

IgE receptor of mast cells signals mediator release and inflammation via adaptor protein 14-3-3 ζ



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Background: Mast cells (MCs) are tissue-resident immune cells that mediate IgE-dependent allergic responses. Downstream of Fc ϵ RI, an intricate network of receptor-specific signaling pathways and adaptor proteins govern MC function. The 14-3-3 family of serine-threonine phosphorylation-dependent adapter proteins are known to organize intracellular signaling. However, the role of 14-3-3 in IgE-dependent activation remains poorly defined.

Objective: We sought to determine whether 14-3-3 proteins are required for IgE-dependent MC activation and whether 14-3-3 is a viable target for the treatment of MC-mediated inflammatory diseases.

Methods: Genetic manipulation of 14-3-3 ζ expression in human and mouse MCs was performed and IgE-dependent mediator release assessed. Pharmacologic inhibitors of 14-3-3 and 14-3-3 ζ knockout mice were used to assess 14-3-3 ζ function in a MC-dependent *in vivo* passive cutaneous anaphylaxis (PCA) model of allergic inflammation. Expression and function of 14-3-3 ζ were assessed in human nasal polyp tissue MCs.

Results: IgE-dependent mediator release from human MCs was decreased by 14-3-3 ζ knockdown and increased by 14-3-3 ζ overexpression. Deletion of the 14-3-3 ζ gene decreased IgE-dependent activation of mouse MCs *in vitro* and PCA responses *in vivo*. Furthermore, the 14-3-3 inhibitor, RB-11, which impairs dimerization of 14-3-3, inhibited cultured MC and polyp tissue MC activation and signaling downstream of the Fc ϵ RI receptor and dose-dependently attenuated PCA responses.

Conclusion: IgE/Fc ϵ RI-mediated MC activation is positively regulated by 14-3-3 ζ . We identify a critical role for this p-Ser/Thr-binding protein in the regulation of MC Fc ϵ RI signaling and IgE-dependent immune responses and show that this

pathway may be amenable to pharmacologic targeting. (J Allergy Clin Immunol 2023;152:725-35.)

Key words: 14-3-3, adaptor protein, anaphylaxis, Fc ϵ RI signaling, IgE, mast cells, nasal polyp

Mast cells (MCs) are multifaceted effector cells of the immune system. They are strategically located in close proximity to blood vessels, neurons, and lymphatic vessels, which allows them to serve as sentinel immune cells acting as a first line of defense after encountering infectious agents.¹ Although the beneficial role of MCs in immunity has been the focus of much recent research, MCs are best known as the primary effector cells in type I hypersensitivity reactions, such as allergic rhinitis, allergic asthma, and anaphylaxis.² The release of a vast array of powerful biological mediators upon the activation of their high-affinity receptors for IgE (Fc ϵ RI) can dramatically affect the extracellular milieu and impact tissue homeostasis.^{3,4}

The propagation of Fc ϵ RI signaling requires the spatiotemporal organization of membrane-bound and intracellular signaling molecules that coordinate input-specific downstream cellular responses.⁵ An important class of intracellular signaling molecules are adaptor proteins that serve as activation-dependent molecular scaffolds that regulate and assemble context-specific signaling networks. Historically, phosphorylation of tyrosine residues on transmembrane receptors and organization of signaling complexes by p-Tyr-specific adaptor proteins, including LAT, Grb2, Gab2, and SLP-76, were viewed as the major events leading to multiprotein complex assembly and immediate IgE-dependent activation of MCs.^{6,7} Serine-threonine phosphorylation has also been found to regulate immediate responses in a limited range of immune cells, including T cells;⁸ however, the role of serine-threonine phosphorylation in signaling complex formation and MC activation is poorly understood. We previously identified the WW domain-containing p-Ser/Thr-binding protein Nedd4-2 as an inhibitor of IgE-mediated MC activation.⁹ However, the functions of many p-Ser/Thr-binding proteins and domains, including 14-3-3, forkhead-associated domains, and WD40 repeats in MC activation, remain unexplored.

14-3-3 proteins are a 7-membered family of adaptor proteins that were one of the first molecules to be recognized as distinct p-Ser/Thr-binding proteins.¹⁰ 14-3-3 proteins regulate cellular pathways by affecting protein localization and by altering protein modifications that affect signal transduction. Recent studies have indicated a link between 14-3-3 protein levels and chronic inflammatory disorders.^{11,12} 14-3-3 has been shown to interact with T-bet in human CD8⁺ T cells to restrict T-bet's translocation into

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Abbreviations used

BMCMC:	Bone marrow–derived cultured MC
DNP:	2,4-Dinitrophenol
ERK:	Extracellular signal-regulated kinase
HCMC:	Human cultured MC
HMEM:	Hanks minimum essential medium
HSA:	Human serum albumin
LAMP1:	Lysosomal-associated membrane protein 1
MC:	Mast cell
PCA:	Passive cutaneous anaphylaxis
PI3K:	Phosphatidylinositol 3-kinase
PIPES:	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PPI:	Protein–protein interaction
siRNA:	Small interfering RNA
WT:	Wild type

the nucleus, thus promoting type 2 cytokine production.¹³ 14-3-3 was also found to mediate Toll-like receptor activation of extracellular signal-regulated kinase (ERK)-1/2 mitogen-activated protein kinase kinases and TNF secretion by macrophages.¹⁴

The association of 14-3-3 with client proteins occurs through defined phospho-peptide motifs, 2 of which (RSXpSXP and RXXXpSXP) are highly conserved and recognized by all 14-3-3 isoforms.¹⁵ 14-3-3 isoforms share high sequence similarity, but emerging evidence indicates that they perform different cellular functions via the formation of homo- and heterodimers.¹⁶ The inherently dynamic nature of 14-3-3 as p-Ser/Thr-binding proteins places them at the center of an adaptive signaling hub.¹⁷ We previously reported that the dimerization of 14-3-3 proteins is regulated by the phosphorylation of serine residue 58 (Ser58), which is buried within the dimer interface (numbering relates to the ζ isoform). Once Ser58 is phosphorylated, the dimeric structure of the 14-3-3 is disrupted and its function inhibited.^{9,10}

In this study, we investigated how IgE/Fc ϵ RI-mediated MC function can be regulated by 14-3-3. We show that 14-3-3 ζ is the dominant 14-3-3 isoform expressed in MCs and that 14-3-3 ζ protein expression is increased after IgE cross-linking and in MCs in airway tissue from patients with chronic rhinosinusitis with nasal polyps compared to MCs in healthy sinus tissue. We further show that 14-3-3 ζ is required for MC activation and intracellular signaling downstream of IgE cross-linking and that pharmacologic inhibition of 14-3-3 dimerization decreases MC activity *in vitro* and blocks IgE-induced passive cutaneous anaphylaxis (PCA) *in vivo*. These findings indicating that the IgE–Fc ϵ RI–14-3-3 ζ pathway is a novel target for the treatment of IgE-dependent, MC-mediated allergic disease.

METHODS

Degranulation assay and mediator release measurement

Ten to 12 weeks' culture of human cultured MCs (HCMCs) and 8 weeks' culture of mouse bone marrow–derived cultured MCs (BMCMCs; for details of their generation, see the Methods section in this article's Online Repository available at www.jacionline.org) were sensitized with IgE anti-2,4-dinitrophenol (DNP) mAb (SPE-7; 2 μ g/mL for BMCMCs or human myeloma IgE [0.5 μ g/mL; Calbiochem]) for HCMCs and incubated 16 hours at 37°C. After 16 hours' IgE sensitization, BMCMCs or HCMCs (10⁶ cells/mL) were resuspended in Tyrode buffer (129 mmol NaCl, 8.4 mmol glucose, 10 mmol HEPES, 5 mmol KCl, 1 mmol MgCl₂, 1.4 mmol CaCl₂, and 1% BSA at pH 7.4) and then

incubated with RB-11 (0.05–10 μ mol) for 2 hours at 37°C. Cells were then activated with DNP with human serum albumin (HSA) (10–1000 ng/mL; BMCMCs) or anti-human IgE Ab (1 μ g/mL; HCMCs) for 30 minutes. Histamine, Cys-LT, and prostaglandin D₂ (PGD₂) levels in supernatants and corresponding cell lysates (histamine only) were measured using histamine (Beckman Coulter) or Cys-LT EIA and PGD₂ enzyme immunoassay (Cayman Chemicals) kits according to the manufacturers' instructions. For measurement of β -hexosaminidase release, supernatants and corresponding cell lysates were incubated with the substrate *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (aka pNAG) solution in citrate buffer at 37°C for 1 hour, followed by addition of glycine buffer. Absorbance was measured with a microplate reader at 405 nmol with reference filter at 620 nm. Histamine and β -hexosaminidase release are expressed as a percentage of total cellular content of histamine (histamine release [%]) and β -hexosaminidase (degranulation [%]).

Topical RB-11 application and IgE-dependent PCA

For PCA, both ears of mice received a single topical application of RB-11 (7.5 μ g or 15 μ g per ear) diluted in vehicle (100% EtOH, propylene glycol, and water mixed at a 2:1:1 vol/vol/vol ratio), or of vehicle alone. Eight hours later, mice from all groups were injected intradermally with 20 μ L of IgE anti-DNP mAb at a concentration of 5 μ g/mL (ie, 100 ng dose) diluted in Hanks minimum essential medium (HMEM)-piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (Sigma-Aldrich) to one ear and equal volume of vehicle to the other ear. After 16 hours' IgE sensitization, all mice were injected intravenously via the tail vein with 2 mg/mL of DNP-HSA (Sigma-Aldrich) diluted in 100 μ L of sterile 0.9% saline (ie, dose of 200 μ g). Ear thickness was measured using a dial thickness gauge (model G-1A, Ozaki Manufacturing) before (baseline) and at intervals after intravenous antigen challenge.

RESULTS

Fc ϵ RI-mediated MC activation is reduced by 14-3-3 ζ deficiency

To explore the role of 14-3-3 proteins in MCs, we first interrogated our existing data set of gene expression in human MCs.¹⁸ We found that MCs express high amounts of the gene encoding 14-3-3 ζ compared to other 14-3-3 isoforms. Moreover, we found that the gene encoding 14-3-3 ζ (*YWHAZ*) is uniquely upregulated after IgE-mediated activation of MC (Fig 1, A), indicating that the ζ isoform of 14-3-3 may play an important role in MC function. To investigate the role of 14-3-3 ζ in Fc ϵ RI-mediated responses, we utilized human HCMCs together with gene silencing using small interfering RNA (siRNA) oligonucleotide targeting 14-3-3 ζ . The knockdown efficacy of the siRNA was ~50% at 72 hours after transfection as determined by immunoblot (Fig 1, B) and did not affect the expression of other 14-3-3 isoforms that expressed in MCs (see Fig E1 in the Online Repository available at www.jacionline.org). The siRNA-mediated knockdown of 14-3-3 ζ expression significantly reduced histamine release from HMC compared to control siRNA treatment (Fig 1, C).

To determine if the function of 14-3-3 ζ is conserved in mouse MCs, we used BMCMCs derived from bone marrow of mice lacking the 14-3-3 gene (14-3-3 $\zeta^{-/-}$ mice).¹⁹ We first analyzed IgE/antigen-dependent MC activation in IgE-sensitized BMCMCs activated by the specific antigen DNP-HSA and found that selective loss of 14-3-3 ζ resulted in a marked reduction in degranulation, shown by the release of the β -hexosaminidase (Fig 1, D). Decreased MC activation was also supported by diminished production of the proinflammatory cytokine TNF- α and chemokine CCL2 from 14-3-3 $\zeta^{-/-}$ BMCMCs compared to wild-type (WT) littermate BMCMCs after IgE sensitization and DNP-HSA stimulation (Fig 1, E and F). The MC numbers and surface expression

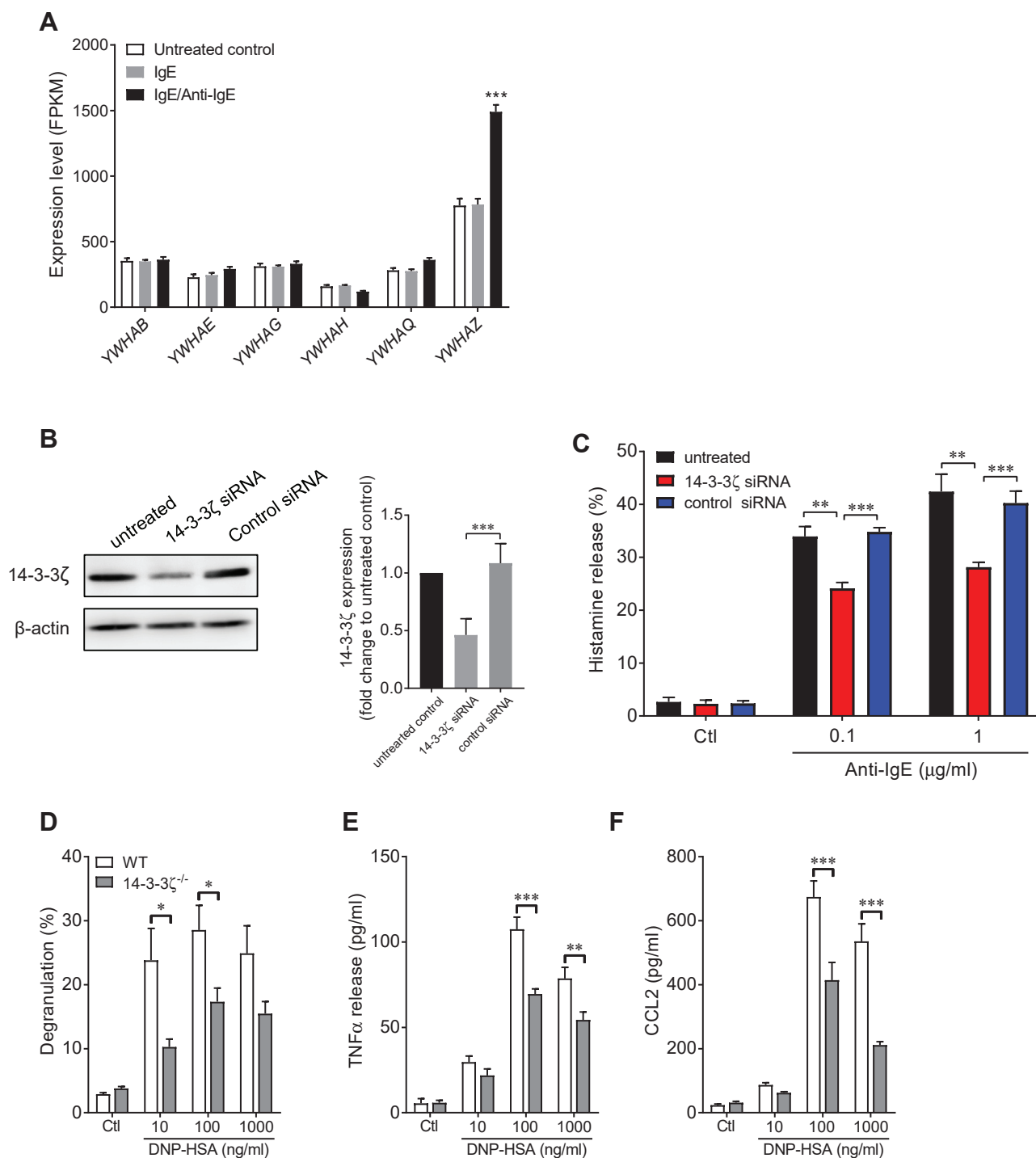


FIG 1. 14-3-3 ζ deficiency downregulates IgE-mediated MC activation. **(A)** RNA sequencing-based gene expression values (fragments per kilobase of transcript per million fragments mapped, aka FPKM) for each 14-3-3 isoform in HCMC. Data are extracted from table S2 of Cildir et al.¹⁸ **(B) Left,** Immunoblot of 14-3-3 ζ expression in HCMCs at 72 hours after gene silencing with 14-3-3 ζ siRNA. **Right,** Quantification of immunoblot band intensities measured by Image Lab software and data were normalized to β -actin and then to unstimulated control. **(C)** HCMCs transfected with 14-3-3 ζ and scrambled control siRNA were sensitized with myeloma IgE followed by stimulation with anti-IgE for histamine release. **(D-F)** WT and 14-3-3 ζ ^{-/-} BMCMCs were sensitized with IgE anti-DNP antibody, then stimulated with indicated concentrations of DNP-HSA for measurement of release of **(D)** β -hexosaminidase (1 hour), **(E)** TNF- α , and **(F)** CCL2 (6 hours). Ctl, IgE-sensitized only control. Data are expressed as means $\pm$ SEMs from 3 (C) to 4 (B, D-F) independent experiments. * P < .05, ** P < .01, *** P < .001 for indicated comparison, (B) 1-way ANOVA, and (C-F) 2-way ANOVA with Bonferroni post-test.

of c-Kit and FcεRI were similar between 14-3-3ζ^{-/-} and WT BMCMCs after 6 weeks of culture (see Fig E2 in the Online Repository available at www.jacionline.org), indicating that 14-3-3ζ^{-/-} BMCMCs develop normally *in vitro*. Taken together, our results demonstrate an important role for 14-3-3ζ in FcεRI-mediated MC degranulation and cytokine/chemokine release.

Human MC signaling and activation in response to IgE cross-linking is controlled by 14-3-3ζ

Our results suggest that 14-3-3ζ may be a potential target for the control of MC activation in allergic inflammation. Recently, different chemical classes of 14-3-3 protein–protein interaction (PPI) modulators have been developed as potential therapeutics, including 14-3-3 PPI stabilizers, as well as phosphonate and non-phosphonate type 14-3-3 PPI inhibitors that bind to 14-3-3 and compete with client protein binding.²⁰ We have recently developed 14-3-3–targeting molecules that disrupt 14-3-3 protein dimerization, which is essential for their adaptor function.^{21,22} Using one such inhibitor of 14-3-3, RB-11, we found that IgE/antigen-mediated histamine release from HCMCs was markedly reduced by 14-3-3 inhibition in a dose-dependent manner (Fig 2, A). A similar effect was observed with a second 14-3-3 inhibitor, RB-12 (see Fig E3, A, in the Online Repository available at www.jacionline.org). We also found that 14-3-3 inhibition not only reduced the rapid secretion of MC granules but also *de novo* synthesis of lipid mediators, including cysteinyl leukotrienes (Cys-LTs) and PGD₂ (Fig 2, B and C) and the production of proinflammatory cytokines such as IL-8 and TNF-α (Fig 2, D and E, and see Fig E3, B and C). We examined caspase-3 activation, a characteristic marker of apoptosis in MCs,²³ and found that incubation of HCMCs with RB-11 in a dose–response and time-course study did not induce HMC apoptosis (see Fig E4 in the Online Repository), although it was capable of inducing apoptosis in the Jurkat leukemia cell line.²¹ These data indicate that RB-11 inhibits MC effector function without affecting the viability of MCs.

Inhibition of 14-3-3 by RB-11 also inhibited mediator release after IgE-dependent activation of the human MC line LAD2, and overexpression of 14-3-3ζ (see Fig E5, A, in the Online Repository available at www.jacionline.org) amplified IgE/antigen-induced degranulation in LAD2 cells, an effect that was suppressed dose dependently by RB-11 (Fig E5, B). To ascertain the effect of 14-3-3 inhibition on non-IgE-mediated MC activation, HCMCs and LAD2 cells were incubated with RB-11 before stimulation with compound 48/80 or the neuropeptide substance P, both of which induce MC degranulation. Interestingly, 14-3-3 inhibition did not alter human MC activation triggered by either compound 48/80 or substance P, suggesting that 14-3-3 is required specifically for IgE-dependent signaling in MCs (Fig 3).

Our data demonstrate an important role for the ζ isoform of 14-3-3 in FcεRI-mediated MC activation. However, it remains unknown if other 14-3-3 isoforms exhibit functional redundancy. If RB-11 inhibits IgE/antigen activation of MC via isoforms additional to 14-3-3ζ, an additive effect of RB-11 over that found in 14-3-3ζ^{-/-} MCs would be expected. We therefore incubated BMCMCs generated from 14-3-3ζ^{-/-} mice with RB-11 before IgE/antigen stimulation and measured β-hexosaminidase release. Unlike WT BMCMCs, RB-11 did not alter IgE-dependent degranulation and cytokine release in the absence of 14-3-3ζ in 14-3-3ζ^{-/-} BMCMCs (see Fig E6, A, in the Online Repository

available at www.jacionline.org). This result strongly suggests that 14-3-3ζ is the key 14-3-3 isoform that regulates FcεRI-mediated MC activation and degranulation.

ERK1/2 and Akt phosphorylation are facilitated by 14-3-3ζ in IgE-activated MC

To investigate the mechanism of action of RB-11 in the inhibition of MC function, we first confirmed that treatment of HCMCs with RB-11 and RB-12 resulted in the phosphorylation of Ser58 that inhibits 14-3-3 dimerization (Fig 4, A). We next examined the FcεRI signaling pathway in HCMCs treated with RB-11. Surprisingly, we found that phosphorylation of the FcεRI and membrane-proximal signaling molecules Fyn, Hck, and Syk; membrane transport protein LAT1; and docking protein Gab2 were unaffected by RB-11 treatment. In contrast, phosphorylation of both ERK1/2 and Akt in response to IgE/antigen were reduced by ~50% after treatment with RB-11 (Fig 4, B). We next examined FcεRI signaling in LAD2 cells overexpressing 14-3-3ζ. We found that overexpression of 14-3-3ζ resulted in increased ERK1/2 and Akt phosphorylation in response to IgE-mediated activation. Moreover, RB-11 treatment strongly inhibited phosphorylation of both ERK1/2 and Akt in IgE-activated control (empty vector) and 14-3-3ζ-overexpressing LAD2 cells (Fig E5, C). These data indicate that in IgE-mediated MC activation, 14-3-3 functions downstream of LAT/Gab2, where it is required for ERK1/2 and Akt activity.

IgE-mediated PCA is regulated by 14-3-3ζ

Immunologic activation of MCs is essential for the pathophysiology of acute allergic reactions and anaphylaxis.²⁴ To evaluate the role of 14-3-3 proteins in skin inflammation, we used a MC-dependent model of IgE-induced PCA and assessed the effect of 14-3-3 inhibition on PCA responses using the 14-3-3 inhibitor RB-11 and 14-3-3ζ^{-/-} mice. WT mice developed tissue swelling in the IgE-αDNP-injected DNP-HSA challenged ear that peaked at 30 minutes and resolved by 6 hours after DNP-HSA challenge. Inhibition of 14-3-3 function by RB-11 treatment dose-dependently reduced PCA tissue swelling responses (see Fig E7, A, in the Online Repository available at www.jacionline.org). Skin irritation or ear swelling was not observed in any of the HMEM-PIPES vehicle-injected control ears with or without topical application of RB-11 (Fig E7, B). We next evaluated PCA responses in 14-3-3ζ^{-/-} mice and found that ear tissue swelling was substantially suppressed in the absence of 14-3-3ζ. As expected, no ear swelling was observed in the ears that received HMEM-PIPES control (Fig 5, A). Moreover, 14-3-3ζ^{-/-} mice exhibited diminished IgE/antigen-mediated vascular permeability, as evidenced by decreased Evans blue dye extravasation in the IgE-sensitized ear skin (Fig 5, B). Importantly, RB-11 treatment did not further reduce tissue swelling and vascular permeability in 14-3-3ζ^{-/-} mice, suggesting a specific positive regulatory role for the 14-3-3ζ isoform in IgE-mediated allergic responses.

14-3-3ζ deficiency or inhibition did not affect MC development (Fig E2) or survival (Fig E4) *in vitro*. Similarly, we did not observe any differences in MC numbers in ear tissues between WT and 14-3-3ζ^{-/-} mice, or after RB-11 treatment (Fig 5, C). Moreover, we observed a normal level of circulating neutrophils and mononuclear phagocytes in 14-3-3ζ^{-/-} mice at baseline (see Fig E8 in

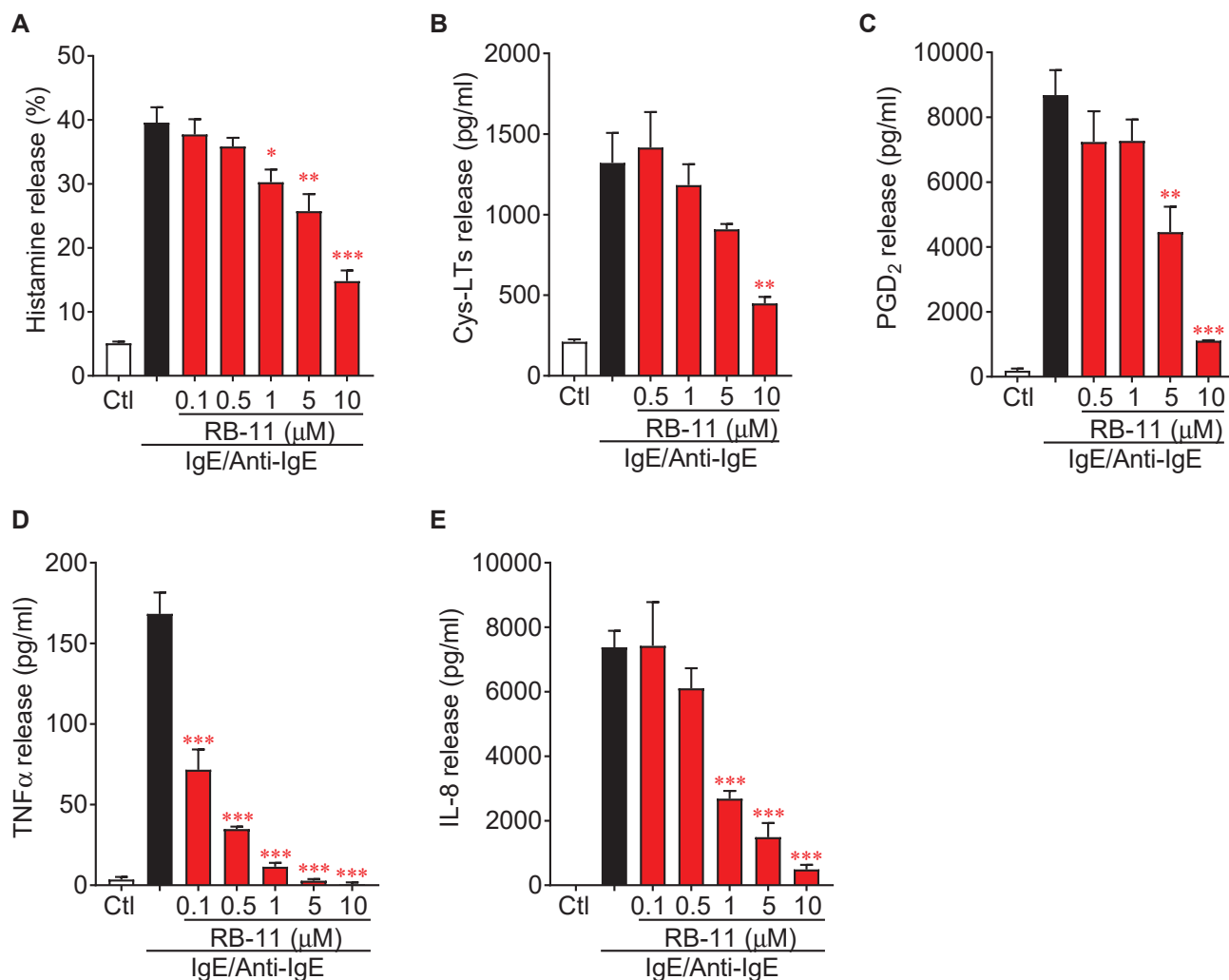


FIG 2. The 14-3-3 inhibitor RB-11 dose-dependently suppresses IgE/anti-IgE-mediated HCMC activation. IgE-sensitized HCMCs were incubated with RB-11 for 2 hours before stimulation with anti-IgE for (A) histamine, (B) Cys-LTs, (C) PGD₂, (D) TNF-α, and (E) IL-8 release. Ctl, IgE-sensitized only control. Data are expressed as means ± SEMs from 3 (A-C) or 4 (D and E) independent experiments. **P* < .05, ***P* < .01, ****P* < .001 compared to IgE/anti-IgE treatment, 1-way ANOVA with Dunnett post-test.

the Online Repository available at www.jacionline.org). However, attenuated MC degranulation (6 hours, Fig 5, D) and transcription of histidine decarboxylase (*Hdc*, 2 hours, Fig 6, A) was observed in WT mice treated with RB-11 and *14-3-3ζ*^{-/-} mice in the IgE-sensitized ears after antigen challenge compared to antigen-challenged WT mice treated with vehicle control. This is consistent with the upregulation of *Hdc* mRNA after MC degranulation to replenish the histamine content in degranulated MCs.²⁵ At 6 hours after antigen stimulation, we observed diminished levels of the proinflammatory mediators TNF and IL-6 in the IgE-sensitized ears of *14-3-3ζ*^{-/-} mice (Fig 6, B and C) compared to WT controls, which correlated with reduced neutrophil and mononuclear phagocytes infiltration, as determined by flow cytometry (Fig 6, D-F). Additionally, the inflammatory milieu and cellular inflammation in IgE-sensitized ears in WT and *14-3-3ζ*^{-/-} mice treated with RB-11 were found to be similar to the IgE-sensitized ears in *14-3-3ζ*^{-/-} mice, indicating that the 14-3-3ζ isoform is uniquely required for MC-dependent anaphylaxis *in vivo* (Fig 6). Taken together, our data show that the p-Ser/

Thr binding adaptor protein 14-3-3ζ is selectively required for optimal FcεRI pathway signaling after IgE cross-linking on the surface of MCs and that inhibition of 14-3-3ζ suppresses MC mediator release to limit excessive allergic reactions and pathology *in vivo*.

Targeting 14-3-3ζ reduces activation of human nasal polyp MCs

Chronic rhinosinusitis with nasal polyps is a chronic inflammatory sinus disease associated with MC expansion and activation.^{26,27} Local B-cell activation and immunoglobulin class-switch recombination trigger the synthesis of local IgE, and that contributes to IgE-dependent activation of MCs within nasal polyps.²⁸⁻³⁰ To further assess the clinical significance of the 14-3-3ζ pathway in inflammatory disease, we collected human nasal polyp tissues and measured 14-3-3ζ protein expression by immunofluorescence staining. Intriguingly, strong expression of 14-3-3ζ protein was detected in tryptase-positive MCs (Fig 7, A),

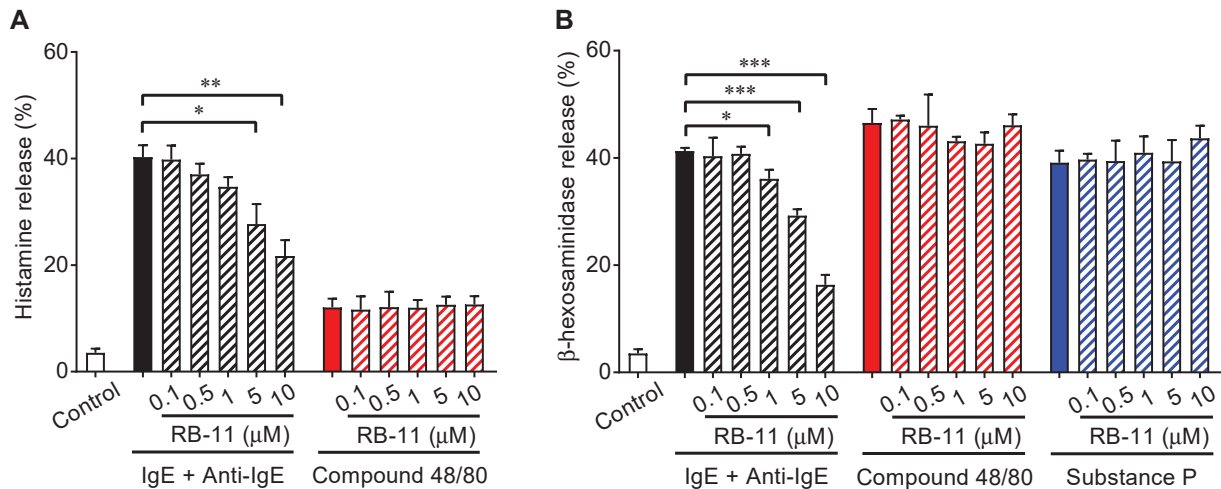


FIG 3. 14-3-3 inhibition does not affect non-IgE-mediated activation of H1MC. IgE-sensitized (A) H1MCs or (B) LAD2 human MC line was incubated with RB-11 for 2 hours before stimulation with anti-IgE, compound 48/80, or substance P and measurement of histamine or β-hexosaminidase release. Data are expressed as means ± SEMs from 3 independent experiments. * $P < .05$, ** $P < .01$, *** $P < .001$ compared to IgE/anti-IgE treatment, 1-way ANOVA with Dunnett post-test.

and MCs in diseased nasal polyp tissue expressed significantly more than MCs from healthy sinus tissue (Fig 7, A and B). To determine if inhibiting 14-3-3 could reduce the activation of MCs from diseased human tissue, we isolated FcεRI⁺c-Kit⁺ double-positive MCs from nasal polyp and activated them by IgE cross-linking in the presence of the 14-3-3 inhibitor RB-11. We found that 14-3-3 inhibition reduced cell surface expression of the activation marker lysosomal-associated membrane protein 1 (LAMP1) on IgE/antigen-activated nasal polyp MCs (Fig 7, C). These data indicate that 14-3-3 is enriched in MCs from diseased nasal polyp tissue, where it facilitates IgE-dependent MC activation.

DISCUSSION

We herein define the p-Ser/Thr-binding adaptor protein 14-3-3ζ as a novel regulator of IgE-dependent MC activation and identify 14-3-3ζ as a target for inhibition of MC-dependent anaphylaxis and inflammation. Previous studies have focused on the role of p-Tyr-binding adaptor proteins in regulating FcεRI-mediated responses in MCs,^{6,7} and phosphorylation of proteins on serine and threonine residues was initially thought to regulate protein function largely through allosteric modifications rather than by directly mediating PPIs.³¹ However, it is now becoming evident that signaling molecules and modular domains can specifically bind to p-Ser/Thr-containing motifs, recruiting these substrates into protein-protein signaling complexes in response to phosphorylation by Ser/Thr kinases.¹⁰ Our data allow us to identify 14-3-3ζ as a core component of the FcεRI signaling cascade that links receptor-proximal and cytoplasmic signaling by FcεRI.

As a result of the high degree of 14-3-3 protein homology, it was initially thought that 14-3-3 isoforms were redundant in function, and in the absence of one 14-3-3 isoform, others would compensate.³² However, in-depth studies of the 14-3-3 phylogenetic tree suggest that all isoforms evolved before the divergence of mammals and that the orthologs have a higher homology across species compared to isoforms of the same species, suggesting that individual 14-3-3 isoforms have unique and fundamental roles.³³

For example, overexpression of 14-3-3ζ in T cells induced biased T-cell polarization that favors regulatory T-cell proliferation.³⁴ We show here the existence of an isoform-specific increase in 14-3-3ζ transcript expression after IgE-mediated MC activation and an increase in 14-3-3ζ protein expression in nasal polyp tissue MCs. Alterations in 14-3-3 expression have been documented in cancer, neurodegenerative diseases, and chronic inflammatory disorders.^{11,12,35} However, the exact mechanism of 14-3-3 regulation and the physiologic function of changes in 14-3-3 expression are not well understood. Notably, 14-3-3 proteins in plants have been shown to display rapid changes in abundance during pathogen infection and the plant hypersensitive response, and to subsequently interact or associate with known regulators of plant defense.^{36,37} These studies indicate that changes in 14-3-3 expression may be a highly conserved immunoregulatory mechanism. The activation-dependent upregulation of 14-3-3 expression that we observed may support rapid and persistent MC responses in chronic allergic inflammation, and warrants further investigation.

During FcεRI-mediated MC activation, the initial events that promote signaling are organized around the receptor itself (ie, receptor phosphorylation), but the formation of novel docking sites for signaling proteins on the receptor is not sufficient for signal amplification and downstream events. The adapter proteins LAT, Grb2, and Gab2 are essential for signal propagation and are important for IgE-dependent allergic and anaphylactic immune responses.^{6,7} Our study demonstrates, for the first time, an essential role of 14-3-3ζ as an adaptor protein to propagate FcεRI-mediated signaling in both human and mouse MCs. We found that inhibition of 14-3-3 protein function did not interfere with the early signaling events during FcεRI cross-linking in MCs. Instead, phosphorylation of the serine-threonine kinase Akt and ERK1/2, which are major mediators of phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase kinase pathways, respectively, and are essential for optimal MC degranulation and cytokine synthesis upon FcεRI stimulation,^{38,39} were attenuated by 14-3-3 inhibition.

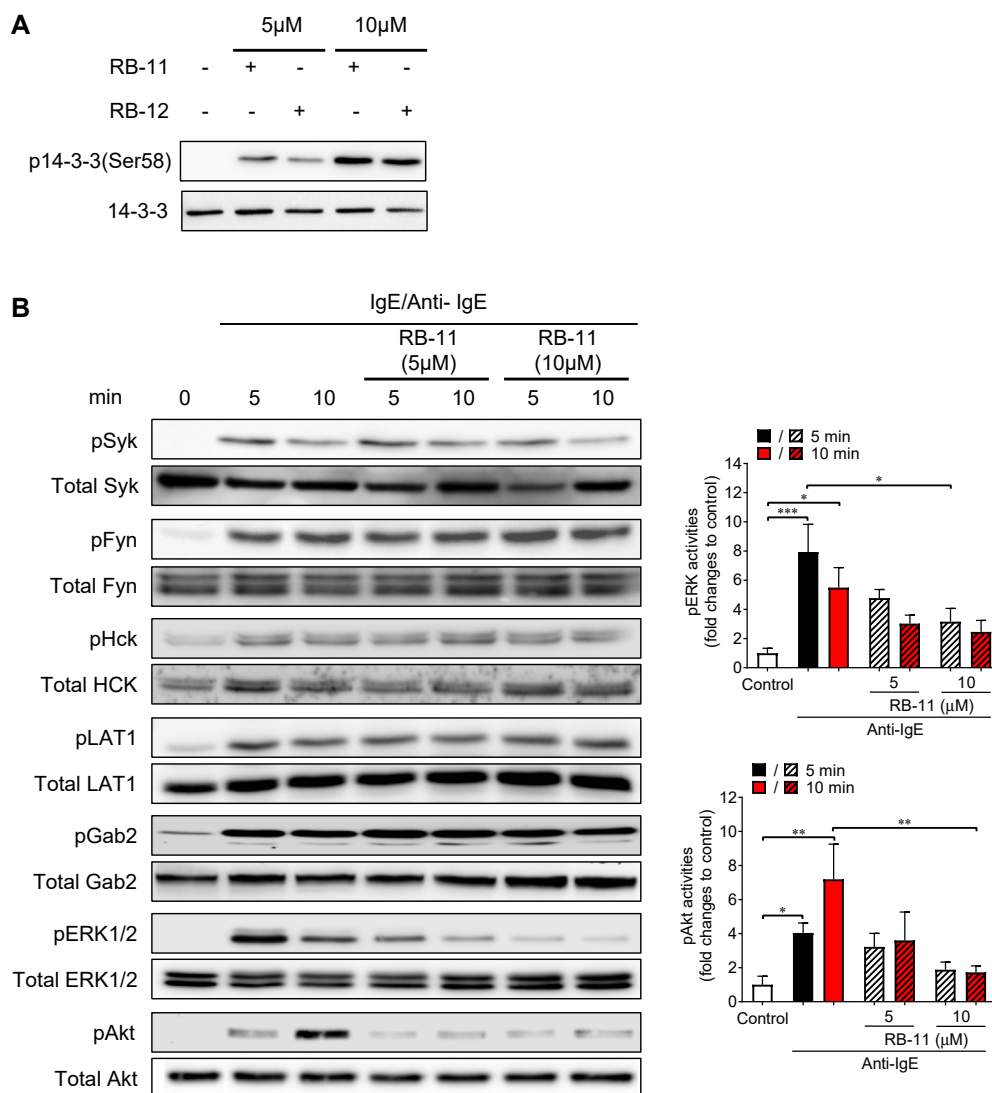


FIG 4. Disruption of 14-3-3 dimerization by 14-3-3 inhibitor diminishes ERK1/2 and Akt signaling in HCMCs. Immunoblot analysis of **(A)** Ser58 phosphorylation of 14-3-3 in HCMCs after incubation with RB-11 or RB-12 for 2 hours; data are representative of 3 independent experiments with similar results. **(B) Left**, Phosphorylated (p-)Fyn, Hck, Syk, LAT1, Gab2, ERK1/2, and Akt signaling in whole cell lysates prepared from IgE-sensitized and anti-IgE-stimulated HCMCs at indicated time points; data are representative of 2 to 5 independent experiments with similar results. **Right**, Quantification of immunoblot band intensities measured by Image Lab software; data were normalized to total amount of corresponding protein and then to unstimulated control. Data are expressed as means $\pm$ SEMs from 5 independent experiments. * $P < .05$, ** $P < .01$ for indicated comparisons, 2-way ANOVA with Bonferroni post-test.

Several lines of evidence have demonstrated that PI3K is a strong candidate for being a 14-3-3 ζ client protein. 14-3-3 ζ was found to interact with the p85 subunit of PI3K, which plays an important role in Akt phosphorylation and activation of the PI3K/Akt pathway, and the Ser83 motif on p85 is likely the major motif mediating 14-3-3 ζ binding.⁴⁰ Additionally, the ERK1/2 signaling pathway is reported to be activated in a PI3K-dependent manner upon IgE-mediated eicosanoid production in MCs,³⁹ and this PI3K-mediated simultaneous activation of Akt and ERK pathways is essential for optimal MC degranulation.⁴¹ Furthermore, 14-3-3 is well known as a Raf-binding protein and regulator of downstream ERK activation. In this context, 14-3-3 ζ binding is dependent on the phosphorylation of serine residues on Raf and on the integrity of the 14-3-3 dimer.^{42,43} These

findings may provide mechanistic explanation of how inhibition of 14-3-3 function represses IgE-mediated Akt and ERK activation in MCs. It should be noted that although we did not detect any differences in MC activation in response to the MC activators compound 48/80 and substance P, we previously reported that the Tyr179 residue of 14-3-3 ζ was involved in mediating IL-3-induced Akt activation and regulation of BMCMC survival,⁴⁴ indicating that 14-3-3 ζ may also have other roles in regulating MC biology.

A recent study reported a multitissue transcriptome data analysis on asthma-relevant tissue and used differentially expressed genes to identify candidate asthma therapeutics and computationally predict effective drug repurposing. Using the connectivity map (CMap) analysis approach, they explored

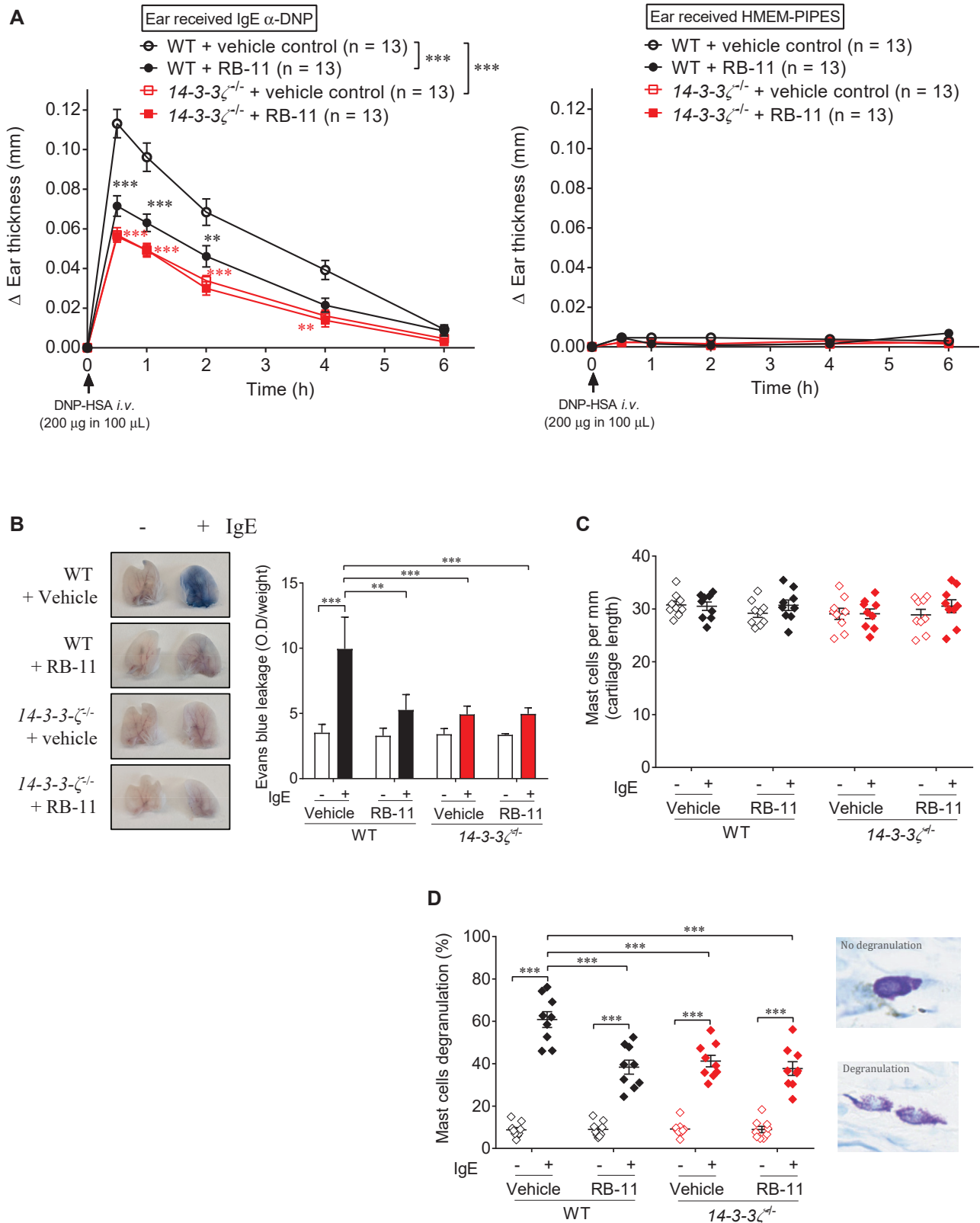


FIG 5. 14-3-3 ζ is required for IgE-mediated, MC-dependent PCA. RB-11 or vehicle was topically applied to ear pinnae of WT and 14-3-3 $\zeta^{-/-}$ mice 6 hours before intradermal injection of anti-DNP IgE. Sixteen hours later, DNP-HSA was administered by intravenous injection. Changes (Δ) in ear thickness in **(A)** IgE-sensitized ear and vehicle control ear at 0 to 6 hours. **(B)** Evans blue dye extravasation (ear tissue weight adjusted) quantified by absorption at 610 nm in vehicle or IgE anti-DNP-treated ear pinnae at 30 minutes after intravenous (tail vein) DNP-HSA (containing Evans blue dye) administration. Representative ears shown for each group of mice tested. **(C)** Dermal MC numbers in ear pinnae of mice at completion of PCA experiments. **(D)** Percentage of MCs in vehicle or IgE-sensitized ear exhibiting degranulation 6 hours

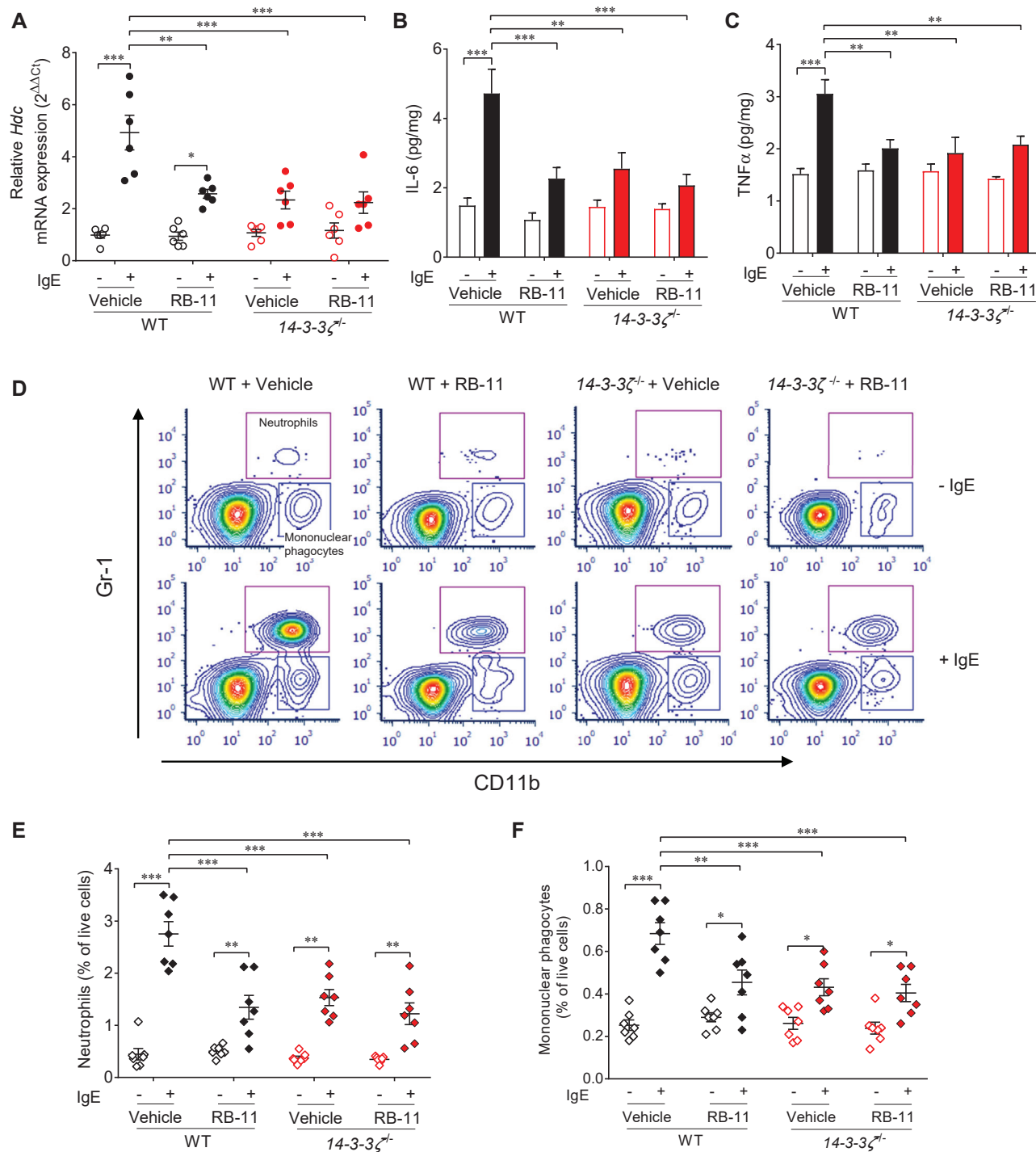


FIG 6. Inhibition of 14-3-3 ζ activity restrains skin inflammation. Ear tissues of WT and 14-3-3 $\zeta^{-/-}$ mice were collected at (A) 2 hours and (B-E) 6 hours after DNP-HSA challenge for immune analyses. (A) mRNA was extracted from ear tissues, and expression of *Hdc* was detected by real-time PCR. (B and C) IL-6 and TNF- α in ear lysates were detected by ELISA and normalized to weight of tissues. (D and E) Single cell suspensions were prepared from ear tissue; neutrophils and mononuclear phagocytes were detected by flow cytometry. Data are expressed as means $\pm$ SEMs from 3 independent experiments performed with 2 to 3 mice per group in each experiment. ** $P < .01$, *** $P < .001$ compared to vehicle treatment, 2-way ANOVA with Bonferroni post-test.

after intravenous DNP-HSA challenge. Data are expressed as means $\pm$ SEMs, 3 to 4 independent experiments performed with 2 to 4 mice per group in each experiment. ** $P < .01$, *** $P < .001$ compared to WT mice received vehicle treatment, 2-way ANOVA with Bonferroni post-test.

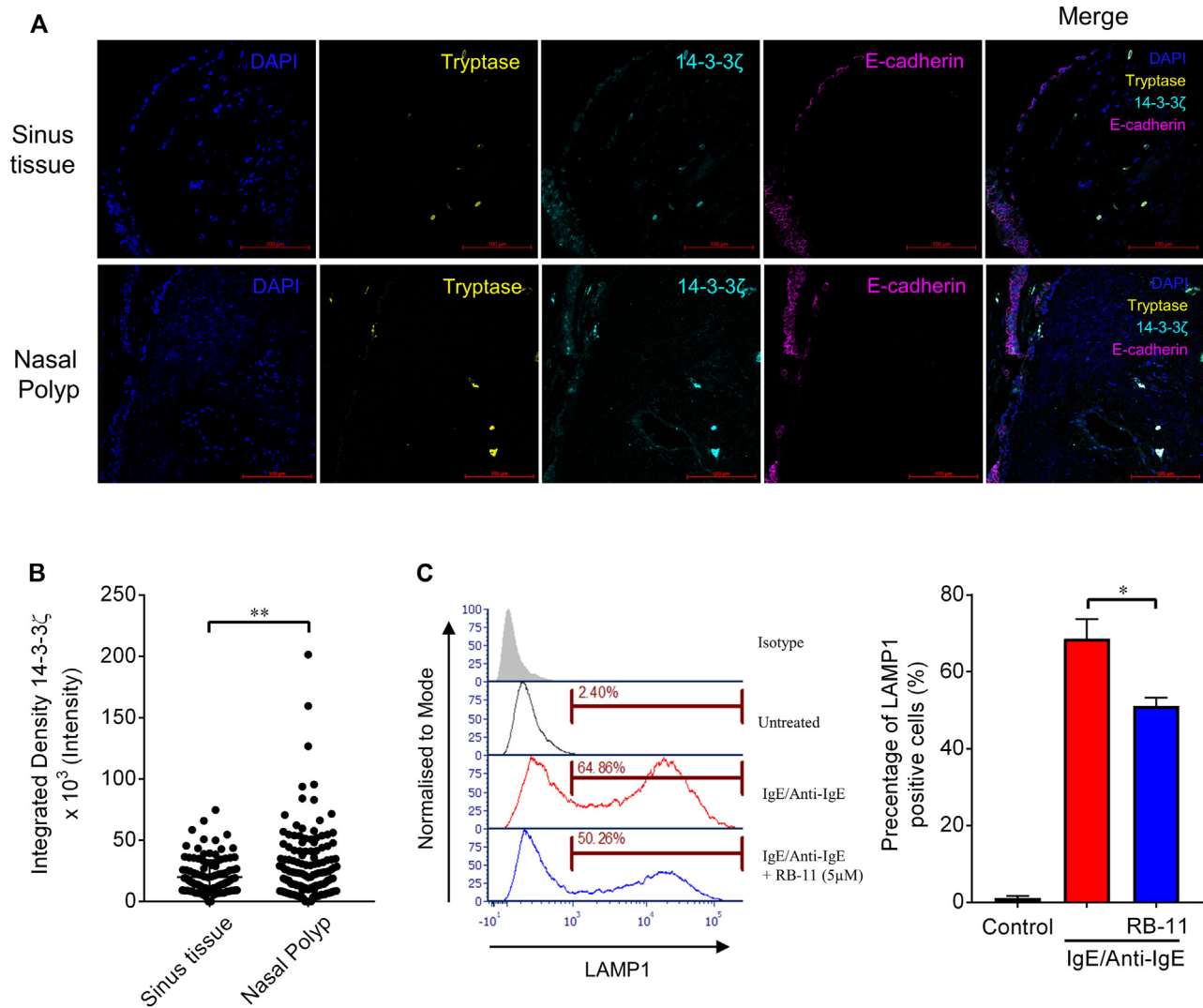


FIG 7. 14-3-3ζ is increased in human nasal polyp MCs and regulates nasal polyp MC activation. **(A)** Representative confocal immunofluorescence images of sinus and nasal polyp tissues stained with anti-tryptase (yellow), anti-14-3-3ζ (turquoise), and anti-E-cadherin (magenta) and 4',6-diamidino-2-phenylindole (aka DAPI) (blue). Scale bar = 100 μm. **(B)** Integrated density of 14-3-3ζ immunofluorescence of tryptase-positive MCs on tissues from 4 or 5 patients per donor per group and 4 to 6 fields per tissue. ** $P < .01$, unpaired t test. **(C)** Left, Stacked histograms of cell surface LAMP1 expression on isolated nasal polyp MCs at 10 minutes after anti-IgE stimulation in different treatment groups from 1 representative experiment. Right, Percentages of LAMP1-positive MCs in each treatment group. Data (means ± SEMs) are pooled from 3 independent experiments, each of which gave similar results. * $P < .05$, unpaired t test.

potential drugs for asthma treatment by systematically mining functional connections between asthma disease, differentially expressed genes, and perturbagens. 14-3-3ζ chemical perturbagen was identified as one of the top-ranking perturbagens that can potentially reverse the asthma signature at a multitissue level, including tissues from nasal epithelial cells, airway epithelium, and bronchial biopsy.⁴⁵ Given the well-recognized harmful role of IgE-mediated MC activation in allergy, anaphylaxis, and inflammation, there is an urgent need to identify efficient strategies that can limit the detrimental function of MCs in such settings. Our findings identify 14-3-3ζ as a key regulator of IgE-mediated MC activation and show that this pathway is amenable to pharmacologic targeting to inhibit MC-mediated pathology.

Moreover, the increase in 14-3-3ζ expression in diseased tissue MCs highlights the potential clinical application of 14-3-3 inhibition in inflammatory diseases where 14-3-3 is selectively enriched in MCs.

DISCLOSURE STATEMENT

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Key messages

- FcεRI-mediated MC activation is positively regulated by 14-3-3ζ.
- The 14-3-3 inhibitor RB-11 disrupts 14-3-3 dimerization and IgE-mediated MC activation.
- Targeting 14-3-3ζ attenuates IgE/antigen-mediated cutaneous anaphylaxis in mice.

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METHODS

Study approval

This study was approved by the Central Adelaide Local Health Network human research ethics committee (HREC/18/CALHN/381 and HREC/18/CALHN/133). For all *in vivo* experiments, age-matched male mice 6 to 12 weeks old were used, and all mice were bred in house at the UniSA Animal Care Facility (Adelaide, Australia). Experiments were performed in compliance with the ethical guidelines of the National Health and Medical Research Council of Australia, with approval from the UniSA animal ethics committee (AEC21/16 and U21/21, South Australia). *14-3-3ζ*^{-/-} mice have been previously described.^{E1}

Generation of mouse BMCMCs

WT and *14-3-3ζ*^{-/-} BMCMCs were obtained by culturing bone marrow cells from the femurs and tibias of 6-week-old BALB/c WT or *14-3-3ζ*^{-/-} mice in Dulbecco modified Eagle medium (Gibco; Thermo Fisher Scientific) supplemented with 10% fetal calf serum (Bovogen) and 20% WEHI-3 conditioned medium (supplemented to 3–4 ng/mL IL-3 with recombinant mouse IL-3 [Shenandoah Biotechnology]) for 5 to 7 weeks. After 6 weeks' culture, >95% of the cells were identified as MCs by May Grünwald-Giemsa staining and flow cytometric analysis (FceRI⁺c-Kit⁺).^{E2}

Generation of HCMCs

HCMCs were generated as previously described.^{E3} Briefly, the human buffy coat was diluted with sterile phosphate-buffered saline (PBS) at a ratio of 1:1, then layered gently over Histopaque-1077 (1.7 g/L; Sigma-Aldrich). After centrifugation (600 × *g*, 30 minutes), the interface containing mononuclear cells were collected, and the remaining red blood cells were disrupted with hemolytic solution (155 mmol NH₄Cl, 10 mmol KHCO₃, 0.1 mmol EDTA 2Na). CD34⁺ progenitor cells were enriched by positive immunomagnetic selection using CD34 MicroBeads and an autoMACS Separator (Miltenyi Biotec) according to the manufacturer's instructions. Isolated CD34⁺ cells were transferred into 12-well plates at a density of 5 × 10⁶ cells/mL in Iscove modified Dulbecco medium (Life Technologies; Thermo Fisher Scientific), 50 μmol 2-mercaptoethanol (Life Technologies), 1% penicillin–streptomycin (Life Technologies), 0.1% BSA (Sigma-Aldrich), 100 ng/mL recombinant human (rh) stem cell factor, 50 ng/mL rhIL-6, and 5 ng/mL rhIL-3 (all rh cytokines from Shenandoah Biotechnology) and placed in a CO₂ incubator at 37°C. The cytokine-supplemented medium was replaced weekly, and rhIL-3 was omitted from the medium after the first 2 weeks of culture. HCMCs were subsequently maintained for a further 8 weeks before being used in different studies, at which time the population contained over 96% HCMCs.

siRNA transfection of HCMCs

HCMCs at 10 to 12 weeks old were transfected with a mixture of 3 independent stealth siRNAs to 14-3-3ζ (HSS111442, HSS111443, and HSS111444; Invitrogen; Thermo Fisher Scientific) using the Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) according to the manufacturer's instructions, and cells were used in assays 72 hours after transfection.

Transduction of 14-3-3ζ into LAD2 human MCs

The human MC line LAD2 was the kind gift of Dr Arnold Kirshenbaum from the US National Institute of Allergy and Infectious Diseases.^{E4} Stable LAD2 cells overexpressing 14-3-3ζ were generated as follows. Human *14-3-3ζ* cDNA was cloned using gateway recombination into pDEST/N-SF-TAP (the kind gift of C. J. Gloeckner),^{E5} which fuses the N-terminus of *14-3-3ζ* with an epitope tag that has been used previously to express and isolate the native interactors of 14-3-3ε. The N-SF-TAP-*14-3-3ζ* fusion was then subcloned into a third-generation lentiviral vector, pLvEIG (the kind gift of S. C. Barry), which positioned the *14-3-3ζ* coding region 5' of IRES-GFP. Recombinant lentivirus was generated as previously described^{E6} and LAD2 transduced using fresh viral supernatant containing

4 μg/mL polybrene. Transduced cells were subsequently sorted for green fluorescent protein by flow cytometry and SF-TAP 14-3-3ζ protein expression confirmed by immunoblot.

Immunoblot

For the signaling studies, HCMCs were incubated with human myeloma IgE (0.5 μg/mL) for 16 hours at 37°C in a CO₂ incubator, centrifuged at 180 × *g* for 5 minutes, and resuspended in Tyrode buffer (2 × 10⁶ cells per sample). HCMCs were then incubated with RB-11 for 2 hours at 37°C before being activated with 1 μg/mL anti-IgE for 5 or 10 minutes at 37°C. The reaction was quenched by adding 1 mL ice-cold buffer, followed immediately by centrifugation at 180 × *g* for 5 minutes at 4°C. Cells were then lysed in 100 μL ice-cold lysis buffer containing 50 mmol Tris base, 100 mmol NaCl, 5 mmol EDTA, 10 mmol Na₂P₂O₇, 1% Triton X-100, 2 mmol NaF, and 1× complete protease inhibitors cocktail (Roche). Total cell lysates were separated with SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline that contained 0.1% Tween buffer; they were then probed with antibodies raised in rabbit against the phosphorylated form of ERK1/2 (Thr202/Tyr204, Cell Signaling Technology), Akt (Ser473, Cell Signaling Technology), Syk (Tyr525/526, Cell Signaling Technology), LAT1 (Tyr191, EMD Millipore), Fyn (Tyr530, Abcam), Hck (Tyr521, Thermo Fisher Scientific), and Gab2 (Tyr452, Cell Signaling Technology) at a dilution of 1:500 to 1000 overnight at 4°C. Membranes were then probed with horseradish peroxidase-conjugated antibody against rabbit IgG (1:3000 dilution), and bands were visualized with enhanced chemiluminescence reagent (Bio-Rad Laboratories) with a ChemiDoc MP Imaging System (Bio-Rad Laboratories). Membranes were then stripped and reprobed with antibodies against the total form of Fyn, Hck, Syk, LAT1, Gab2, Akt, and ERK1/2 (all from Cell Signaling Technology). The band intensity was quantified by Image Lab software (Bio-Rad Laboratories).

For 14-3-3 isoform detection, total cell lysate transferred to nitrocellulose membranes was probed with antibodies against 14-3-3β (Cell Signaling Technology), 14-3-3γ (Cell Signaling Technology), 14-3-3ε (Santa Cruz Biotechnology), 14-3-3η (R&D Systems), 14-3-3τ (Santa Cruz Biotechnology), and 14-3-3ζ (in-house-generated mouse mAb), followed by antibody against β-actin (Cell Signaling Technology).

Evans blue dye extravasation

The PCA reaction was elicited as described with the exception that DNP-HSA (200 μg in 100 μL of sterile 0.9% saline containing 1% Evans blue dye [Gurr-Searle Diagnostic]) was administered intravenously (tail vein). Mice were humanely killed 30 minutes after intravenous DNP-HSA injection, and whole ears were obtained and weighed. To extract the Evans blue dye, the ears were diced into pieces (~1–2 mm²) in an Eppendorf tube and incubated in 0.3 mL formamide at 55°C overnight. Samples were then centrifuged at 18,000 × *g* for 15 minutes, and 100 μL of supernatant was quantified by absorption at 610 nm on a plate reader. Data were expressed as optical density/weight (g).

Histology and quantification of MC numbers

Mice were humanely killed by CO₂ inhalation. Samples of ear pinnae were fixed in 10% buffered formalin, processed, and embedded in paraffin, and 4 μm sections were cut. Dewaxed tissue sections were stained with 0.2% toluidine blue (pH 1.0, Sigma-Aldrich) for 90 seconds at room temperature and then rinsed under tap water. Sections were dehydrated with xylene and mounted with DPX mounting media. An image of the ear section was captured by a Hamamatsu NanoZoomer (40× magnification, Adelaide Microscopy Facility) and exported by NDP View v2 software. Ear pinnae MCs (cytoplasmic granules that appear purple) and MC degranulation were counted by Fuji ImageJ software (1.50e, imagej.nih.gov/ij). Numbers of MC were expressed per horizontal ear cartilage field length (mm).

Flow cytometry on ear skin immune cells

Ear tissues were split into dorsal and ventral sections and digested with Dispase II solution (final concentration 2 U/mL) in RPMI 1640 medium at 37°C

for 60 minutes. The dermis was then separated from the epidermis with forceps, and tissues were cut into small pieces with scissors before incubation with collagenase IV (final concentration 0.2 mg/mL) and DNase I (final concentration 0.05 mg/mL) in RPMI 1640 medium at 37°C for 60 minutes. Digested tissues were passed through a 70 μ m nylon cell strainer (Falcon) to obtain single-cell suspensions. Cells were washed in flow cytometry buffer (PBS with 2% fetal bovine serum) before incubation with Live/Dead Aqua stain (1:1000 dilution, Molecular Probes) and anti-mouse CD16/CD32 antibody (1:100 dilution, eBioscience; Thermo Fisher Scientific) for 15 minutes and then incubated on ice for 30 minutes with anti-mouse Gr-1 phycoerythrin (eBioscience) and anti-mouse CD11b fluorescein isothiocyanate (eBioscience). Flow cytometry data was acquired on a Gallios flow cytometer (Beckman Coulter) and analyzed with FCS Express 6 (De Novo Software).

Measurement of cytokines in ear skin lysates

Ear skin lysates were prepared in PBS containing protease inhibitors as previously described. TNF and IL-6 protein levels in the supernatants were measured by ELISA (eBioscience), according to the manufacturer's instructions. Total protein levels in the supernatants were measured by a Bio-Rad Dc protein assay, according to the manufacturer's instructions (Bio-Rad Laboratories). Data were expressed as picogram of cytokine per milligram of protein (pg/mg).

Immunofluorescence staining

Nasal polyp tissues were fixed in 10% buffered formalin, processed and embedded in paraffin and 4 μ m sections were cut. Antigen retrieval was performed on dewaxed sections by boiling slides in a pressure cooker for 15 minutes at 110°C in 10 mmol citrate buffer (pH 6). Sections were blocked in normal goat serum in CAS-Block (Thermo Fisher Scientific) for 1 hour in a humidified chamber at room temperature then rabbit anti-tryptase antibodies (Abcam, 1:100) were added overnight in blocking buffer at 4°C. Sections were washed in distilled water and Alexa Fluor-488 goat anti-rabbit secondary antibody (Thermo Fisher Scientific, 1:200), Alexa Fluor 555 mouse anti-E-cadherin (BD Pharmingen, 1:200) and Alexa Fluor 647 mouse anti-14-3-3 ζ (1:50, In-House generated mouse mAb^{E7}) were added and incubated for 2 hours at room temperature in the humidified chamber in the dark. After final wash with distilled water and sections were mounted in ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were acquired using 40 $\times$ (C Apo 40 $\times$ /1.2 W DICII) objectives at room temperature on a Zeiss LSM 700 confocal system and recorded by ZEN 2011 (Black Edition) software (Carl Zeiss Microscopy) equipped with 405 nm (5 mW), 488 nm (10 mW), 555 (10 mW), and 639 nm (5 mW) lasers. All quantification was performed by ImageJ software (imagej.nih.gov/ij) thresholding on the corresponding isotype control, and a minimum of 3 fields of view were averaged for every nasal polyp tissue.

MCs isolation from human nasal polyp tissues

Nasal polyp specimens were cut into small pieces and were enzymatically digested for 45 minutes in RPMI 1640 with collagenase IV (final concentration 0.2 mg/mL) and DNase I (final concentration 0.05 mg/mL) at 37°C in a hybridization incubator with rotation. Cold medium was added to stop the reaction. Enzymatically digested tissue pieces were mechanically disrupted using a syringe and filtered through a 70 μ m nylon filter (BD Biosciences). Red-blood cells were lysed and washed with FACS buffer (PBS with 2% fetal bovine serum). Cells were then with Live/Dead Aqua stain (1:1000 dilution, Molecular Probes) and anti-mouse CD16/CD32 antibody (1:100 dilution, eBioscience) for 15 minutes and then incubated on ice for 30 minutes with anti-human CD117 phycoerythrin (1:100 dilution, eBioscience) and anti-human Fc ϵ RI α fluorescein isothiocyanate (1:100 dilution, eBioscience), then washed as above before cell sorting. Fc ϵ RI⁺/CD117⁺ cells were sorted using the Beckman Coulter MoFlo Astrios EQ High-Speed Cell Sorter and cultured in complete Iscove modified Dulbecco medium (as used in HCMC culture) until further experiments.

Statistical analysis

One-way ANOVA or 2-way ANOVA with Dunnett or Bonferroni post-test was used for multiple comparisons. GraphPad Prism v5.0d software was used. Asterisks above a group indicate the level of significance of that group relative to the control (* P < .05, ** P < .01, *** P < .001).

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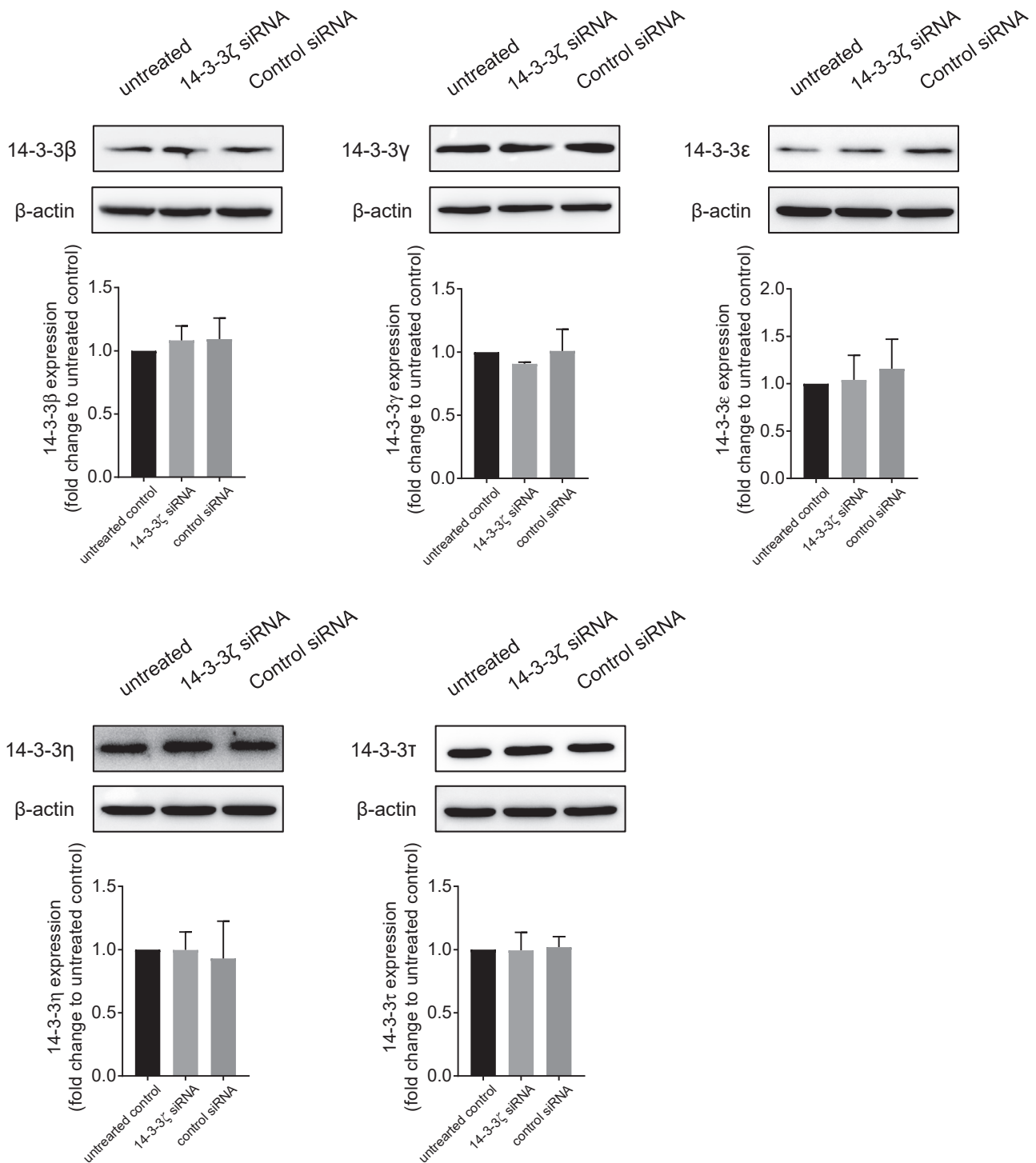


FIG E1. 14-3-3 ζ siRNA knockdown did not affect other 14-3-3 isoform expression in MCs. Immunoblots of 14-3-3 isoforms expression in HMCs at 72 hours after gene silencing with 14-3-3 ζ siRNA. Quantification of immunoblot band intensities was measured by Image Lab software, and data were normalized to β -actin and then to unstimulated control.

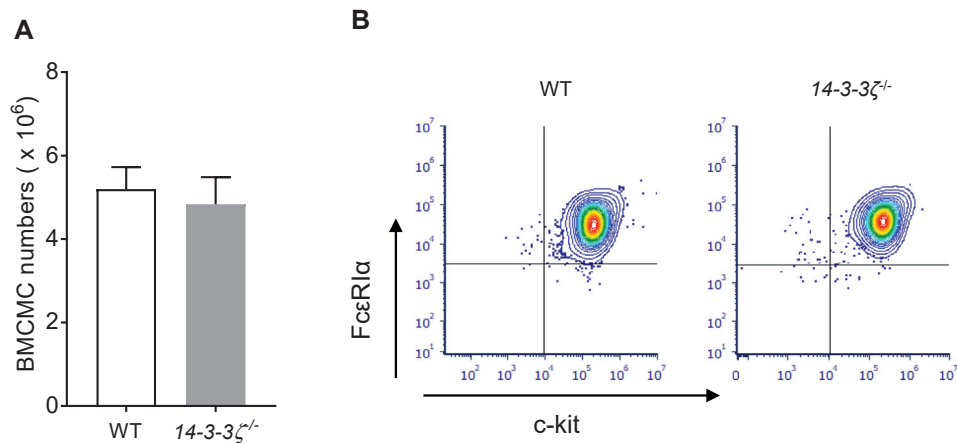


FIG E2. 14-3-3 ζ does not affect MC growth and development. **(A)** BMCMC numbers. **(B)** Representative flow plots on Fc ϵ R1 α and c-Kit expression. Data are means $\pm$ SEMs from 6 independent experiments.

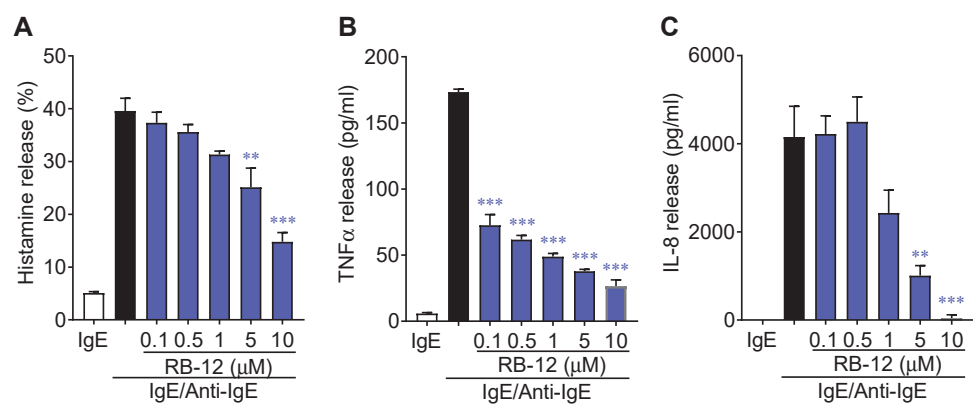


FIG E3. An 14-3-3 inhibitor, RB-12, dose-dependently suppresses IgE/anti-IgE-mediated HCMC activation. IgE-sensitized HCMCs were incubated with RB-12 for 2 hours before stimulation with anti-IgE for **(A)** histamine, **(B)** TNF- α , and **(C)** IL-8 release. Data are means $\pm$ SEMs from 3 or 4 independent experiments performed. ** $P < .01$, *** $P < .001$ compared to IgE/anti-IgE treatment, 1-way ANOVA with Dunnett post-test.

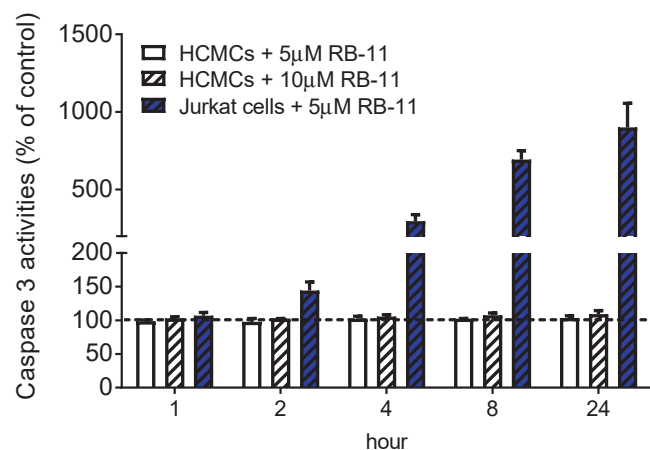


FIG E4. 14-3-3 inhibition does not induce apoptosis in HCMCs. HCMCs were incubated with RB-11 at different concentrations for indicated time points before staining with NucView 488 caspase-3 substrate and subsequent analysis by flow cytometry. Data are normalized to no treatment control and are means $\pm$ SEMs from 3 independent experiments.

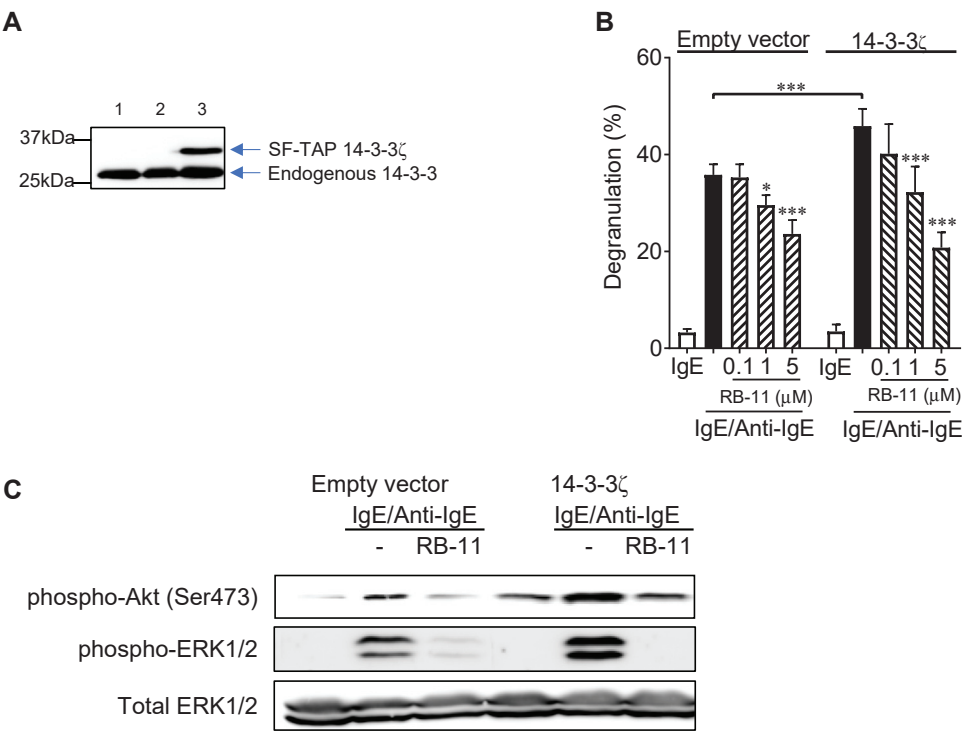


FIG E5. 14-3-3 ζ overexpression enhances MC activation and Fc ϵ RI-signaling. **(A)** Immunoblot on 14-3-3 ζ expression in LAD2 human MCs. (1) Nontransduced LAD2. (2) LAD2 transduced with empty vector; LAD2 transduced with SF-TAP 14-3-3 ζ . **(B)** IgE-sensitized LAD2 HCMCs were incubated with RB-11 for 2 hours before stimulation with anti-IgE for release of β -hexosaminidase. Data are means $\pm$ SEMs from 3 independent experiments performed. ** $P < .01$, *** $P < .001$ compared to IgE/anti-IgE treatment, 1-way ANOVA with Dunnett post-test. **(C)** Phosphorylated ERK1/2 and Akt signaling in whole cell lysates prepared from myeloma IgE-sensitized and anti-IgE-stimulated (5 minutes) LAD2. Data are representative of 3 independent experiments performed with similar results.

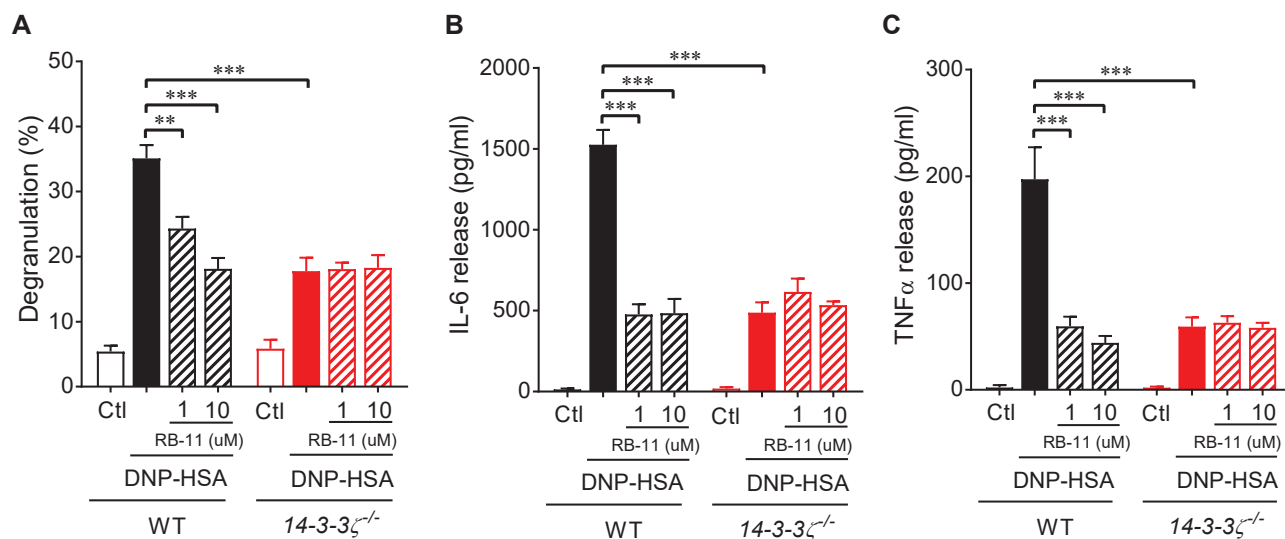


FIG E6. 14-3-3 ζ is the key 14-3-3 isoform that controls IgE-dependent BMCMC activation. IgE-sensitized WT and 14-3-3 ζ ^{-/-} BMCMCs were incubated with RB-11 for 2 hours before stimulation with DNP-HSA for (A) β -hexosaminidase, (B) IL-6, and (C) TNF- α release. Data are means $\pm$ SEMs from 4 independent experiments. ** P < .01, *** P < .001 for indicated comparisons, 2-way ANOVA with Bonferroni post-test.

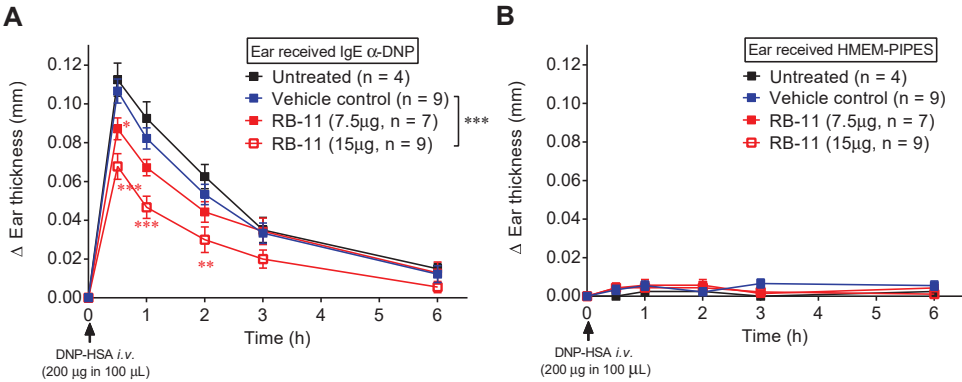


FIG E7. 14-3-3 inhibitor dose-dependently represses IgE-mediated MC-dependent PCA. RB-11 (7.5 or 10 μ g per ear) or vehicle was topically applied to ear pinnae of WT mice 6 hours before intradermal injection of anti-DNP IgE. Sixteen hours later, DNP-HSA was administered through intravenous injection. Changes (Δ) in ear thickness in (A) IgE-sensitized ear and (B) vehicle control ear at 0 to 6 hours. Data are means $\pm$ SEMs from 3 independent experiments performed with 1 to 3 mice per group in each experiment. ** P < .01, *** P < .001 compared to WT mice receiving vehicle treatment, 2-way ANOVA with Bonferroni post-test.

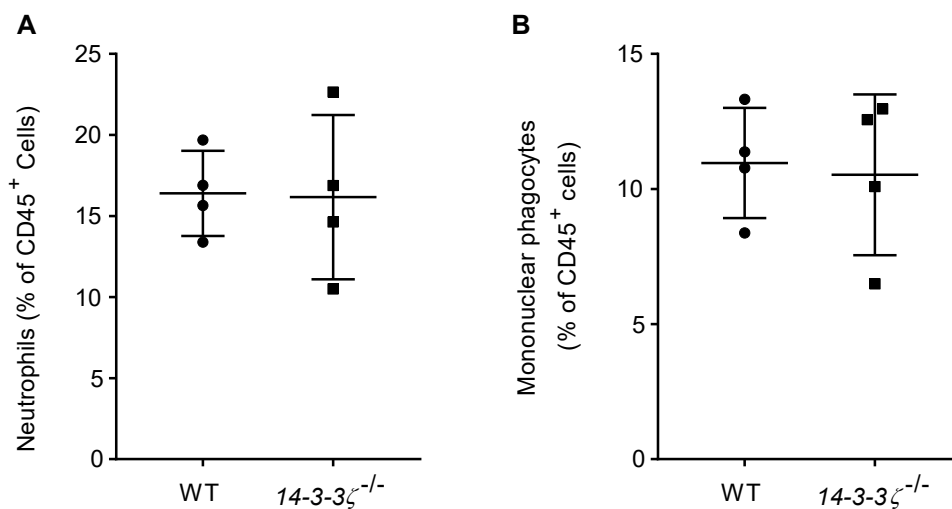


FIG E8. Normal circulating neutrophil and mononuclear phagocytes levels in 14-3-3 ζ ^{-/-} mice. Blood neutrophils and mononuclear phagocytes were detected by flow cytometry. Data are means $\pm$ SEMs from 2 independent experiments performed with 2 mice per group in each experiment.