

Exon skipping of FcεRIβ eliminates expression of the high-affinity IgE receptor in mast cells with therapeutic potential for allergy

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Allergic diseases are driven by activation of mast cells and release of mediators in response to IgE-directed antigens. However, there are no drugs currently available that can specifically down-regulate mast cell function in vivo when chronically administered. Here, we describe an innovative approach for targeting mast cells in vitro and in vivo using antisense oligonucleotide-mediated exon skipping of the β-subunit of the high-affinity IgE receptor (FcεRIβ) to eliminate surface high-affinity IgE receptor (FcεRI) expression and function, rendering mast cells unresponsive to IgE-mediated activation. As FcεRIβ expression is restricted to mast cells and basophils, this approach would selectively target these cell types. Given the success of exon skipping in clinical trials to treat genetic diseases such as Duchenne muscular dystrophy, we propose that exon skipping of FcεRIβ is a potential approach for mast cell-specific treatment of allergic diseases.

mast cell | allergy | IgE receptor | oligonucleotides | dermatitis

Asthma and related allergic diseases affect up to 1 in 10 people in developed countries, and about 10% of patients with asthma cannot be controlled with current therapeutic approaches. The most widely used therapies for asthma rely upon dampening the inflammatory response with either oral or inhaled glucocorticosteroids and relaxing the constricted airway smooth muscle cells with β-adrenoreceptor agonists. However, high doses of steroids when needed for severe asthma are associated with undesirable side effects and inhaled β-adrenoreceptor agonists can increase the risk of death from asthma if not used in combination with glucocorticosteroids. It is suggested that β-adrenoreceptor agonists may promote underlying inflammation that could contribute to the airway wall remodeling seen in asthma (1). Other clinical approaches that aim for more long-term alleviation of symptoms, including desensitization with incremental increases in dose of allergen or hyposensitization to induce immune tolerance, have proven beneficial for some, but not all, patients, and serious adverse effects can occur (2).

In addition to asthma, other allergic diseases have similar treatment strategies and also have an unmet clinical need. An example is atopic dermatitis (AD), which like asthma, is also a multifactorial disease with complex pathophysiology, remains incurable, and affects 10–20% of children in the United States (3). The two major symptoms of AD are inflammatory lesions and severe pruritus. Both the prevalence and the economic burden of AD are increasing worldwide. Symptomatic treatment with topical and/or systemic glucocorticosteroids or calcineurin inhibitors are still considered the gold standard. However, due to the known adverse effects of these drugs, prescription rates are low and patient compliance is often poor (3). Thus, there is still a need for new therapeutic approaches with a better safety profile.

An approach for intervention in allergic inflammation that we have pursued is based on the finding that the gene loci 11q12-q13 are strongly linked to allergy and asthma susceptibility and that the membrane spanning 4A (MS4A) gene family is clustered around 11q12-q13 (4–7). Moreover, the genes *MS4A1* (encoding the protein CD20) and *MS4A2* [encoding the protein FcεRIβ (β-subunit of the high-affinity IgE receptor)] are associated with the activation

and proliferation of B cells (8) and mast cells (9–11), respectively, and are therefore considered potential candidates for the linkage of these genetic regions with allergy. In particular, FcεRIβ contributes to IgE-dependent mast cell signaling by trafficking the FcεRI receptor complex to the cell surface and amplifying FcεRI-induced signaling (12, 13). The first transmembrane domain of FcεRIβ is required for trafficking the receptor complex (14), whereas the C-terminal immunoreceptor tyrosine-based activation motif (ITAM) amplifies signaling (15). Thus, a report that polymorphisms in *MS4A2* were associated with development of asthma gained interest (16), but studies into the functional consequence of mutations in *MS4A2* did not appear to affect the function of FcεRIβ (17). However, we have identified the expression of a truncated isoform of FcεRIβ (t-FcεRIβ) that lacks exon 3 of *MS4A2*, which encodes the first two transmembrane domains of FcεRIβ (10). This truncated isoform does not traffic to the plasma membrane (9, 10) and appears to function in intracellular rather than plasma transmembrane signaling (9). Therefore, our hypothesis is that perturbation of *MS4A2* splicing could lead to disproportionate expression of the t-FcεRIβ isoform at the expense of full-length (FL) FcεRIβ isoform and thus perturb trafficking of the FcεRI receptor complex to the plasma membrane as well as mast cell responses to IgE-directed antigens.

Here, we have examined whether manipulation of *MS4A2* splicing favors t-FcεRIβ formation, disrupts FcεRI expression and signaling, and has functional consequences. We identified that forced expression of t-FcεRIβ using antisense oligonucleotide (AON)-mediated exon skipping of *MS4A2* exon 3 eliminated expression of FcεRI in

Significance

We identified an innovative use for the technique of antisense oligonucleotide-mediated exon skipping to specifically target and down-regulate IgE receptor expression in mast cells. Exon skipping is typically used as part of personalized medicine, where a mutant exon is skipped after sequencing the patients' affected genes. Our approach, however, targets a nonmutated gene and an exon that is critical for surface IgE receptor expression. It does not require a personalized approach with genetic sequencing or multiple iterations of oligonucleotides that would require clinical trials. Furthermore, the diseases to be treated with this technology are ideal for local delivery of the oligonucleotides by aerosols or topical cream formulations.

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Conflict of interest statement: Carrying out this study resulted in the filing of US Provisional Patent Application No. 62/289,447 by G.C. and D.D.M.

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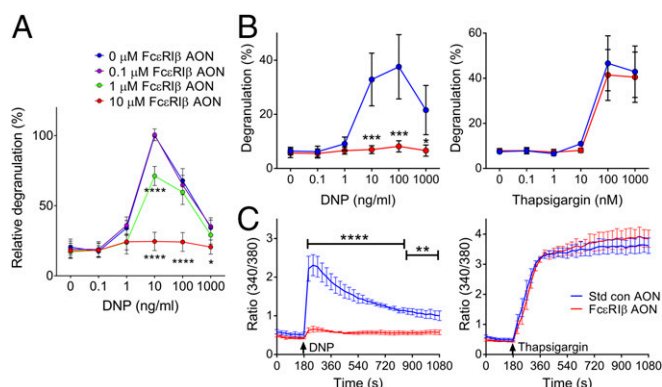


Fig. 2. Transfection of FcεRIβ AONs eliminates mast cell responsiveness to IgE. (A) Transfection of FcεRIβ AON in mouse BMMCs results in a dose-dependent reduction in degranulation in response to antigen challenge. Data are the mean $\pm$ SEM from five experiments. (B) Degranulation of BMMCs in response to either antigen (DNP, *Left*) or thapsigargin (*Right*). Standard control AON is in blue, and FcεRIβ AON is in red. (C) Ratiometric calcium signaling was performed on the same cells as in B. The arrowhead denotes the time of the stimulant addition. Transfection of FcεRIβ AON (red lines) eliminates both degranulation (B, *Left*) and the calcium response (C, *Left*) to antigen without affecting the degranulatory or calcium responses to thapsigargin (B and C, *Right*). Data are the mean $\pm$ SEM from four experiments. Statistical analyses were carried out as ANOVA with Tukey's posttest, comparing each DNP concentration to the corresponding DNP concentration with 0 μM AON (A) or FcεRIβ AON to Std Con AON for each concentration (B) or time point (C). * P < 0.05, ** P < 0.01, **** P < 0.0001.

reduced by FcεRIβ exon skipping (Fig. 3A, C, and D). In contrast to PLCγ1, the AKT and ERK pathways are activated by thapsigargin, but neither AKT nor ERK phosphorylation was affected by FcεRIβ exon skipping (Fig. 3A, C, and D).

Low-level activation of mast cells can result in the production of cytokines without evidence of acute signaling events. One such example is IgE alone, which did not elicit rapid phosphorylation of PLCγ1, AKT, or ERK (Fig. 3A–D), but did cause release of the cytokine GM-CSF after 6 h (Fig. 3E). Nevertheless, this release, as well as that induced by IgE plus antigen to induce FcεRI aggregation, was blocked by FcεRIβ exon skipping without affecting thapsigargin-induced GM-CSF release in BMMCs (Fig. 3E).

We also examined the effects of exon skipping on antigen-mediated BMMC migration (28). Standard control AON-treated BMMCs that were sensitized with IgE migrated toward antigen (DNP-BSA), but FcεRIβ AON-treated BMMCs did not (Fig. 3F). However, BMMC migration mediated through KIT, the receptor for stem cell factor (SCF) was not reduced and if anything it was enhanced by FcεRIβ exon skipping, although not to a statistically significant extent (Fig. 3F). Collectively, these data

indicate that FcεRIβ exon skipping selectively and substantially abrogates FcεRI-dependent responses in BMMCs.

Survival and Proliferation. In addition to the classical view that IgE binding to FcεRI on mast cells primes mast cells for activation by bivalent/multivalent antigens, it is now recognized that IgE binding to FcεRI leads to activation of mast cells to release pro-survival cytokines that maintain viability of the cells (29–31). In the absence of external supporting cytokines, mast cells rapidly undergo apoptosis, which is suppressed by the addition of IgE. Therefore, elevation of tissue IgE during allergic diseases, such as the lung in asthma or the skin in AD, could contribute to increased mast cell numbers by promoting cytokine release and mast cell survival. We therefore tested whether FcεRIβ exon skipping would also eliminate the pro-survival effect of IgE on mast cells. BMMCs were deprived of the culture growth-promoting cytokine, IL-3, for 24 h after transfection with AONs. Over the course of 72 h, IgE almost completely protected mast cells from cell death and apoptosis after withdrawal of IL-3 in control AON-treated BMMCs (Fig. S1). However, treatment of BMMCs with FcεRIβ AON, which resulted in loss of surface FcεRI expression (Fig. S1), eliminated the protective effect of IgE after IL-3 withdrawal (Fig. S1). The apparent decrease in FcεRI expression in the presence of IgE we believe reflects the decreased affinity of MAR-1 for FcεRI once it is occupied by IgE (32). Taken together, FcεRIβ exon skipping could potentially suppress the pro-survival effect of elevated IgE in vivo and thus the increase in mast cell population as well as reduce IgE-dependent degranulation in allergic disease.

We next examined the effects of FcεRIβ exon skipping (day 0) on mast cell proliferation in the presence of IL-3, while monitoring both surface FcεRI expression (Fig. S2) and proliferation using a CellTrace Violet dilution assay (Fig. S2). The majority of the proliferation occurred between day 5 and day 7 with a population of cells appearing with diluted CellTrace dye at day 7 (Fig. S2). There was no difference in proliferation in the absence or presence of FcεRIβ exon skipping at either 5 or 7 d. There was a population of BMMCs treated with FcεRIβ AON that began to express FcεRI on the surface at day 7, despite all of the cells at day 5 being negative for surface FcεRI (Fig. S2), suggesting that these cells were regaining FcεRI expression. Gating the populations of cells based on surface FcεRI expression and plotting CellTrace Violet fluorescence demonstrated that the cells expressing surface FcεRI were the cells that had proliferated (Fig. S2). These data most likely indicate that, although overall proliferation is not affected by FcεRIβ AON treatment, BMMCs that do proliferate dilute the FcεRIβ AON between daughter cells, thus reducing exon-skipping efficacy.

In Vivo Passive Cutaneous Anaphylaxis. As proof-of-concept of therapeutic utility, we sought evidence that the FcεRIβ exon-skipping approach is effective in vivo, having in mind the possibility of

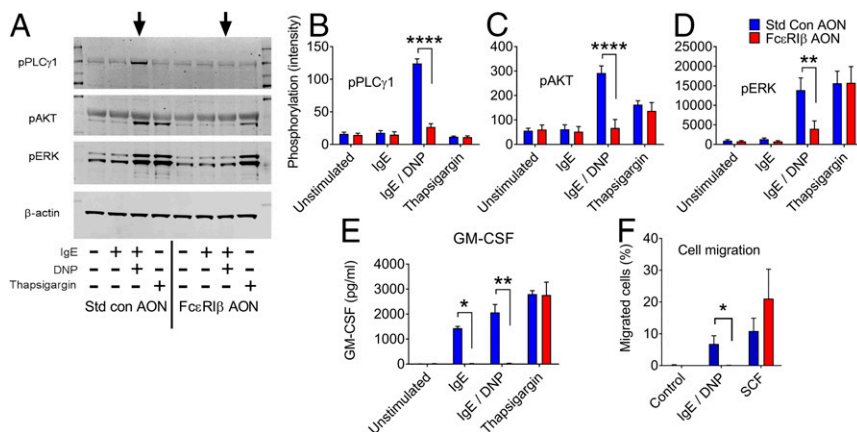


Fig. 3. Transfection of FcεRIβ AONs eliminates IgE-dependent cell signaling, cytokine production, and migration. (A) Immunoblots from mouse BMMCs transfected with either standard control AON (left four lanes) or FcεRIβ AON (right four lanes) and stimulated with the indicated molecules. (B–D) Combined quantification of phosphorylation data for standard control AON (blue bars) or the FcεRIβ AON (red bars)-transfected BMMCs. (E) ELISA data for GM-CSF release after 6 h. (F) BMMC migration with FcεRIβ AON treatment. Data are the mean $\pm$ SEM from three (B–E) or four (F) experiments. * P < 0.05, ** P < 0.01, **** P < 0.0001.

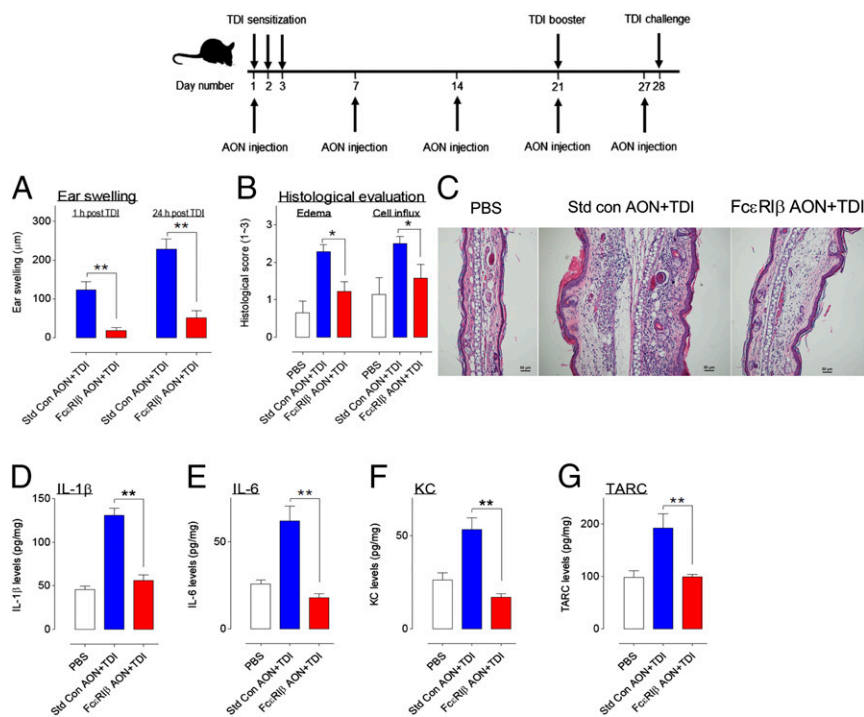


Fig. 5. Repeated administration of FcεRIβ AONs during sensitization reduce the allergic dermatitis response in vivo. (A) Ear-swelling effects of mice treated with either standard control Vivo-Morpholino AON (blue bars) or Vivo-Morpholino FcεRIβ AON (red bars) on TDI-induced allergic dermatitis. Ear swelling was calculated by a comparison of the ear thickness before and after (1 and 24 h) TDI challenge. (B) Histological evaluation of ear skin sections stained with H&E and assessed for edema and cellular infiltrate at 24 h post-TDI challenge. (C) Representative H&E histology sections of ear skin. (D–G) Down-regulated cytokine levels in ear skin of mice treated with either standard control Vivo-Morpholino AON (blue bars) or Vivo-Morpholino FcεRIβ AON (red bars) 24 h following challenge with TDI. Concentrations of IL-1β, IL-6, KC, and TARC were determined by ELISA. Data are the mean ± SEM from eight mice for each group. ***P* < 0.01.

considered the innovative approach of forcing the cell to produce an alternative splice variant of FcεRIβ (t-FcεRIβ) that does not associate with FcεRIα or FcεRIγ. The current study indicates that this approach works efficiently by eliminating surface FcεRI expression and function, rendering mast cells completely unresponsive to IgE. This has significant implications on not only immediate mast cell activation and the wheal and flare reaction, but also on cytokine release and the late-phase allergic response, which we demonstrate is also reduced in an allergic dermatitis in vivo model. In addition, because IgE and FcεRI act as pro-survival and chemoattractant factors for mast cells (28, 40), AON-induced exon skipping of FcεRIβ might reduce mast cell numbers in affected tissues by reducing mast cell recruitment and survival, thus increasing the efficacy of the drug. We are currently examining this in chronic allergy models. Importantly, we demonstrate FcεRIβ exon skipping in vivo as a proof-of-concept for further development of AON-mediated altered splicing of FcεRIβ as a therapeutic approach for allergic diseases.

Our studies provide important proof-of-concept data for developing AON-induced exon skipping of FcεRIβ as a therapeutic

and/or preventative treatment for allergic diseases. Our in vitro and in vivo mouse studies demonstrate surprisingly potent efficacy in vitro with a “knockout” effect and a more variable, but still impressive effectiveness in vivo with some mice exhibiting no histological evidence of inflammation 24 h after TDI challenge in the allergic dermatitis model. Although we demonstrate that efficient exon skipping can be achieved in skin mast cells in vivo, a limitation is that effective delivery of AONs into mast cells in skin is limited by diffusion of drug within the skin and the surface area of skin that can be infiltrated by intradermal injection. Systemically administered AONs distribute poorly into the skin and topical administration would be more desirable delivery mechanism for skin disorders. The AON system that we describe here is probably best suited for topical application. To develop this approach further, we need to identify effective methods of delivery over larger surface areas of skin and into other tissues of interest such as the airways. An additional limitation is that the sequence of the human *MS442* gene is less conducive to exon skipping than the mouse *Ms4a2*. Although the experimental dermatitis model illustrated the full potential of the AON approach in mice, it is apparent that more refined AON configurations might be needed to achieve this in humans.

Despite these challenges, we believe that this approach has the potential for clinical application. AON-mediated exon skipping is emerging as a promising gene therapy approach with the advantage that it does not alter the genome and is reversible with cessation. Exon skipping has been effectively used for rare genetic defects where frameshift mutations in genomic DNA lead to loss of protein function (for reviews, see refs. 18–20). Indeed, two new drug applications for the AON drugs Drisapersen (BioMarin Pharmaceutical) and Eteplirsen (Sarepta Therapeutics) to treat Duchenne muscular dystrophy have been filed with the Food and Drug Administration. However, exon skipping is predominantly used for rare genetic disorders that require gene sequencing, a personalized gene therapy approach, and separate clinical trials and approvals for each exon targeting AON, causing a burden on development. We have devised an AON approach to alter splicing of FcεRIβ with the potential of treating allergic conditions such as asthma and atopic dermatitis, which are amenable to inhaled or topical treatments, respectively, and not associated with a specific genetic defect, thus eliminating the need for a personalized approach. Our data indicate that this could be a viable approach for

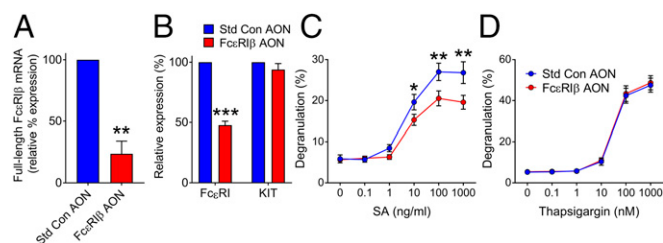


Fig. 6. Transfection of FcεRIβ AONs into human LAD-2 mast cells reduces FcεRI expression and IgE-dependent degranulation. (A) Quantitative RT-PCR of FL FcεRI mRNA expression in human LAD-2 mast cells with transfection of FcεRIβ AON results in about 75% exon skipping. (B) FcεRIβ exon skipping in LAD-2 cells results in about 50% reduction in surface FcεRI with this AON construct. (C) IgE-dependent LAD-2 cell degranulation is significantly reduced with FcεRIβ exon skipping, but thapsigargin-induced degranulation is unaffected (D). Data are the mean ± SEM from three (A), five (B), and seven (C and D) experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

treatment of allergic diseases and a mast cell-specific therapeutic strategy that, we believe, has the potential to translate into the clinic.

Materials and Methods

For additional information on methods, see *SI Materials and Methods*.

Experiments on mice carried out at NIH were conducted under a protocol approved by the Animal Care and Use Committee at National Institute of Allergy and Infectious Diseases (NIAID), NIH. BMDCs were developed from bone marrow obtained from femurs of C57BL/6J mice (The Jackson Laboratory), as previously described (41).

Transfection of Mast Cells with AONs. Transfection was achieved using the Nucleofector II and Cell Line Kit V (Lonza) as described (9), and program X-001 was used. AONs were purchased from Gene-Tools LLC.

Allergic Dermatitis TDI Model. Female 6-wk-old BALB/cAnN were purchased from Charles River Laboratories. All aspects of the current study were conducted in accordance with the Animal Care and Use Program of the North Carolina State University (Institutional Animal Care and Use Committee Protocol No. 13-111-B).

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Supporting Information

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SI Materials and Methods

Mast Cell Culture. Experiments on mice carried out at National Institutes of Health (NIH) were conducted under a protocol approved by the Animal Care and Use Committee at National Institute of Allergy and Infectious Diseases (NIAID), NIH. Bone marrow-derived MCs (BMMCs) were developed from bone marrow obtained from femurs of C57BL/6J mice (The Jackson Laboratory), as described (41). LAD-2 human mast cells were cultured, as described (42), in StemPro-34 medium containing StemPro-34 Nutrient Supplement, L-glutamine (2 mM), penicillin (100 U/mL)/streptomycin (100 µg/mL) (Gibco) with 100 ng/mL recombinant human SCF added (Peprotech). One-half of the medium supplemented with SCF was changed every 7 d.

Transfection of Mast Cells with Antisense Oligonucleotides. For transfection of mast cells with antisense oligonucleotides (AONs), 2×10^6 mast cells were used for each transfection. Transfection was achieved using the Nucleofector II and Cell Line Kit V (Lonza) as described (9), and program U-025 was used for LAD-2 cells, and X-001 was used for mouse BMMCs. Cell viability was monitored during experiments, and there was no evidence of cytotoxicity with AON transfection. To determine transfection efficiency, 10 µM FITC-conjugated standard control 25-mer AON was used (purchased from Gene-Tools). All other AONs were not conjugated.

AONs. AONs were designed to target exon 3 of mouse *Ms4a2* (NM 013516.2) and constructs purchased from Gene-Tools LLC. A region within the splicing acceptor region was targeted with the following sequence: 5'-GTGTTGCCTGTGGAAAA-CATGAATT-3'. For human *MS4A2*, the following AON was used: 5'-CACAAATATGGCTCCCCAGAATGGA-3'. The standard control AON provided by Gene-Tools LLC had the following sequence: 5'-CCTCTTACCTCAGTTACAATTTATA-3'. For in vivo studies, the Vivo-Morpholino chemistry described below was used and purchased from Gene-Tools.

RT-PCR. BMMCs were transfected as described above. After 24 h, mast cells were washed twice in ice-cold PBS and total RNA was isolated using the RNeasy plus mini-kit (QIAGEN) according to the manufacturer's instructions with inclusion of the QIAshredder step. RT-PCR was carried out using the QIAGEN One-Step RT-PCR kit. The primers used were designed to amplify the ORF of FcεRIβ mRNA with the following sequences: forward, 5'-ATGGACACAGAAAATAGGAGCA-3', and reverse, 5'-TGAATCAACTGGA-GAAGATGTTT-3'. For human FcεRIβ mRNA, the following primers were used: forward, 5'-ATGGACACAGAAAAGTAA-TAGGAG-3', and reverse, 5'-TTATAAATCAATGGGAGGA-GAC-3'. Quantitative RT-PCR was carried out as described (9).

Degranulation Assays. To measure degranulation, mast cells were transfected as described above. Cells were cultured for 32 h to allow for loss of surface FcεRI expression, and then sensitized with 100 ng/mL anti-DNP IgE (SPE7 clone) (Sigma) for BMMCs, or biotinylated human myeloma IgE for LAD-2 cells, and incubated overnight (16 h). Degranulation was assayed by β-hexosaminidase release as described (43).

Calcium Imaging. Changes in cytosolic Ca²⁺ levels were determined following loading of the cells with Fura-2 AM ester (Molecular Probes) as described (44). BMMCs were transfected as described above. Cells were cultured for 32 h and then sen-

sitized with 100 ng/mL anti-DNP IgE (SPE7 clone) for 16 h. Fluorescence was measured at two excitation wavelengths (340 and 380 nm) and an emission wavelength of 510 nm. The ratio of the fluorescence readings was calculated following subtraction of the fluorescence of the cells that had not been loaded with Fura-2 AM.

Cytokine Production Assays. BMMCs were transfected as described above. Cells were cultured for 32 h, and then BMMCs were sensitized with 100 ng/mL anti-DNP IgE (SPE7 clone) for 16 h (DNP-stimulated cells only), and some cells were not sensitized with IgE (all other conditions). Cytokine release was assayed at a cell concentration of 1×10^6 BMMCs per mL as described (43). Mouse GM-CSF was measured using Duo-Set ELISAs (R&D Systems) according to the manufacturer's instructions.

Migration Assay. BMMCs were transfected as described above. Cells were cultured for 32 h, and then some cells were sensitized with 100 ng/mL anti-DNP IgE (SPE7 clone) for 16 h (to measure migration toward DNP) and some cells were not sensitized with IgE (to measure migration toward SCF). Migration was assayed using Transwell chambers as described (45, 46) except Transwells with 5-µm pores were used for BMMCs instead of 8-µm pores for human cells. A total of 2×10^5 BMMCs was loaded into the top chamber, and the percentage of cells that migrated to the bottom chamber was calculated.

Cell Signaling. BMMCs were transfected as described above. Cells were cultured for 32 h and then BMMCs were sensitized with 100 ng/mL anti-DNP IgE (SPE7 clone) for 16 h (DNP-stimulated cells only), and some cells were not sensitized with IgE (all other conditions). BMMCs were stimulated, and signaling was carried out according to previous protocols using human mast cells (9, 39, 47). Briefly, 5×10^5 BMMCs were stimulated with the indicated stimulus in 100 µL of Hepes buffer plus 0.04% BSA for 2 min. At 2 min, boiling lysis buffer was added, and samples were syringed to disrupt genomic DNA as described (9). Immunoblotting was carried out as described (8) using the phosphorylation-specific antibodies: anti-PLCγ1 (Tyr783), anti-Akt (Ser473), and anti-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technologies). Anti-β-actin clone AC-15 (Sigma-Aldrich) was used as a loading control. IRDye (680RD or 800CW)-labeled secondary antibodies (1:20,000) were used to label both phosphorylated and total proteins simultaneously. Membranes were visualized and quantified using an Odyssey CLx imager (LI-COR Biosciences).

Proliferation Assay. BMMCs were washed once with PBS. A total of 2×10^6 BMMCs was stained with CellTrace Violet (Invitrogen) according to the manufacturer's instructions. Briefly, cells were stained for 15 min, followed by addition of 5× volume of complete RPMI medium 1640 with 10% (vol/vol) FBS at 37 °C. BMMCs were pelleted and transfected as described above. BMMCs were cultured in complete RPMI 1640 supplemented with 30 ng/mL recombinant mouse IL-3 at 37 °C for 5–7 d and stained with Aqua live/dead (Invitrogen) and FITC-FcεRIα (eBioscience). Flow cytometry was performed on an LSRII flow cytometer. Dead cells were gated out using the Aqua live/dead stain with an additional gate set for the single-cell population.

Apoptosis Assay. BMMCs (2×10^6) were transfected with standard control AON or FcεRIβ AON as indicated above. To allow sufficient time for the AON to be effective, BMMCs were incubated for 24 h in complete RPMI medium 1640 containing 30 ng/mL recombinant mouse IL-3. Flow cytometry for surface

