells treated with shRNA to knockdown a particular gene of interest, etc.) and compare these levels to those observed on appropriate control (wild-type) mast cells. Moreover, if the gene or protein you are studying is involved in endocytosis or membrane trafficking events, ubiquitination pathways, or other signaling pathways downstream of the FcεRI, it may be important to look at FcεRI stability, recycling, and antigen-induced internalization in addition to baseline expression levels. This chapter describes a number of flow cytometry-based assays to investigate cell surface FcεRI expression and dynamics in bone marrow-derived cultured mast cells (BMCMCs), the most commonly used model system for studying mast cells *in vitro*. For detailed protocols on how to generate and culture BMCMCs and assess maturity, please *see* Kalesnikoff and Galli 2011 [29]. Although we developed these protocols using BMCMCs, they could easily be adapted and used to studying FcεRI dynamics on other mast cell types or other cells that express FcεRI.

The protocols described herein can be used as a starting point to examine the effect of intracellular receptor trafficking inhibitors, genetic deficiencies (knockout or knockdown studies), or other receptor perturbations on FcεRI receptor dynamics. These dynamics are extremely important when trying to compare IgE-dependent processes between two mast cell populations (e.g., mast cells lacking a particular gene of interest generated from knockout mice

(or via shRNA knockdown studies) versus wild-type mast cells), which require Fc ϵ RI expression and receptor dynamics to be similar between populations. If preliminary experiments reveal that two mast cell populations have different baseline Fc ϵ RI expression levels, the protocols described herein serve as a foundation to explore the underlying biologic processes responsible for this difference in Fc ϵ RI expression. We outline an assay to examine surface half-life of Fc ϵ RI (or other receptors of interest) by examining the decay of surface Fc ϵ RI after blocking protein synthesis (using cycloheximide) or blocking exit from the endoplasmic reticulum (using brefeldin A). Recycling of Fc ϵ RI may also contribute to altered surface expression; thus, we describe a method to examine both the extent and kinetics of Fc ϵ RI recycling using fragment antigen-binding (Fab) fragments that selectively label Fc ϵ RI without inducing receptor aggregation. Conversely, if preliminary experiments reveal that two mast cell populations have similar Fc ϵ RI expression levels, but there is concern that stimulation-induced internalization may be perturbed (e.g., due to alterations in Rab- or ubiquitination-dependent pathways), we outline steps to determine the kinetics of stimulation-induced Fc ϵ RI internalization. Because differences in this very proximal event can have a profound influence on the extent of mast cell activation [10], stimulation-induced internalization experiments are also important to perform on mast cell populations with different baseline Fc ϵ RI expression levels.

We focus herein on flow cytometry-based assays to study Fc ϵ RI dynamics for a number of reasons. First, this system allows for quick and user-friendly experiments with equipment that is ubiquitous in most research settings. One only needs a few antibodies, standard reagents used in many assays, and a flow cytometer (a machine that is becoming more available to individual labs and becoming increasingly easier to operate). Second, flow cytometry permits the scientist to perform analyses on small quantities of cells (an important advantage when working with rare cell populations or freshly isolated cells, such as peritoneal mast cells). Moreover, flow cytometry allows the scientist to look at individual cells (a property intrinsic to flow cytometry) in a population of cells and then pool the single-cell data to generate average (mean) values for a population of cells being studied (this is the standard in other techniques that have been used in examining protein trafficking events, including subcellular fractionation to assess receptor localization in different compartments, surface radiolabeling, or surface biotinylation experiments). By examining the distribution of the protein of interest in a population (and not just the population average), the investigator may gain additional insight about the kinetics of a phenomenon or be better suited to choose the correct method by which to analyze the data (comparing means, medians, or sub-grouping cell populations). Third, flow cytometry has the

advantage of allowing the scientist to exclude dead or dying cells (which have drastically different receptor dynamics) from analyses with the use of vital dyes such as propidium iodide (PI). Finally, with more sophisticated experiments than those described herein, one can compare a number of different cell populations in one experiment or even in the same tube. This is important when studying freshly isolated cells *ex vivo* because one can use surface protein expression to identify cell populations of interest and exclude potentially confounding cell populations. Further, for transgene experiments, cells transfected or transduced with bi-cis-transonic vectors expressing fluorophores that can be analyzed with the flow cytometer's filter set (e.g., GFP, CFP, mTomato, or mCherry) allow for the simultaneous comparison of initially homogenous populations in the same staining sample or experimental group, and these populations can be separated later for analysis.

2 Materials

A flow cytometer with a 488 nm fixed argon laser and three-color detection capability (i.e., FL1, FL2, and FL3) is essential for the methods described below. Using APC or Alexa Fluor® 647-conjugated antibodies, the flow cytometry will require a flow cytometer with argon and red diode laser (635 nm) with four-color detection capability. For the materials and methods described below, we used a FACSCalibur® flow cytometer (BD Biosciences).

2.1 Assessing Baseline FcεRI Expression in BMCMCs

1. Flow cytometry (FACS) buffer: phosphate buffered saline (PBS) supplemented with 2 % fetal bovine serum (FBS) (*see Note 1*).
2. 5 mL polystyrene round bottom tubes (FACS tubes) or other tubes compatible with the flow cytometer instrument (*see Note 2*).
3. Purified anti-mouse CD16/32, clone 93 (*see Note 3*).
4. APC (allophycocyanin)-conjugated anti-mouse FcεRIα, clone MAR-1 (*see Note 4*).
5. APC-conjugated isotype control antibody (if desired).
6. Propidium iodide (PI): prepare a 1 mg/mL stock in PBS.

2.2 Stimulation-Induced Internalization of FcεRI-Bound IgE

1. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.1 % bovine serum albumin (BSA) (*see Note 5*).
2. DMEM supplemented with 10 % FBS.
3. Purified IgE (*see Note 6*).
4. 15–50 mL conical polystyrene tubes.
5. 1.5 mL capped microcentrifuge (Eppendorf) tubes for stimulation.

6. FACS buffer (*see* Subheading 2.1 and **Note 1**).
7. 5 mL FACS tubes (*see* **Note 2**).
8. APC-conjugated streptavidin (*see* **Note 4**).
9. Biotin rat anti-mouse IgE (clone R35-72).
10. Propidium iodide (PI) stock: 1 mg/mL solution in PBS.

2.3 *FcεRI* Stability Assay

1. DMEM supplemented with 0.1 % BSA.
2. DMEM supplemented with 10 % FBS.
3. FACS buffer (*see* Subheading 2.1 and **Note 1**).
4. 5 mL FACS tubes (*see* **Note 2**).
5. Cycloheximide (CHX) stock solution: 100 mg/mL in dimethyl sulfoxide (DMSO).
6. Brefeldin A (BFA) stock solution: 10 mg/mL in DMSO.
7. Purified anti-mouse CD16/32, clone 93 (*see* **Note 3**).
8. APC-conjugated anti-mouse FcεRIα, clone MAR-1 (*see* **Note 4**).
9. PI stock solution: 1 mg/mL in PBS.

2.4 *FcεRI* Recycling Assay

1. DMEM supplemented with 0.1 % BSA.
2. DMEM supplemented with 10 % FBS.
3. FACS buffer (*see* Subheading 2.1 and **Note 1**).
4. 5 mL FACS tubes (*see* **Note 2**).
5. Purified anti-mouse CD16/32, clone 93 (*see* **Note 3**).
6. Alexa Fluor® 647 (AF647)-conjugated monovalent streptavidin (SA) and unlabeled monovalent SA, generated from pET21a-Streptavidin-Dead (Addgene plasmid number 20859; *see* **Note 7**).
7. Biotinylated anti-mouse FcεRIα, clone MAR-1 antigen-binding (Fab) fragments: prepare 0.1–1 mg/mL stock solution in PBS (*see* **Note 8**).
8. PI stock solution: prepare 1 mg/mL in PBS.

3 Methods

3.1 Assessing Baseline *FcεRI* Expression in BMCMCs

1. Approximately 5×10^4 – 5×10^5 cells are required for each condition (there will be two or three conditions for each population of BMCMCs being studied, as described in **step 3**, Subheading 3.1). Wash cells with 3 mL 4 °C FACS buffer (*see* **Note 1**) in a 5 mL FACS tube (*see* **Note 2**). Centrifuge at $500 \times g$, 5 min, 4 °C. Aspirate the supernatant down to the pellet.
2. Prepare FcγR blocking solution: dilute anti-mouse CD16/CD32, clone 93 (*see* **Note 3**), 1/200 in FACS buffer (2 μg/mL final

concentration of α -CD16/ α -CD32). Add 10 μ L Fc γ R blocking solution to each pellet (resuspend pellet or flick tube to agitate). This step blocks nonspecific binding of antibodies. Incubate for 5 min on ice.

3. Prepare anti-mouse FcεRI α staining solution: dilute APC-conjugated anti-mouse FcεRI α (*see Note 4*) 1/200 in FACS buffer. Add 20 μ L anti-mouse FcεRI α staining solution to the desired tubes from **step 2**. For each BMCMC population, it is best to have one tube of cells that receives the APC-conjugated anti-mouse FcεRI α staining solution (“stained”) and one tube of cells that does not receive staining solution (“unstained”) or a tube that receives an APC-conjugated isotype control antibody (*see Note 9*). Incubate for 15–30 min on ice protected from light. If working with peritoneal mast cells, *see Note 10*. *Important*: From this step forward, protect samples from direct light (use lid on ice bucket).
4. Wash cells with 3 mL 4 °C FACS buffer. Centrifuge at 500 $\times g$, 5 min, 4 °C. Aspirate the supernatant.
5. Prepare propidium iodide (PI) staining solution: dilute PI stock 1/1,000 in FACS buffer (final PI concentration 1 μ g/mL). Resuspend pellet in 200 μ L PI staining solution.
6. Acquire cell-associated APC and PI fluorescence on a flow cytometer (no compensation is usually necessary for these two fluorophores).
7. Analyze results using data analysis software. We use FlowJo software (Tree Star, Ashland, OR), but other software, such as software that comes with the flow cytometer, can be used.

3.2 Stimulation-Induced Internalization of FcεRI-Bound IgE

1. Approximately 1×10^5 cells are required for each condition or time point (*see Note 11*). Remove the desired number of BMCMCs and wash with 10 mL of pre-warmed (37 °C) DMEM+10 % FCS. Centrifuge at 500 $\times g$, 5 min, 20–25 °C (room temperature (RT)). Aspirate the supernatant.
2. Prepare IgE solution: dilute monoclonal mouse IgE (*see Note 6*) to a concentration of 1–2 μ g/mL in DMEM+10 % FCS. Resuspend cell pellet in appropriate volume of IgE solution to achieve the desired concentration of 1×10^6 cells/mL. Transfer cells to the appropriate size tissue culture dish (e.g., 24-well plate). Incubate for 16–18 h at 37 °C in a tissue culture incubator. This step “preloads” the mast cells with IgE.
3. Transfer cells from culture dish or wells to a 15 mL tube. Wash cells with 5–10 mL of 20–25 °C (room temperature) DMEM+0.1 % BSA to remove excess/unbound IgE. Centrifuge at 500 $\times g$, 5 min, 4 °C. Aspirate the supernatant.
4. Repeat wash step with 5–10 mL 4 °C DMEM+0.1 % BSA. Centrifuge at 500 $\times g$, 5 min, 4 °C. Aspirate the supernatant.

5. Resuspend pellet to achieve 2×10^6 cells/mL concentration in 4 °C DMEM+0.1 % BSA. Remove an aliquot (1×10^5 cells; 50 μ L) for unstained control; add cells to a 5 mL FACS tube (*see Note 2*) containing 3 mL of 4 °C FACS buffer and keep on ice (until all time points have been collected in **step 10**, and then proceed to **step 11**, Subheading 3.2).
6. To the remaining cells, add biotin rat anti-mouse IgE antibody to a final concentration of 1 μ g/mL (stock is 500 μ g/mL $\therefore$ 1/500 dilution). Incubate for 1 h on ice. The antibodies will cross-link IgE-bound Fc ϵ RI α receptors.
7. Wash cells with 5–10 mL 4 °C DMEM+0.1 % BSA. Centrifuge at $500 \times g$, 5 min, 4 °C. Aspirate the supernatant. Repeat **step 7**, once more.
8. Resuspend at 1×10^6 cells/mL in pre-warmed (37 °C) DMEM+0.1 % BSA. Immediately remove an aliquot (1×10^5 cells; 100 μ L) for 0 min time point; add cells to a FACS tube containing 3 mL of 4 °C FACS buffer and keep on ice (until all time points have been collected in **step 10**; then proceed to **step 11**, Subheading 3.2).
9. Transfer the remaining cells to a 1.5 mL microcentrifuge (Eppendorf) tube and place tube in a 37 °C water bath. Remove aliquots (1×10^5 cells each; 100 μ L each) at 5, 15, 30, 60, and 90 min; add cells to a 5 mL FACS tube containing 3 mL of 4 °C FACS buffer and keep on ice (until all time points have been collected).
10. Centrifuge all samples at $500 \times g$, 5 min, 4 °C. Aspirate the supernatant.
11. Prepare streptavidin staining solution: dilute APC-conjugated streptavidin (*see Note 4*) 1/200 in FACS buffer. Add 20 μ L streptavidin staining solution to all samples. Incubate for 20 min on ice protected from light. *Important:* From this step forward, protect samples from direct light (use lid on ice bucket).
12. Wash cells with 3 mL 4 °C FACS buffer. Centrifuge at $500 \times g$, 5 min, 4 °C. Aspirate the supernatant.
13. Prepare propidium iodide (PI) staining solution: dilute PI stock 1/1,000 in FACS buffer (final PI concentration 1 μ g/mL). Resuspend pellet in 200 μ L PI staining solution.
14. Acquire cell-associated APC and PI fluorescence on a flow cytometer (no compensation is usually necessary for these two fluorochromes).
15. Analyze results using data analysis software. To calculate percent Fc ϵ RI internalization, we use the flow cytometry data analysis software to calculate mean fluorescence intensity (MFI) of the APC channel for each time point (after gating out

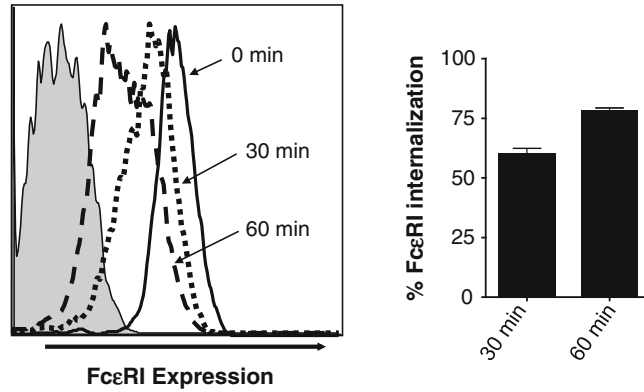


Fig. 1 FcεRI internalization assay. BMCMCs were sensitized with 1–2 μg/mL IgE, and then stimulated with 1 μg/mL biotinylated anti-mouse IgE antibodies (α-IgE) for the indicated times, as described in Subheading 3.2. Surface α-IgE was assessed by streptavidin (SA)-APC fluorescence and analyzed by flow cytometry, as described in Subheading 3.2. The histogram (*left panel*) shows representative results obtained from a single batch of BMCMCs; gray represents SA-APC staining only. The bar graph (*right panel*) shows the mean + SEM of percentage FcεRI internalization determinations from three separate batches of BMCMCs

PI-positive/dead cells). Using the MFI for each time point, calculate percent internalization using the following equation: $[MFI(\text{time } X) - MFI(\text{time } 0')]/MFI(\text{time } 0')$. An abbreviated time course histogram and compiled results are depicted in Fig. 1.

3.3 FcεRI Stability Assay

1. Approximately 2×10^5 BMCMCs are required for each condition or time point (*see Note 12*). Remove the desired number of BMCMCs and wash with 10 mL of warm (37 °C) DMEM + 10 % FCS. Centrifuge at $500 \times g$, 5 min, 20–25 °C (RT). Aspirate the supernatant.
2. Resuspend at 1×10^6 cells/mL in 37 °C DMEM + 10 % FCS. Immediately remove two aliquots (1×10^5 cells each; 100 μL each) for unstained control and 0 min time point; add cells to a 5 mL FACS tube containing 3 mL of 4 °C FACS buffer and centrifuge at $500 \times g$, 5 min, 4 °C. Aspirate the pellet, and then proceed to **step 2** of the protocol listed in Subheading 3.1 to assess FcεRI expression (do not add APC-conjugated anti-mouse FcεRIα (*see Note 4*) antibodies to unstained control in **step 3**, Subheading 3.1).
3. Split the remaining volume of cells into three equal portions (for treatment with vehicle (DMSO), cycloheximide, or brefeldin A); transfer cells to the appropriate size tissue culture dish (e.g., 48-well plate). To the vehicle (DMSO)-treated cells, add a volume of DMSO to generate a final concentration of DMSO equal to that of the highest concentration of DMSO

being used with cycloheximide or brefeldin A treatment (we typically use 1 % DMSO). To the cycloheximide-treated cells, add cycloheximide to a final concentration of 1.5 $\mu\text{g}/\text{mL}$ (*see* **Note 13**). To the brefeldin A-treated cells, add brefeldin A to a final concentration of 50–100 $\mu\text{g}/\text{mL}$ (*see* **Note 13**). Transfer cells to 37 °C tissue culture incubator.

4. Remove aliquots (1×10^5 cells each; 100 μL each) from all treatment groups (vehicle [DMSO], cycloheximide, and brefeldin A) at 2, 4, 6, and 12 h (or other predetermined time points); add cells to a 5 mL FACS tube containing 3 mL of 4 °C FACS buffer, and centrifuge at $500 \times g$, 5 min, 4 °C. Aspirate the supernatant, and then proceed to **step 2** of the protocol listed in Subheading 3.1 to assess Fc ϵ RI expression. Given the long intervals between time points, stain each sample as it becomes available.
5. Analyze results using data analysis software. Calculate MFI of the APC channel (*see* **Note 4**) for each time point (after gating out PI-positive/dead cells). To generate decay curves, first, calculate percent Fc ϵ RI expression relative to baseline using the following equation: $(\text{MFI time } X / \text{MFI time } 0) \times 100$. Pool data from three or more experiments to generate decay curves using data analysis software (we use Prism (GraphPad, La Jolla, CA), but other software can be used). Compare decay curves between experimental groups at each time point to look for differences in experimental groups. An example of a typical Fc ϵ RI stability time course after exposure to brefeldin A or cycloheximide is depicted in Fig. 2.

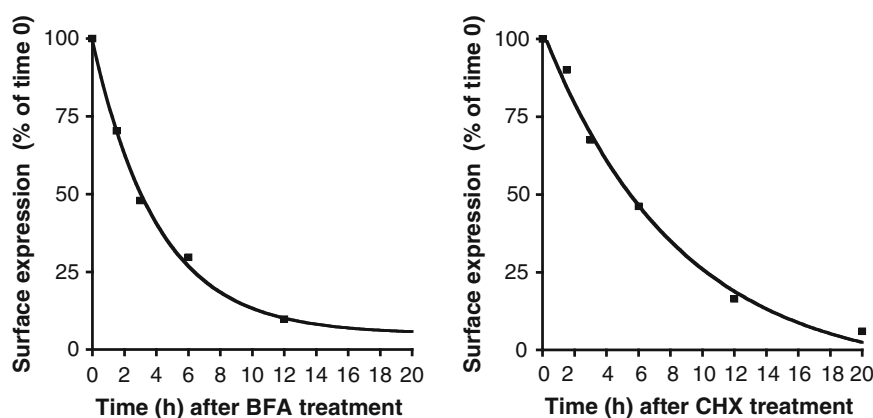


Fig. 2 Fc ϵ RI α half-life assay. Fc ϵ RI α half-life was measured in BMCMCs by exposing cells to 100 $\mu\text{g}/\text{mL}$ brefeldin A (BFA; *left panel*) or 1.5 $\mu\text{g}/\text{mL}$ cycloheximide (CHX; *right panel*) for the indicated times, and surface Fc ϵ RI was assessed by flow cytometry as described in Subheading 3.3. Expression relative to time 0 was calculated for the indicated time points. Data from three experiments were pooled, averaged, and fit to exponential decay curves

3.4 FcεRI Recycling Assay

1. Approximately 1×10^5 BMCMCs are required for each condition or time point (*see Note 14*). Remove the desired number of BMCMCs and wash with 10 mL of DMEM+10 % FCS (at 20–25 °C). Centrifuge at $500 \times g$, 5 min, RT. Aspirate the supernatant.
2. Resuspend at 1×10^6 cells/mL in 20–25 °C DMEM+10 % FCS. Remove an aliquot (1×10^5 cells; 100 µL) for unstained control; use a 5 mL FACS tube containing 3 mL of 4 °C FACS buffer and keep on ice (until all time points have been collected in **step 11**; then proceed to **step 12**, Subheading 3.4).
3. Transfer the remaining cells to the appropriate size tissue culture dish (e.g., 48-well plate). Add biotinylated anti-mouse FcεRIα Fab fragments to a final concentration of 0.5 µg/mL. Incubate for 3 h at 37 °C in a tissue culture incubator (*see Note 15*).
4. Transfer cells to a 15 mL tube. Wash cells with 10 mL of DMEM+0.1 % BSA (at 20–25 °C). Centrifuge at $500 \times g$, 5 min, 4 °C. Aspirate the supernatant.
5. Repeat wash step with 4 °C DMEM+0.1 % BSA. Centrifuge at $500 \times g$, 5 min, 4 °C. Aspirate the supernatant.
6. Resuspend at 1×10^6 cells/mL in 4 °C DMEM+0.1 % BSA. Remove an aliquot (1×10^5 cells; 100 µL) to assess baseline surface FcεRI expression; add cells to a 5 mL FACS tube containing 3 mL of 4 °C FACS buffer, and then centrifuge at $500 \times g$, 5 min, 4 °C. Aspirate the pellet and stain this aliquot with AF647-conjugated monovalent streptavidin as outlined in **step 9** (Subheading 3.4), and then wash cells with 3 mL of 4 °C FACS buffer and proceed to **step 12**, Subheading 3.4.
7. To the remaining cells, add unlabeled monovalent streptavidin to a final concentration of 100 µg/mL. Incubate for 30 min on ice; the unlabeled monovalent streptavidin will bind to the biotinylated Fab fragments (*see Note 16*).
8. Wash cells with 4 mL 4 °C DMEM+0.1 % BSA. Centrifuge at $500 \times g$, 5 min, 4 °C. Aspirate the supernatant.
9. Resuspend at 1×10^6 cells/mL in 4 °C DMEM+0.1 % BSA. Add AF647-conjugated monovalent streptavidin to a final concentration of 25 µg/mL. Incubate for 30 min on ice. *Important:* From this step forward, protect samples from direct light (use lid on ice bucket and aluminum foil for tubes in water bath).
10. Remove an aliquot (1×10^5 cells; 100 µL) for 0 min time point (*see Note 17*); add cells to a 5 mL FACS tube containing 3 mL of 4 °C FACS buffer and keep on ice (until all time points have been collected).
11. Transfer the remaining cells to a 1.5 mL microcentrifuge (Eppendorf) tube and place tube in a 37 °C water bath.

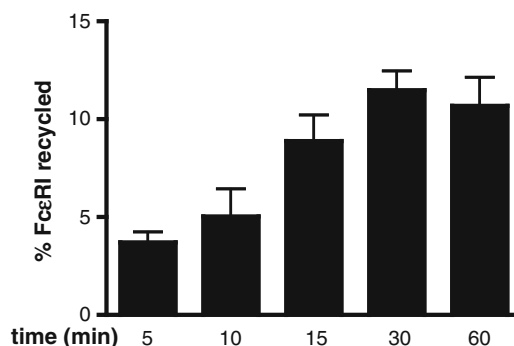


Fig. 3 FcεRI recycling assay. FcεRI recycling was assessed in BMCMCs using biotinylated α-FcεRIα Fab fragments, as described in Subheading 3.4. The bar graph represents mean + SEM of percentage FcεRI recycled determinations from three separate batches of BMCMCs

Remove aliquots (1×10^5 cells each; 100 μL each) at 5, 15, 30, and 60 min; add cells to a 5 mL FACS tube containing 3 mL of 4 °C FACS buffer and keep on ice (until all time points have been collected).

12. Centrifuge tubes at $500 \times g$, 5 min, 4 °C. Aspirate the supernatant.
13. Prepare propidium iodide (PI) staining solution: dilute PI stock 1/1,000 in FACS buffer (final PI concentration 1 μg/mL). Resuspend the pellet in 200 μL PI staining solution.
14. Acquire cell-associated AF647 and PI fluorescence on a flow cytometer (no compensation is usually necessary for these two fluorophores).
15. Analyze results using data analysis software (we use FlowJo software [Tree Star, Ashland, OR], but other software, such as software that comes with the flow cytometer, can be used). Calculate MFI of the AF647 channel (*see Note 4*) for each time point (after gating out PI-positive/dead cells). Using the MFI for each time point, calculate percent recycled FcεRI using the following equation: $[\text{MFI (time X)} - \text{MFI (time 0)}] / \text{baseline MFI}$. An example of the typical results produced is shown in Fig. 3.

4 Notes

1. It is acceptable to use PBS+2 % FBS, PBS+0.5 % FBS, PBS+0.1 % BSA, or PBS as FACS buffer for flow cytometry staining. Prepare FACS buffer fresh (on the day of experiment) or add 0.1 % sodium azide (NaN_3) to FACS buffer and store at 4 °C.

2. If you have a limited number of cells (e.g., less than 5×10^4), you can use a microcentrifuge (Eppendorf) tube so that the pellet can be visualized (if compatible with the flow cytometer being used). Wash volumes will need to be adjusted accordingly (depending on tube capacity).
3. Anti-mouse CD16/CD32 clone 93 or clone 2.4G2 antibodies can be used to block mast cell expressed Fc binding to antibodies used for flow cytometry.
4. Anti-mouse FcεRIα (MAR-1) antibodies or streptavidin can be coupled to other convenient fluorophores. We chose APC (or similar excitation/emission spectra fluorophores such as Alexa Fluor® 633 or Alexa Fluor® 647)-conjugated antibodies or streptavidin for our experiments to minimize compensation-induced changes from green- and red-emitting fluorophores that may effect the mean fluorescence intensities (MFI) that we compute and compare between populations.
5. It is acceptable to culture or stimulate BMCMCs in Iscove's Modified Dulbecco's Medium (IMDM) for all protocols that refer to DMEM.
6. A growing field of data shows that different mouse monoclonal IgE molecules vary in their ability to induce signaling events (and survival) in mouse mast cells in the absence of known antigen. Of the IgE antibodies studied so far, the mouse monoclonal anti-DNP IgE, clone SPE-7 (Sigma), displays the strongest signaling potential in mast cells [30, 31] and has been called highly cytokinergic [32], whereas other IgE clones (e.g., H1 ε-206 and H1 ε-26) that minimally activate mast cells [33, 34] have been called poorly cytokinergic [32]. When studying FcεRI dynamics, it is important to know whether you are using a poorly or highly cytokinergic IgE, since different mouse monoclonal IgE molecules may cause differences in FcεRI surface expression, internalization, and receptor cycling (e.g., SPE-7 can stimulate receptor internalization in the absence of known antigen). We used poorly cytokinergic IgE (i.e., H1 ε-26) for our studies, but it may be useful and interesting to examine the effects of highly cytokinergic IgE molecules in certain model systems.
7. Monovalent streptavidin is necessary in the FcεRIα flow cytometric recycling assay to label biotinylated anti-FcεRIα Fab fragments without cross-linking the FcεRI receptors. Cross-linking the FcεRI receptors would likely induce signaling-mediated internalization of the receptor and interfere with the study of FcεRIα dynamics. Normal or wild-type streptavidin cannot be used because it can bind up to four biotin moieties. The Ting Lab [35] has generated and made available a modified streptavidin molecule that only has one biotin

binding site, thus eliminating the possibility of streptavidin-induced cross-linking of biotinylated Fc ϵ RI α Fabs. Using the protocol listed in the nature protocol exchange site <http://www.nature.com/protocolexchange/protocols/413> and the Addgene vector (plasmid number 20859), one can generate and purify these extremely useful molecules. We labeled our monovalent streptavidin with AF647 using the Alexa Fluor[®] 647 protein labeling kit (Invitrogen; catalog #A20173), but monovalent streptavidin can be coupled to other convenient fluorophores, such as AF633 or APC (*see* **Note 4**).

8. To generate anti-mouse Fc ϵ RI α monovalent Fab fragments, we used the Pierce Fab Micro Preparation Kit (catalog #44685), as per manufacturer's instructions. Briefly, 100 μ g of whole anti-mouse Fc ϵ RI α , clone MAR-1, antibody was digested with immobilized papain for 16 h in digestion buffer. Undigested antibodies and Fc fragments were removed by Protein A spin columns. The flow-through fraction, containing the Fab fragments, was purified with desalting spin columns (to remove cysteine and EDTA) and analyzed by reducing and nonreducing polyacrylamide gel electrophoresis (PAGE) to confirm the absence of contaminating F(ab')₂ fragments. The purified Fab fragments were conjugated to NHS-biotin using the Pierce EZ-Link Sulfo-NHS-Biotinylation Kit (catalog #21925), as per manufacturer's instructions. The concentration of the biotinylated anti-mouse Fc ϵ RI α Fab fragments was determined by measuring UV absorbance at 280 nm. Dilute biotinylated anti-mouse Fc ϵ RI α Fab fragments in PBS to prepare a 0.1–1 mg/mL stock solution.
9. To prepare APC-conjugated isotype control antibody staining solution, dilute APC-conjugated isotype control antibody 1/200 in FACS buffer. Add 20 μ L staining solution to desired tubes (as described in Subheading 3.1). If anti-mouse Fc ϵ RI α antibodies are conjugated to a fluorophore other than APC (*see* **Note 4**), be sure to use an isotype control antibody conjugated to the same fluorophore.
10. If using purified peritoneal mast cells (or BMCMCs that have been incubated with IgE), the best way to accurately assess total Fc ϵ RI levels is to stain the cells with anti-mouse Fc ϵ RI α antibodies (as described in Subheading 3.1) plus anti-IgE antibodies coupled to the same fluorophore as the anti-mouse Fc ϵ RI α antibodies. It appears that when IgE is bound to its receptor, Fc ϵ RI α is no longer recognized or is recognized at much lower efficiency by anti-mouse Fc ϵ RI α , clone MAR-1, antibodies (perhaps IgE binding causes a conformational change in the Fc ϵ RI α chain or steric effects that occlude antibody binding). Alternatively, one can use this information to assess the IgE-bound versus free pools of Fc ϵ RI α on the

surface of mast cells by staining with anti-mouse FcεRIα antibodies and anti-IgE antibodies coupled to different fluorophores.

11. For each population of cells being studied, a typical experiment requires an unstained control and cells that have been activated with anti-IgE for 0, 5, 15, 30, 60, and 90 min at 37 °C. Thus, we typically used 7×10^5 cells/experiment when studying stimulation-induced internalization of IgE-bound FcεRIα ($\sim 1 \times 10^5$ cells for each condition or time point).
12. For each population of cells being studied, a typical experiment requires an unstained control and cells that have been at 37 °C for 0, 2, 4, 6, and 12 h. For the 2-, 4-, 6-, and 12-h time points, cells were treated with vehicle (DMSO), cycloheximide, or brefeldin A in separate wells. Thus, we typically used 3×10^6 cells/experiment when studying FcεRI (2×10^5 cells for each condition or time point).
13. When initiating studies with cycloheximide and brefeldin A, it is worthwhile to test a range of concentrations to determine the lowest concentration that will exhibit effects, given that both of these compounds are documented to be toxic to cells.
14. For each population of cells being studied, a typical experiment requires an unstained control and cells that have been at 37 °C for 0, 5, 15, 30, and 60 min. Thus, we typically used 6×10^5 cells/experiment when studying FcεRI recycling (1×10^5 cells for each condition or time point).
15. Biotinylated anti-mouse FcεRIα Fab fragments will bind to FcεRI receptors expressed on the surface of BMCMCs. The extended incubation time at this step allows a number of rounds of FcεRI recycling to occur; FcεRIα-bound Fab fragments will be internalized during the normal turnover of the FcεRI complex. Thus, at the end of this step, the FcεRI complexes undergoing intracellular recycling and those expressed on the surface of BMCMCs will be labeled with biotinylated anti-mouse FcεRIα Fab fragments.
16. This step will effectively saturate or block the biotin moieties on the Fab fragments bound to FcεRI complexes on the surface of the BMCMCs; thus, only Fab fragments bound to intracellular FcεRI complexes will be free to bind the AF647-conjugated monovalent streptavidin added in subsequent steps.
17. AF647-conjugated monovalent streptavidin will bind to biotinylated anti-mouse FcεRIα Fab fragments on the surface of the BMCMCs that were not blocked with unlabeled monovalent streptavidin. Typically, the fluorescence observed at the 0 min time point is similar to that observed in the unstained control; however, fluorescence can be higher at the 0 min time point. As intracellular FcεRIα-bound Fab fragments reach the surface of the cell (as observed when the cells are incubated at 37 °C

in subsequent steps), the AF647-conjugated monovalent streptavidin will bind to the biotinylated anti-mouse FcεRIα Fab fragments and increase cell-associated AF647 fluorescence. An important control experiment is to incubate cells that have not been exposed to Fab fragments with AF647-conjugated monovalent streptavidin to ensure that the pinocytosis does not contribute substantially to cell-associated AF647 fluorescence during the time frame of your experiment.

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