



Exon Skipping of FcεRIβ for Allergic Diseases

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Abstract

Mast cells are key effector cells in allergic inflammation and consequently are ideal targets for new therapeutics. The high-affinity IgE receptor complex, FcεRI, plays a critical role in mast cell and basophil activation by allergens to drive the immediate allergic inflammatory response. The β subunit of FcεRI is critical for trafficking the FcεRI complex to the cell membrane and amplifies the FcεRI signaling cascade. We have utilized splice switching antisense oligonucleotides to force expression of a truncated isoform of FcεRIβ, which we have shown does not associate with the FcεRI complex. This approach eliminates surface FcεRI expression in mast cells by targeting protein–protein interactions. Exon skipping has several therapeutic applications, and our findings demonstrate a novel application to alter receptor trafficking and dampen allergic inflammation. Here, we describe the methods of exon skipping in mast cells and the assays used to examine the responses of mast cells in vitro and in vivo.

Key words Mast cell, Allergic diseases, Allergic inflammation, FcεRI, Exon skipping

Abbreviations

AON	Antisense oligonucleotide
APC	Allophycocyanin
BMMC	Bone marrow-derived mast cells
BSA	Bovine serum albumin
dH ₂ O	Deionized H ₂ O
DMSO	Dimethylsulfoxide
DNP-HSA	Dinitrophenyl-human serum albumin
FcεRI	High-affinity IgE receptor
FcεRIβ	β subunit of the FcεRI complex
FITC	Fluorescein isothiocyanate
PBS	Phosphate-buffered saline

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PE	Phycoerythrin
PNAG	p-nitrophenyl N-acetyl- β -D-glucosamide
rhSCF	Recombinant human stem cell factor
t-Fc ϵ RI β	Truncated variant of Fc ϵ RI β lacking exon 3

1 Introduction

Asthma and related allergic diseases are common and prevalence in the developed world is increasing [1, 2]. The most widely used treatment strategies for allergy and asthma are insufficient and most therapies for asthma rely upon dampening inflammation with glucocorticosteroids and/or relaxing airway constriction with β -agonists. Adverse effects of high steroid doses, variable patient compliance, and the heterogeneity and complex pathophysiology of allergic diseases limit the treatment efficacy, and thus new therapeutic approaches remain a critical need. Mast cells play a key role in the immediate allergic response by releasing pro-inflammatory mediators in response to allergens (for reviews see [3, 4]). Many emerging therapies for allergic diseases target specific inflammatory mediators rather than the cells producing those mediators. Targeting single mediators in an inflammatory response only removes a fraction of the inflammatory “pool” of mediators. Therefore, targeting mast cells that are the source of many key inflammatory mediators would represent a more desirable therapeutic target for allergic diseases.

A principal mechanism of mast cell activation is through IgE-dependent stimulation of mast cells in response to allergens. Allergen-specific IgE antibodies bind to tetrameric high-affinity receptors for IgE (Fc ϵ RI) on the mast cell surface. Crosslinking Fc ϵ RI-bound IgE with specific multimeric allergens triggers Fc ϵ RI aggregation and a phosphorylation cascade, resulting in calcium influx and the subsequent release of stored and newly generated pro-inflammatory mediators (for reviews see [5, 6]). As an essential participant in IgE-induced mast cell activation, Fc ϵ RI represents a point for therapeutic intervention and thus identifying molecular mechanisms that regulate Fc ϵ RI signaling are of great interest, but identifying targets that are specific to Fc ϵ RI signaling are difficult.

The Fc ϵ RI tetramer complex consists of α , β , and two γ subunits, all of which fulfill specific functions. Of particular interest is Fc ϵ RI β , since the full length variant of this subunit is responsible for trafficking the Fc ϵ RI receptor complex to the mast cell surface, thus facilitating Fc ϵ RI-mediated mast cell activation as well as amplifying signaling directly by recruiting Src family kinases [7–11]. In contrast, the naturally occurring truncated variant of Fc ϵ RI β (t-Fc ϵ RI β) lacks exon 3, which encodes the first two trans-membrane domains necessary for protein–protein interactions and

trafficking of the FcεRI complex to the plasma membrane [9, 12, 13]. Since FcεRIβ expression is limited to mast cells and basophils, we devised a method to target FcεRIβ and disrupt FcεRI trafficking by utilizing therapeutic antisense oligonucleotide (AON)-mediated exon skipping as an approach to specifically target these cell types. Exon skipping of FcεRIβ forces expression of t-FcεRIβ isoform, which we have shown does not associate with the FcεRI complex [12, 13]. Thus, FcεRIβ AON delivery to mast cells eliminates surface FcεRI expression rendering mast cells completely unresponsive to IgE in vitro with markedly diminished early and late phase allergic inflammation in vivo when tested in a mouse model of allergic dermatitis [14]. These results indicate that FcεRIβ AON delivery to mast cells could offer a novel therapeutic strategy for allergic diseases.

In this chapter, we describe the protocol for targeted FcεRIβ exon-skipping in mast cells and assays to examine the downstream effects of AON-induced FcεRIβ truncation in mast cell function and FcεRI trafficking. These protocols are suitable for the mast cell line LAD-2, but can be easily adapted for primary mast cells from both rodent and human origin.

2 Materials

2.1 Transfection of Mast Cells with Antisense Oligonucleotides

1. StemPro-34 complete medium (GIBCO, Grand Island, NY): 13 mL StemPro-24 Nutrient Supplement, L-glutamine (2 mM), penicillin G (100 IU/mL), streptomycin (100 µg/mL) in 500 mL StemPro-34 serum-free medium.
2. Recombinant human stem cell factor (rhSCF).
3. Amaxa Nucleofector cell line Nucleofection kit V (Lonza, Switzerland).
4. Antisense oligonucleotides. In our examples, we use morpholino oligonucleotides from Gene-Tools LLC. For mouse FcεRIβ the following AON was used 5'-GTGTTGCC TGTGGAACATGAATT-3'. For human FcεRIβ the following AON was used 5'-CACAAATATGGCTCCCCAG AATGGA-3'.
5. For adapting the protocol for mouse bone marrow-derived mast cells (BMMCs) *see* **Note 1**.

2.2 Monitoring Mast Cell Degranulation Response by β-Hexosaminidase Assay

1. Biotin-conjugated human myeloma IgE (*see* **Note 2**).
2. Streptavidin.
3. Thapsigargin.
4. HEPES buffer: 10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na₂HPO₄·7H₂O, 5.6 mM glucose, 1.8 mM CaCl₂·2H₂O, 1.3 mM Mg²⁺ (as MgCl₂ or MgSO₄) in distilled

or deionized H₂O (dH₂O). To make 1 L, dissolve components except the calcium and magnesium salts in 900 mL dH₂O: 2.38 g HEPES, 8.00 g NaCl, 0.200 g KCl, 0.103 g Na₂HPO₄·7H₂O, 1.008 g glucose. Adjust pH to 7.4 using concentrated NaOH. Add 0.265 g of CaCl₂·2H₂O and 1.3 mL of a 1 M MgCl₂ solution. Adjust volume to 1 L with dH₂O. Sterilize by filtration (pore size: 0.22 μM) and store at 4 °C.

5. HEPES buffer with 0.04% (w/v) bovine serum albumin (BSA): Add 0.04 g BSA fraction V (>96% purity) to 100 mL HEPES buffer. Warm up to 37 °C and mix well.
6. p-Nitrophenyl N-acetyl-β-D-glucosamide (PNAG) (Sigma-Aldrich).
7. Citrate buffer (pH 4.5): citric acid (40 mM), Na₂HPO₄·7H₂O (20 mM). To make 286 mL Dissolve 2 g citric acid and 1.53 g Na₂HPO₄·7H₂O in 200 mL dH₂O. Adjust pH to 4.5 using NaOH. Adjust volume to 286 mL with dH₂O.
8. Glycine (400 mM, pH 10.7).
9. Triton X-100 (0.1%).
10. V-bottomed 96-well plates.
11. Flat-bottomed 96-well plates.
12. For adapting the protocol for BMMCs these additional materials are required as well as those described in **Note 1**. Mouse anti-dinitrophenyl (DNP) IgE, clone SPE-7. DNP-human serum albumin (HSA).

2.3 Monitoring Mast Cell Response by Calcium Assay

1. Fura-2 AM ester.
2. Dimethyl sulfoxide.
3. HEPES buffer with 0.04% BSA (*see* Subheading 2.2).
4. HEPES buffer with 0.04% BSA and sulfinpyrazone (0.3 mM). To make 100 mL: Dissolve 12.13 mg sulfinpyrazone in 100 mL HEPES buffer with 0.04% BSA.
5. Materials for LAD-2 sensitization (*see* Subheading 2.2).
6. Flat-bottom black 96-well plate.
7. Fluorescence plate reader capable of rapid 2 channel real time fluorescence with injectors and heated chamber. Examples given in this protocol used the BioTek Neo2 multimode plate reader with Fura-2 filter set and dual injectors.
8. For adapting the protocol for BMMCs these additional materials are required as well as those described in **Note 1**. Mouse anti-dinitrophenyl (DNP) IgE, clone SPE-7. DNP-human serum albumin (HSA).

2.4 Monitoring FcεRI Expression by Flow Cytometry

1. Phosphate-buffered saline (PBS).
2. Phycoerythrin (PE) anti-human FcεRI antibody (*see Note 3*).
3. Allophycocyanin (APC) anti-human CD117 antibody.
4. For adapting the protocol for BMMCs these additional materials are required as well as those described in **Note 1**. Allophycocyanin (APC) anti-mouse CD117 antibody, 0.2 mg/mL. FITC anti mouse FcεRIα (clone MAR1).

2.5 Measuring Mast Cell Function In Vivo

1. Mouse anti-dinitrophenyl (DNP) IgE, clone SPE-7.
2. DNP-human serum albumin (HSA).
3. Evans Blue solution (1% in saline) (Sigma).
4. Syringes (23G for IV injections, 29G for intradermal ear injections).
5. Formamide.
6. Heating lamp.
7. Mouse restrainer.
8. Gauze cloth (100 μm pore size).
9. TissueRuptor tissue homogenizer (Qiagen).
10. RNALater (Qiagen).
11. RNAEasy Plus total RNA isolation kit (Qiagen).
12. QIASHredders (Qiagen).
13. Vivo-morpholino antisense oligonucleotides (Gene-Tools, LLC). For mouse FcεRIβ the following AON was used 5'-GTGTTGCCTGTGGAAAACATGAATT-3'. For human FcεRIβ the following AON was used 5'-CACAAATATGGCTCCCCAGAATGGA-3'.

3 Methods

3.1 Transfection of Mast Cells with Antisense Oligonucleotides

In order to assess the activity of antisense oligonucleotides on mast cells it is necessary to ensure that the oligonucleotides are efficiently transfected into the cells and that diffuse localization is achieved rather than endosomal localization. Transfection of mast cells with standard lipid-based transfection protocols can result in strong endosomal localization indicating trapping of the oligonucleotides in endosomes where they will be nonfunctional. However, nucleofection of oligonucleotides results in efficient and reproducible transfection with diffuse cytoplasmic and nuclear localization. This protocol describes the procedure for nucleofection of oligonucleotides into mast cells, but this method can also be adapted for transfection of cDNA or siRNA applications. This protocol describes the procedure for LAD-2 mast cells, but the protocol can be modified for BMMCs (*see Notes 1 and 4*) or other mast cells.

1. Remove 2×10^6 LAD-2 cells from culture (*see Note 5*) for each transfection and place into a 15 mL centrifuge tube.
2. Centrifuge at $250 \times g$ for 5 min at room temperature and aspirate the supernatant.
3. Gently resuspend the pellet in 100 μ L of prewarmed (37°C) nucleofection solution V with supplement added.
4. Add up to 1 nmol of antisense oligonucleotide construct per 2×10^6 cells and transfer to a nucleofector cuvette.
5. Run the appropriate nucleofector program (*see Note 4*).
6. Using a nucleofector pipette, transfer the cells to 4 mL of prewarmed StemPro-34 complete medium (*see Subheading 2.1*) containing 100 ng/mL rhSCF (*see Note 1*) in a 6-well plate and transfer to the incubator (for BMMCs *see Note 6*).

Mast cells are difficult to transfect. Transfection of mast cells with siRNA or plasmid cDNA has been difficult to achieve without the use of lentivirus delivery. However, this method of transfection will work for both siRNA and plasmid cDNA, although we have found this approach to be most effective for splice switching antisense oligonucleotides. We typically achieve >95% transfection efficiency with this method and no evidence of prolonged cytotoxicity is observed with splice switching antisense oligonucleotides. Some cell death (up to 10%) is evident in the first 24 h due to nucleofection (although this is minimized with careful handling of cells), but once these cells have died off the remaining cells exhibit no visible signs of cytotoxicity. Efficient exon skipping can still be observed at 7 days with nondividing or slowly dividing cells, although exon skipping can be reduced after 3 days in rapidly dividing cells. Therefore, the duration and efficacy will depend upon the cell type and the degree of proliferation.

Typical examples of LAD-2 cells and BMMCs transfected with fluorescently labeled antisense oligonucleotides are shown in Fig. 1a. Efficacy of exon skipping in the transfected cells is monitored by RT-PCR with PCR products that span the skipped exon. Efficacy is measured by a shift in PCR product size as shown in Fig. 1b.

3.2 Monitoring Mast Cell Responses to Antisense Oligonucleotide Constructs

In order to assess the efficacy of antisense oligonucleotides to induce exon skipping of Fc ϵ RI β , we measure the functional responsiveness of mast cells to IgE-dependent activation and compare these responses to the degree of exon skipping assessed by RT-PCR. Measurement of mast cell degranulation can be performed using multiple approaches. Our preferred approach is to measure the release of β -hexosaminidase that is stored in mast cell granules and released along with other stored inflammatory mediators during mast cell degranulation. To ensure that exon skipping is specifically targeting IgE-dependent degranulation, we include stimulants

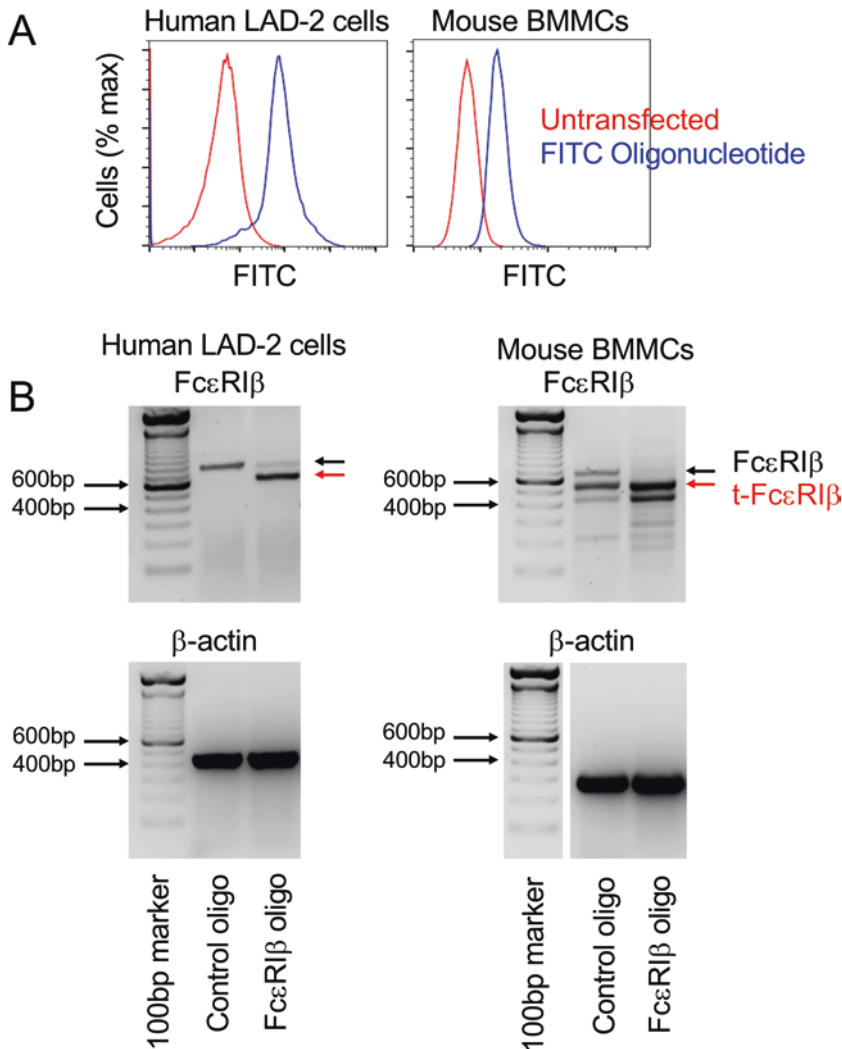


Fig. 1 Analysis of antisense oligonucleotide transfection and efficacy of exon skipping. **(a)** Transfection of antisense oligonucleotides can be monitored using a FITC conjugated oligonucleotide and flow cytometry. Both human LAD-2 cells (left panel) and mouse BMMCs (right panel) demonstrate >95% transfection efficiency of FITC conjugated oligonucleotide (blue lines) when compared to mock treated cells (red lines). X axes are on a log fluorescence scale for the FITC channel in these examples. **(b)** Qualitative RT-PCR analyses of FcεRIβ exon skipping with primers designed to amplify the open reading frame of human FcεRIβ (top left panel) or mouse FcεRIβ (top right panel) mRNA. Exon skipping of exon 3 results in a smaller product. Exon 3 is approximately 150 bp. Control RT-PCR reactions of the reference gene β-actin were included (bottom panels). FcεRIβ exon skipping should be run alongside an appropriate control oligonucleotide as shown

that trigger mast cell degranulation independently of the IgE receptor as controls. In this protocol we will describe using thapsigargin as a control. Thapsigargin induces mast cell degranulation independently from surface receptors by triggering store-operated calcium entry. This protocol describes LAD-2 cells, but the same protocol can be modified for BMMCs (*see Note 7*).

3.2.1 LAD-2 Cell Sensitization and Degranulation

1. Four days after transfection, separate 0.5×10^6 LAD-2 cells and culture overnight (16 h) with biotin-conjugated human myeloma IgE (100 ng/mL) (*see Note 8*).
2. Transfer cells to 15 mL centrifuge tubes and adjust volume to 5 mL with HEPES buffer (*see Note 9*).
3. Centrifuge at $250 \times g$ for 5 min at room temperature and aspirate supernatant.
4. Wash the cells twice in 5 mL HEPES buffer and centrifuge at $250 \times g$ for 5 min at room temperature (*see Note 10*).
5. Resuspend pellet in HEPES-BSA (*see Note 9*) at a concentration of 2×10^4 cells per 90 μ L (0.5×10^6 LAD-2 cells should be resuspended in 2.25 mL).
6. Transfer cells to V-bottom 96-well plate. Each well should contain 90 μ L (*see Note 11*).
7. Prepare 10 $\times$ dilutions of streptavidin and thapsigargin diluted in HEPES-BSA (*see Note 12*).
8. Add 10 μ L of streptavidin or thapsigargin dilutions to corresponding wells.
9. Incubate plate at 37 °C for 30 min (*see Note 13*).
10. Centrifuge plate at $250 \times g$ for 5 min at 4 °C.
11. Transfer 50 μ L of each supernatant to the corresponding wells of a flat-bottom 96-well plate (*see Note 14*) and leave on bench at room temperature until **step 16**.
12. Add 150 μ L of Triton X-100 (0.1%) to each pellet (*see Note 15*) and incubate for 5 min at room temperature to lyse cells.
13. Mix lysates by pipetting and transfer 50 μ L of each lysate to the corresponding wells of a flat-bottom 96-well plate.
14. Add 100 μ L of PNAG to all supernatant and lysate samples.
15. Incubate plates at 37 °C for 90 min (*see Note 16*).
16. Add 50 μ L of glycine to all samples to stop reaction.
17. Measure absorbance at 405 nm (*see Note 17*).

Typical examples of degranulation responses of LAD-2 cells and BMMCs measured by β -hexosaminidase release are shown in Fig. 2. Measuring the effect of antisense oligonucleotides on mast cell function using β -hexosaminidase is a rapid and cost-effective measure of responsiveness to antigen. Mast cell responsiveness can also be monitored by assaying release of eicosanoids or cytokines using commercially available ELISAs. However, measuring cytokine release requires many more cells and longer incubation times.

3.2.2 LAD-2 Cell Sensitization and Calcium Signaling Assay

Mast cell responsiveness can also be measured by assaying calcium signaling. Influx of calcium is critically required for mast cell degranulation. This protocol describes the assay for LAD-2 human

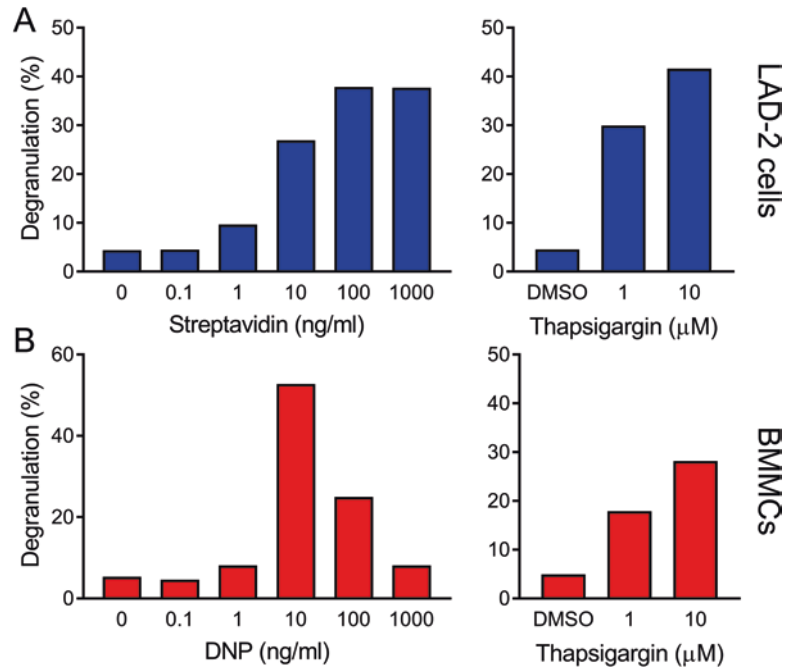


Fig. 2 Analysis of mast cell degranulation by β -hexosaminidase release. (a) Example graphs of β -hexosaminidase release from LAD-2 human mast cells stimulated with a dose-response of streptavidin (left graph) and thapsigargin (right graph) to measure IgE-dependent and receptor-independent mast cell degranulation, respectively. (b) Example graphs of β -hexosaminidase release from mouse BMMCs. For mouse BMMCs DNP is used as the antigen. Data are typical examples expressed as percent release from total β -hexosaminidase calculated from matched cell pellets and supernatants for each well

mast cells, but the procedure can be modified for BMMCs (*see Note 7*) or other mast cells.

1. Twenty four hours after transfection, separate $0.5\text{--}1.0 \times 10^6$ LAD-2 cells and adjust to 1.0×10^6 cells/mL in StemPro-34 complete medium.
2. Culture overnight (16 h) with biotin-conjugated human myeloma IgE (100 ng/mL) (*see Note 8*).
3. Prepare Fura-2 AM by adding 20 μL DMSO to 50 μg Fura-2 AM (*see Note 18*).
4. Add 0.5 μL of Fura-2 AM to 250 μL of cells.
5. Incubate for 30 min in dark at 37 °C.
6. Wash the cells twice in 5 mL of HEPES–BSA–sulfinpyrazone buffer and centrifuge at $250 \times g$ for 5 min at room temperature (*see Note 19*).
7. Resuspend pellet in HEPES–BSA–sulfinpyrazone at a concentration of 10^4 cells in 90 μL per well.

8. Transfer cells into flat-bottom black 96-well plate.
9. Prepare 10× dilutions of streptavidin and thapsigargin diluted in HEPES–BSA (*see Note 12*).
10. Add 10 μ L of streptavidin or thapsigargin dilutions to corresponding wells (*see Note 20*).
11. Assess calcium signaling using the 340–380 nm excitation ratio.

Typical examples of calcium assays with antigen or thapsigargin stimulation in LAD-2 cells and BMMCs are shown in Fig. 3.

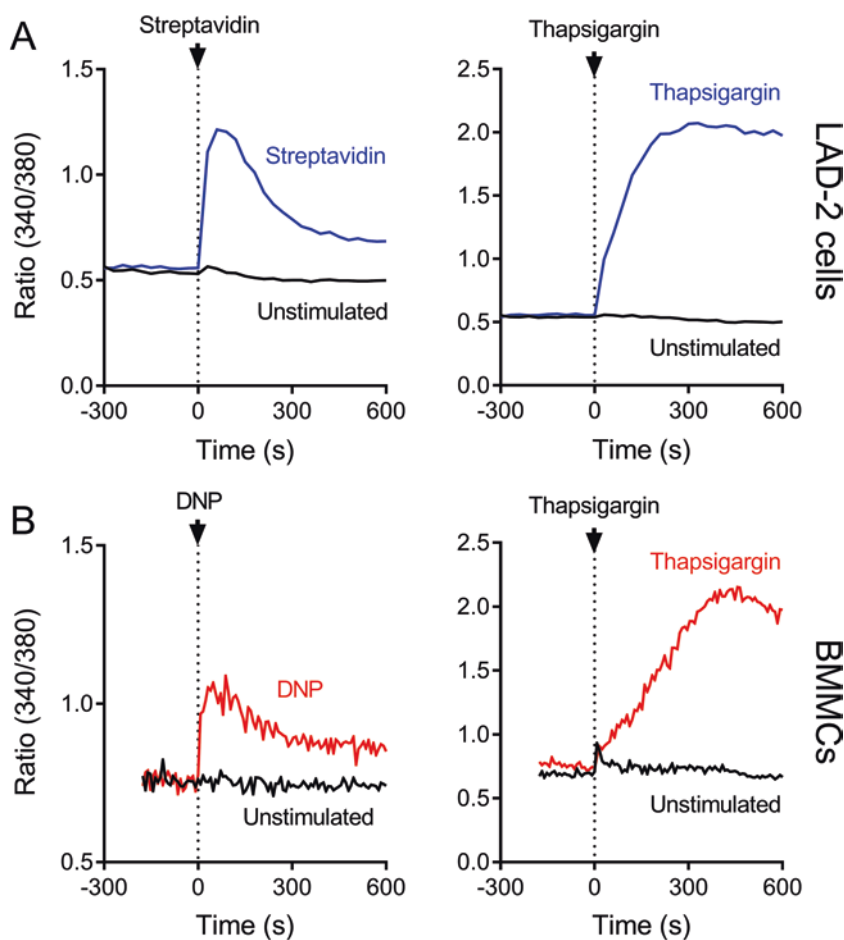


Fig. 3 Analysis of calcium dynamics in suspension mast cells using Fura-2 measurements and real-time fluorescence. (a) Typical ratiometric fluorescence traces of LAD-2 mast cells either stimulated with streptavidin (left panel), thapsigargin (right panel) or vehicle control (black line, both panels). The dotted lines and arrows indicate the point of stimulus addition. (b) The same layout as in a, except BMMCs were used and DNP was used in place of streptavidin. Data in a was acquired every 30 s. Data in b was acquired every 8 s. Optimal timing of data acquisition depends upon experimental design and the number of conditions

3.3 Analysis of Exon Skipping Efficacy with FcεRI Flow Cytometry

Efficacy of FcεRIβ exon 3 skipping can be assessed by measuring surface FcεRIα expression using flow cytometry. The first transmembrane domain of FcεRIβ, encoded by exon 3, is critical for trafficking the FcεRI complex to the cell surface. FcεRIα is the IgE binding subunit and therefore is displayed on the surface. This protocol describes the staining procedures for measuring surface FcεRIα and the growth factor receptor for stem cell factor, KIT (CD117) in LAD-2 cells. This protocol can be easily adapted for BMNCs (*see* **Notes 1** and **4**).

1. Forty eight hours after transfection, transfer 0.2×10^6 LAD-2 cells to 5 mL FACS tubes containing 1 mL PBS (*see* **Note 21**).
2. Centrifuge tubes at $250 \times g$ for 5 min at 4 °C and discard supernatant.
3. Wash cells twice in 1 mL PBS and centrifuge at $250 \times g$ for 5 min at 4 °C (*see* **Note 22**).
4. Resuspend pellet in 100 μL ice-cold PBS and place tubes on ice.
5. Add 4 μL of PE anti-human FcεRI antibody and APC anti-human CD117 antibody to cells and mix by pipetting.
6. Incubate on ice in dark for 1 h.
7. Add 900 μL of ice-cold PBS to each tube.
8. Mix by pipetting and centrifuge at $250 \times g$ for 5 min at 4 °C.
9. Wash cells twice in 1 mL PBS and centrifuge at $250 \times g$ for 5 min at 4 °C.
10. Resuspend pellet in 300 μL ice-cold PBS.
11. Run samples on the flow cytometer.

Typical examples of receptor expression analyses in LAD-2 cells and BMNCs are shown in Fig. 4.

3.4 Measuring Mast Cell Function In Vivo with Passive Cutaneous Anaphylaxis

In order to assess the effects of therapeutics on mast cell function in vivo we utilize mast cell-dependent allergic inflammation models. To avoid the potential for confounding factors from the immune system that can complicate interpretation of data with active sensitization protocols, we favor passive sensitization and anaphylaxis models as initial testing models. The two most common passive anaphylaxis models are Passive Systemic Anaphylaxis (PSA) and Passive Cutaneous Anaphylaxis (PCA) models. Systemic administration of therapeutic oligonucleotides and efficient permeation into tissues such as skin are significant challenges to the therapeutic oligonucleotide field (reviewed in [15]). For this reason, we utilize PCA where local delivery can be achieved and the effects on mast cells can be assessed. This protocol describes the procedure for performing PCA with local administration of antisense oligonucleotides into the skin.

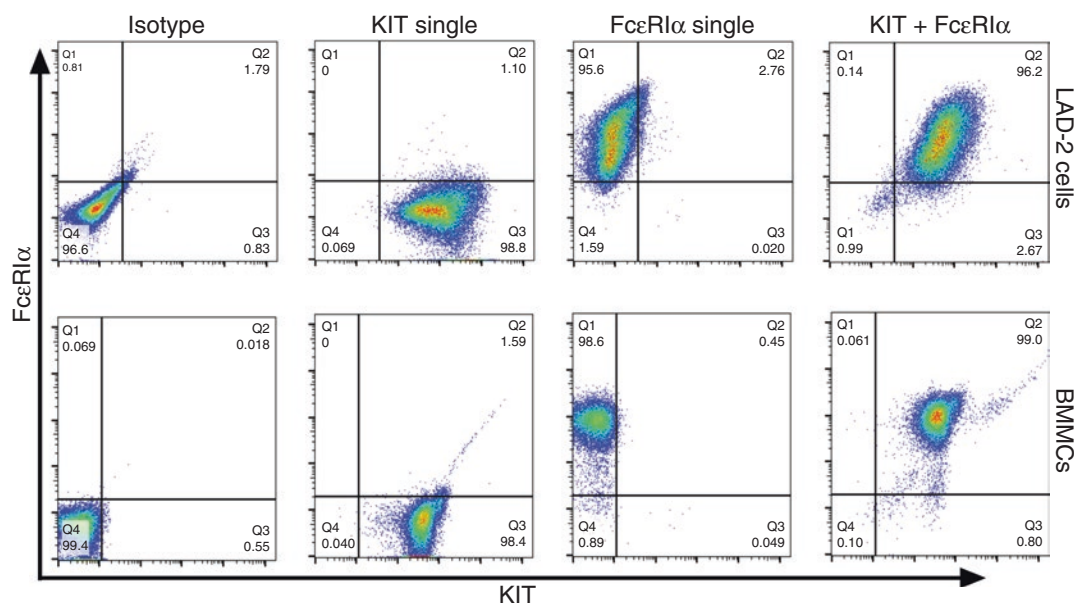


Fig. 4 Analysis of surface FcεRI and KIT receptor expression in mast cells by flow cytometry. Typical examples of surface FcεRI and KIT expression in LAD-2 cells (top panels) and BMMCs (bottom panels) are shown. Density plots for the isotype controls, KIT single stained, FcεRI single stained, and FcεRI and KIT double stained cells are shown (from left to right, respectively). Density plots are gated on the single cell population for each sample and the data are compensated with single stained controls

1. Anesthetize an appropriate number of 2- to 4-month-old age-matched mice (depending on experimental design) using up to 5% isoflurane (*see Note 23*).
2. Inject 10 μ L of 0.5 mM FcεRI β Vivo-morpholino into the dermis of the dorsal side and repeat for the ventral side of both ears of the FcεRI β oligonucleotide treated mice (*see Note 24*).
3. Repeat **step 2** with Standard Control Vivo-morpholino for the control oligonucleotide mice.
4. Repeat **steps 1–3** for the groups of mice every 7 days for at least 2 weeks (*see Note 25*).
5. After treatment of the mice with oligonucleotides has completed, anesthetize the mice as in **step 1** and inject 75 ng of anti DNP-HSA IgE in 10 μ L of sterile PBS into the dermis of the dorsal side and repeat for the ventral side of one ear for both the FcεRI β oligonucleotide and standard control oligonucleotide treated groups.
6. Perform a sham treatment of the opposite ear for all mice using 10 μ L of sterile PBS into the dermis of the dorsal and ventral sides of the ear exactly as performed for the IgE.
7. Twenty four hours after sensitization with IgE, anesthetize the mice as in **step 1** and inject 200 μ L of 1 mg/mL DNP-HSA

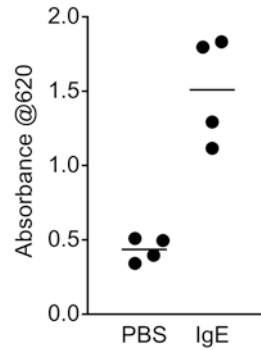


Fig. 5 Analysis of mast cell-mediated immediate inflammation by passive cutaneous anaphylaxis. A typical example of absorbance data obtained from four mice following a PCA reaction. Extravasation of Evans Blue dye measured in the ears of each mouse either sensitized with IgE in one ear or given PBS in the opposite ear

(see **Note 26**) in saline solution containing 0.5% (w/v) Evans Blue dye intravenously (see **Note 27**).

8. Observe the mice for 30 min.
9. Euthanize the mice by CO₂ inhalation followed by cervical dislocation and remove the ears (see **Note 28**).
10. Cut the ears into several pieces and place into 1.5 mL tubes.
11. Add 500 μL of formamide to the tubes and incubate at 55 °C for 4 h (see **Note 29**).
12. Once all of the Evans Blue dye has been extracted from the tissue, filter the samples through 100 μm gauze (see **Note 30**).
13. Pipette 200 μL of filtered sample into each well of a 96 well microplate and measure absorbance at 620 nm.

A typical example of passive cutaneous anaphylaxis experiment is shown in Fig. 5.

4 Notes

1. The materials and methods for culturing BMMCs have been described [16].
2. Biotinylate as described in [17] or by using a commercially available biotinylation kit.
3. This antibody selectively binds FcεRIα, which is the subunit of FcεRI that contains the IgE binding domains, which are displayed on the mast cell surface. FcεRIβ is responsible for trafficking FcεRIα and the receptor complex to the mast cell surface.

4. Transfection of mast cells can be achieved using several approaches. This method describes the use of the Amaxa Nucleofector II. For LAD-2 cells, program U-025 is used, for BMMCs program X-001 is used.
5. LAD-2 cells are cultured in StemPro-34 complete medium containing rhSCF (100 ng/mL).
6. BMMCs can be substituted for LAD-2 cells in this protocol provided that the Amaxa Nucleofector II program used is appropriate (*see* **Note 4**) and that the medium used to culture the cells is appropriate (*see* **Note 1**).
7. To substitute BMMCs for LAD-2 cells in this protocol, mouse anti-DNP IgE should be used instead of human myeloma IgE. The medium for BMMCs (*see* **Note 1**) should be used instead of StemPro medium. The stimulant should be DNP rather than streptavidin. To achieve reproducible results in BMMCs, the cell number per well should be increased from 20,000 for LAD-2 cells to 40,000 for BMMCs.
8. For overnight sensitization with biotin-conjugated IgE, transfected LAD-2 cells are not centrifuged or resuspended in fresh medium, but instead are kept in their original medium. While this is not ideal since it is preferable to remove rhSCF before sensitization, the transfected cells are more susceptible to cell death.
9. HEPES and HEPES-BSA buffer should be kept warm at 37 °C.
10. To avoid spontaneous cell death or degranulation, pipetting should be minimal and gentle.
11. Plate should be kept warm at 37 °C while preparing streptavidin and thapsigargin.
12. To assess LAD-2 cell degranulation, a concentration-dependent response to streptavidin and thapsigargin is assessed. The top concentrations of streptavidin and thapsigargin are 1 µg/mL and 0.65 ng/mL, respectively. A log₁₀ range of 5 dilutions of both streptavidin and thapsigargin should be prepared at 10× the final concentration.
13. During this incubation, PNAG should be defrosted at 37 °C. If precipitate is visible after defrosting, briefly vortex PNAG and return to 37 °C until ready to use.
14. 50 µL of supernatant rather than the entire 100 µL of supernatant is transferred to a separate flat-bottom 96-well plate to avoid disturbing the cell pellet.
15. The 1 in 4 dilution following the addition of Triton X-100 to the pellets should be accounted for in data analysis.
16. The catalysis of PNAG by β-hexosaminidase is a limited reaction, so although 90 min is typically longer than necessary, this

ensures a complete reaction and improves reproducibility between assays.

17. Absorbance is also measured at 620 nm to provide a reference value to which 405 nm absorbance can be normalized. This accounts for variables such as volume discrepancies and bubbles.
18. Fura-2 AM is prepared fresh each day.
19. HEPES–BSA–sulfinpyrazone buffer should be kept warm at 37 °C.
20. Stimulants should be added by injectors in a compatible plate reader with an incubator chamber for rapid and reliable data.
21. Fc ϵ RI and CD117 antibodies are added to separate FACS tubes and compared to unstained cells and isotypes as critical controls.
22. Resuspend LAD-2 cells *via* pipetting rather than vortexing to avoid damaging cells.
23. It is important to both age-match mice for these experiments and to use both male and female mice for comparison.
24. Proper injection and delivery of the Vivo-morpholinos should result in a clean bubble of liquid apparent in the skin. It is important to avoid blood vessels and minimize scratching or damage to the skin.
25. The administration protocol may need to be optimized. Four weeks is long enough to see the maximal effects, but shorter administration protocols may be adequate.
26. The mice should be under a warming lamp during the procedure.
27. Intravenous injection can be achieved by tail vein injection or injection into the retro-orbital sinus.
28. It is important to be consistent with the removal of the ears and to avoid the cartilaginous base of the ears. After removal of the ears, some skin from the surrounding area can be removed and placed into RNALater for analysis of exon skipping by RT-PCR. Skin tissue can be stored at 4 °C overnight in RNALater prior to homogenization with a TissueRuptor and RNA isolation as described [14].
29. It is important to ensure that all Evans Blue dye is extracted from the ear. Incubation times can be increased up to 16 hours if insufficient extraction is achieved with shorter incubation times.
30. It is important to remove any hair or pieces of tissue from all samples as these will affect the absorbance.

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