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Inhibition of Allergic Reactivity through Targeting FcεRI-Bound IgE with Humanized Low-Affinity Antibodies

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Options for effective prevention and treatment of epidemic allergic diseases remain limited, and particularly so for IgE-mediated food allergies. We previously found that mouse low-affinity anti-human IgE mAbs with K_D in the 10^{-6} – 10^{-8} M range were capable of blocking allergic reactivity without triggering immediate allergic mediator release. In this study, we humanized three parent low affinity allergic response inhibitor (LARI) mouse anti-human IgE mAbs and characterized their biological and immunological features, refined the lead candidate for further clinical development, examined their safety profiles, determined their therapeutic efficiency, and explored the mechanism of action potentially responsible for their therapeutic effects. LARI profoundly blocked cat- and peanut-allergic IgE-mediated basophil activation, inhibited acute release of both prestored and newly synthesized mediator from human mast cells, suppressed peanut-specific IgE-mediated passive cutaneous anaphylaxis, and attenuated dansyl IgE-mediated systemic anaphylaxis in human FcεRIα transgenic mice. Safety testing demonstrated that concentrations of LARI well above therapeutic levels failed to trigger immediate release of prestored and newly synthesized allergic mediators, failed to promote robust cytokine/chemokine production from allergic effector cells, and did not elicit allergic reactivity in an animal model of cutaneous and systemic anaphylaxis. Mechanistic studies revealed that LARI downregulated surface FcεRI receptors and IgE via internalization of the IgE/FcεRI, promoted a partial mediator depletion pathway leading to slow release of small amount of mediators, and functioned as a partial antagonist to inhibit FcεRI signaling phosphorylation of Syk, Akt, Erk, and p38 MAPK. These studies demonstrate that targeting surface-bound IgE with LARI profoundly suppresses human allergic reactivity while displaying an excellent safety profile. *The Journal of Immunology*, 2019, 203: 000–000.

Given their increased prevalence and incidence, IgE-mediated allergic disorders have become a major worldwide public health concern (1). This epidemic of IgE-mediated allergic diseases has led to a corresponding interest in developing new therapeutic approaches, including novel immune-based biologic therapies (2, 3). In an effort to develop an effective therapy that would inhibit all IgE mediated reactions and, in particular, severe food allergic reactions, we have proposed the use of specifically designed low affinity anti-IgE Abs to directly target IgE bound to its high affinity receptor on allergic effector cells (4).

Previously, direct targeting of surface-bound IgE with bivalent anti-IgE Abs as an allergy therapeutic was thought not to be

feasible because of the common assumption that anti-IgE Abs would trigger an immediate allergic reaction by cross-linking surface IgE/FcεRI. However, we recently reported that murine prototype low affinity anti-IgE mAbs, with K_D in 10^{-6} – 10^{-8} M range, did not trigger anaphylactic reactivity but instead inhibited the reactivity of human allergic effector cells through weak binding to IgE in the FcεRI (4).

To target human surface-bound IgE with low affinity anti-IgE mAbs as a therapeutic, we have developed a set of humanized low affinity anti-human IgE mAbs designated as low affinity allergic response inhibitors (LARI). We characterized their biological and immunological features, defined their safety profiles, determined their therapeutic effect, and further examined their effects related to their therapeutic mechanism of action. In this article, we will show that the defined LARI has a profound inhibiting effect on allergic reactivity while displaying an excellent safety profile and, in the process, defines a candidate for future clinical development.

Materials and Methods

Abs and reagents

The mAb (FITC and/or PE labeled) to CD123 (Clone 6H6), CD63 (clone H5C6), HLA-Dr (Clone L243), FcεRIα (clone AER-37), CD117 (clone 104D2), Mas-related G-protein coupled receptor X2 (MRGPRX2) (clone K125H4), and Igλ (clone MHL-38) were from BioLegend. PE- and allophycocyanin-labeled Abs to pSyk (y348) (clone moch1ct), pAkt (S473) (clone SNRNR), and pErk 1/2 (T202/y204) (clone MILAN8R) were from eBioscience, and p-p38MAPK (clone 4NIT4KK) was from Invitrogen. Polyclonal goat anti-human IgE (PAE) was from Abcam (Ab9159). Anti-4-hydroxy-3-nitrophenylacetyl (NP)-IgE (clone JW 8/1) was from Bio-Rad. Anti-human IgG mAb (ATCC clone HB60) was purified from mouse ascites. Recombinant mouse IL-3, human IL-3, IL-6, and stem cell factor (SCF) were purchased from PeproTech. The cat allergen Fel d1 and peanut allergen Ara h1, Ara h2, and Ara h6 were obtained from Indoor Biotechnologies.

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Abbreviations used in this article: BAT, basophil activation test; CMC, CD34⁺ hematopoietic stem cell-derived mast cell; EBD, Evans blue dye; FcRn, neonatal Fc receptor; β-hex, β-hexosaminidase; hFcεRIα Tg, human FcεRIα transgenic; LARI, low affinity allergic response inhibitor; LTC₄, leukotriene C₄; MRGPRX2, Mas-related G-protein coupled receptor X2; NP, 4-hydroxy-3-nitrophenylacetyl; PAE, polyclonal goat anti-human IgE; PCA, passive cutaneous anaphylaxis; PO, propylene oxide; SCF, stem cell factor; SPR, surface plasmon resonance.

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Humanization of the mouse anti-human IgE mAb

Mouse anti-human IgE mAbs were produced with standard hybridoma technology (5). Humanization service of the parent low affinity mouse anti-human IgE mAbs p6.2, mE17, and F11 was provided by Genscript using its Ab humanization algorithm. CDR sequences of each V_H and V_L region of the parent founder clones were engrafted into the framework regions of the most homologous human germline sequences. The humanized V_H and V_L were expressed as human IgG₁ (γ1, κ) to make the low affinity anti-human ε specific mAbs, or LARI. To fine-tune IgE binding affinities, additional single mutations were introduced into the humanized V_L derived from the parent founder mE17. Mutated clones without detectable IgE binding were discarded, except for clone E4, which was kept to be used as a negative humanized mAb isotype control when appropriate. All LARI were transiently expressed in HEK-293 cells and purified by protein A affinity column chromatography with >90% purity.

Epitope mapping of LARI

LARI derived from parental founders P6.2, mE17, and F11 were epitope mapped using the CLIP technology (Pepscan Presto BV) (6). A library of overlapped and looped peptide array covering the entire CH1 to CH4 of the IgE H chain was synthesized for epitope mapping. The binding of Ab to each of the synthesized peptides was screened with an ELISA-based approach. The peptide arrays were incubated with primary Ab solution (overnight at 4°C). After washing, the peptide arrays were incubated with an anti-human IgG-peroxidase conjugate for 1 h at 25°C. After washing, the peroxidase substrate 2,2'-azino-di-3-ethylbenzthiazoline sulfonate and 20 μl/ml of 3% H₂O₂ were added. After 1 h, the color development was quantified with a charge coupled device camera and an image processing system. The values ranged from 0 to 3000 MAU with a commercially available Ab 3C9 as a positive control. Box-and-whisker plot and heat map analyses were used to analyze the binding intensity profiles.

Determination of the *t*_{1/2} of LARI

The predicted LARI in vivo *t*_{1/2} was determined using a validated neonatal Fe receptor (FcRn) affinity assay as described (7). The Octet QK (Pall ForteBio) was used for all FcRn binding in vitro assays at 30°C in 96-well solid black plates (655900; Greiner Bio-One). Prior to analysis, all Abs were dialyzed into PBS (pH 6), diluted to 100 μg/ml in PBS (pH 6), and used at a 200-μl volume. Initially, FcRn was immobilized to Ni-NTA-coated biosensors (18-0029; ForteBio) for 180 s at an optimized concentration. After a baseline step, the LARI-FcRn binding rate was determined when the biosensor with immobilized FcRn was exposed to LARI sample in PBS adjusted to pH 6 with HCl for 30 s. Following association, the LARI-FcRn complex was exposed to PBS (pH 7.5) and the rate of the LARI dissociation from FcRn was measured. Each assay was performed in quintuplicate. Each LARI was tested in conjunction with five reference mAbs as a standard that were produced under good manufacturing practice conditions and have reported human in vivo *t*_{1/2} data in healthy individuals. Data analysis was performed using software version 7.0 (Pall ForteBio). Rates were reported in 1/s and the mean K_{on} and K_{off} values of each LARI within an experiment were individually normalized to fold-change values among all tested mAbs. The fold-change FcRn binding rate was determined by averaging the mean fold-change K_{on} and mean fold-change K_{off} values of a mAb. The LARI FcRn binding rates and the accompanying calibration standard with known in vivo *t*_{1/2} were used to generate a linear regression best-fit correlation. The best-fit correlation was then used to predict the expected *t*_{1/2} of the LARI based on the binding rates.

Surface plasmon resonance studies

The affinity of each LARI for IgE was initially performed on a Biacore T2000 instrument and confirmed with Biacore model T200. Human myeloma IgE were immobilized on the CM5 sensor chips by amine coupling. The purified LARI were dissolved in HBS-EP assay buffer containing 0.15 M NaCl, 10 mM HEPES (pH 7.4), 3 mM EDTA, and 0.005% polysorbate 20. The solutions traversed the sensors at a flow rate of 50 μl/min for binding analysis. Binding results were expressed in resonance units. Kinetic studies were analyzed with BIAevaluation Software Version 4.1.

Healthy and allergic subject population

Forty-nine (32 male, 17 female) healthy and 13 previously clinically diagnosed allergic subjects to peanut (two male and two female) and cat (six male and three female) were recruited for this study. The subjects' sensitization status was confirmed by basophil activation testing (BAT) with

corresponding allergen Fel d1 and Ara h126. University of California, Los Angeles institutional review board approved the use of human blood from healthy and allergic subjects.

Culture of human CD34⁺ hematopoietic stem cell derived mast cells

The human CD34⁺ hematopoietic stem cell-derived mast cells (CMC) were developed using the protocol of Yin et al. (8). PBMC (2 × 10⁶) were used to isolate the human CD34⁺ blood hematopoietic stem cells using Easysep Human CD34 Positive Selection Kit (Stemcell Technologies) per manufacturer's instructions. CMC were developed over 8 wk in StemPro-34 SFM medium (Life Technologies) in the presence of SCF and IL-6, plus IL-3 in the first week only. The maturity of CMC was confirmed with surface staining of CD117, FcεRIα, and MRGPRX2.

Basophil activation test

Cat and peanut basophil activation test (BAT) was described previously (4). To determine if LARI could activate basophils, fresh heparinized whole blood (100 μl/test) was incubation for 30 min with various concentrations of LARI. To determine the ability of LARI to block basophil activation, allergic (cat and peanut) subjects' heparinized blood (200 μl/test) was preincubated with LARI or an isotype control at room temperature (22–24°C) with slow agitation (100 RPM) for 24 or 48 h prior to basophil activation using either mixed purified Ara h allergens (Ara h1, h2, and h6 combined) or the cat allergen Fel d1. In some experiments, human IL-3 was used to prime basophils in BAT assay. The cut-off value of the unstimulated basophil CD63 expression was always set as <2% as the background.

Intracellular staining

BD Phosflow (BD Bioscience) protocol was adopted for basophil intracellular staining using heparinized whole blood. The activated basophils were fixed, surface stained with CD123-FITC and HLA-Dr-PerCP, and the erythrocyte lysed with fixation buffer. The perm buffer IV was used for permeabilization, following by intracellular staining with PE- and/or allophycocyanin-labeled phosphor-specific Abs.

Histamine, leukotriene C₄, PG D₂, and β-hexosaminidase release assays

ELISA kits for histamine were from Immuno-Biological Laboratories (IBL-America), and leukotriene C₄ (LTC₄) and PGD₂ were from Cayman Chemical. β-hexosaminidase (β-hex) measurement in triplicate in cell supernatants and cell lysate was used as the indicator of mast cell degranulation. To determine the triggering capacity of LARI, ~4 × 10⁴–5 × 10⁴ IgE-sensitized lung and cultured skin tissue mast cells (9, 10) or CMC were washed and preincubated in a 37°C water bath for 5 min, followed by stimulation of LARI, PAE, or an anti-FcεRIα mAb as the positive control and IgG isotype as the negative control, for 30 min in stimulation buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.38 mM Na₂HPO₄·0.7H₂O, 5.6 mM glucose, 1.8 mM CaCl₂·H₂O, 1.3 mM MgSO₄·0.7H₂O, 0.4% BSA, pH 7.4). The released β-hexosaminidase (β-hex) was quantified by hydrolysis of 45 μl p-nitrophenyl N-acetyl-β-D-glucosamide (Sigma-Aldrich) solution in 0.1 M sodium citrate buffer (pH 4.5) for 90 min at 37°C. After the reaction was stopped with the addition of 150 μl of 0.2 M glycine (pH 10.7), the reaction plates were read at 405 nm (11). The percentage of β-hex release was calculated as a percent of total content using media alone as the background. For LAD2 cell degranulation assay (12), LAD2 cells were sensitized for 20 h with 0.5 μg/ml biotin-conjugated human IgE. Cells were then washed, resuspended in stimulation buffer at 0.01 × 10⁶ per well, and then stimulated with 0.1 μg/ml streptavidin to cross-link the biotinylated IgE or PAE at 1 μg/ml for 60 and 240 min, respectively. The lysates, prepared by lysing the cells in pure water and frozen-thawed three times, were used as the measure of the total content of β-hex to calculate the percentage of the β-hex released.

To determine the inhibitory effects of LARI on mast cell degranulation, various concentrations of LARI or a corresponding isotype control mAb were cultured with the mast cells already sensitized with NP-IgE (for skin mast cells), myeloma IgE, biotinylated myeloma IgE, or recombinant dansyl-specific IgE (13) for 48 h in StemPro-34 SFM medium supplemented with SCF and IL-6. This was followed by challenging the cells with appropriate cross-linker (e.g., NP-BSA at 10 μg/ml, PAE at 1 μg/ml, dansyl-BSA at 22 μg/ml, streptavidin at 0.1 μg/ml, and mastoparan [Anaspec] at 7 μM) in the reaction stimulation buffer for 30 min (skin mast cells and CMC), and 1 and 4 h for LAD2. The reaction supernatants were assayed for β-hex, LTC₄, and PGD₂.

Western blotting

Forty micrograms protein extracted with 4× XT sample buffer (Bio-Rad) from the control and treated LAD2 cells was loaded to 4–12% Criterion Precast Bis-Tris gel (Bio-Rad). Electrophoresis was carried out with 180 V for 15 min and 120 V for 90 min using 1× MOPS buffer, followed by transferring the protein from gel to nitrocellulose membrane using 1× cold Tris/glycine transferring buffer. The membrane was blocked with 5% nonfat milk in 1× TBST at room temperature for 1 h and incubated with rabbit anti p-Erk (9101; Cell Signaling Technology) diluted in 5% nonfat milk at 4°C overnight. After washing three times for 10 min each with TBST, the membrane was incubated for 2 h with HRP-labeled goat anti-rabbit IgG, followed by signal development with x-ray film. The blots were sequentially stripped, blocked, and reprobed with rabbit anti-total Erk Ab (4695; Cell Signaling Technology) and anti-GAPDH Ab (MAB374; MilliporeSigma) as protein loading control for phosphorylation signal normalization. Signal quantification was performed with a densitometry (Bio-Rad).

Multiplex luminex assay

CMC sensitized with dansyl-specific IgE (10 µg/ml) were cultured with various LARI at 5–50 µg/ml for 1, 4, and 48 h, with PAE at 1 µg/ml and dansyl-BSA at 22 µg/ml as the positive controls. The culture supernatants were frozen until assaying for the human cytokines/chemokines with multiple luminex assay using the 38-plex magnetic bead panel (MilliporeSigma). This assay service was provided by Westcoast Biosciences.

Passive cutaneous anaphylaxis

We used our previously established peanut IgE-mediated passive cutaneous anaphylaxis (PCA) assays to assess the ability of LARI to suppress PCA in human FcεRIα transgenic (hFcεRIα Tg) mice (4, 14). The hFcεRIα Tg mice were sensitized intradermally with serially diluted (0.5, 0.25, 0.125, and 0.63 µg/ml) purified IgE containing peanut IgE (4) for 2 h, followed by i.p. injection with LARI or an isotype control at 50 µg/mouse (2 µg/g body weight). Four days later, the mice were injected i.v. with 10 µg whole peanut extract as a challenge allergen mixed with 2% Evans blue dye (EBD) (100 µl/mouse). The mice were euthanized 30 min after allergen challenge, and PCA reactions were photographed. The mouse skin then was dried overnight, and the injected skin areas were cut out, weighted, and EBD extracted overnight in 55°C formamide. The amount of extravagated EBD was quantified with a conventional microtiter ELISA reader at 650 nm. EBD quantity from each PCA spot was normalized per milligram of skin tissue (4). The University of California, Los Angeles animal research committee approved our use of hFcεRIα Tg mice.

Systemic anaphylaxis assay

hFcεRIα Tg mice were sensitized with 40 µg of recombinant dansyl-specific human IgE (4, 13), followed by treatment with LARI E59 (50 µg/mouse) or an isotype control. Four days later, the mice were i.v. challenged with dansyl-BSA (100 µg/mouse). Core body temperature changes and the anaphylaxis clinical score (4) were measured as indicators of systemic allergic reactivity. Animals' core body temperatures were monitored every 5 min for the first hour after dansyl-BSA challenge. The temperature changes were plotted for comparison and statistical analysis to determine the effect of LARI and the isotype control on systemic anaphylaxis (4).

Confocal microscopy

FITC-labeled IgE sensitized CMC were treated with various testing condition for 4 and 24 h, respectively, followed by fixation, permeabilization, and intracellular FcεRIα staining using the protocol previously described (4). The processed cells were then fixed with 0.5 ml of 2% formaldehyde, and spun to the poly-lysine coated glass slide using cytospin at 1000 rpm for 5 min. The cells on the slides were stained with a drop of Vectashield mounting medium with DAPI (Vector Laboratories). The slides were examined with Leica SP2-IP-FCS confocal microscope.

Electron microscopy

Various concentrations of E59, along with PBS and LARI E4 as an isotype control and PAE at 1 µg/ml as a positive control, were injected intradermally in a 40-µl volume into skin on the back of hFcεRIα Tg mouse. Thirty minutes later, the animals were euthanized. About a 1 cm by 1 cm area around the injected skin site was resected and fixed in 0.1 M phosphate buffer, 0.9% NaCl (pH 7.2) containing 2% paraformaldehyde, and 2.5% glutaraldehyde for 1 h at room temperature, followed by fixing the specimen at 1% OsO₄ in 0.1 M PBS 1 h. After washing three times for

10 min with ddH₂O, the specimens were dehydrated for 15–20 min each with 50, 70, 95, and 100% ethanol. The samples then were transferred to propylene oxide (PO) two times for 10–15 min, followed by transferring to 1:1 mix PO/epon for 1 h, and then transfer to 1:2 mix PO/epon for 4 h before leaving them overnight. The samples were then transferred to fresh epon and placed in vacuum for 4 h, followed by polymerizing at 60°C overnight. The treated samples were then embedded and processed in the standard fashion. The grids were examined with a JEOL 100CX transmission electron microscope.

Statistics

Data are expressed as mean ± 1 SEM or 1 SD when appropriate. Unless indicated in the figure legend, Student *t* test was used to determine the statistical significance for the paired data, and two-tailed ANOVA analysis was used to determine the unpaired data using GraphPad Prism 8.01. A statistically significant difference was defined as *p* < 0.05, which was indicated with one asterisk, whereas a significant difference of *p* < 0.01 was indicated with double asterisks in the appropriate figures.

Results

Characteristics of the humanized low affinity anti-IgE mAb LARI

Three mouse founder clones, P6.2, F11, and mE17, were humanized to human IgG₁ (γ1, κ) by engrafting the V_H and V_L CDR regions of the low affinity mouse anti-human IgE mAbs into the framework of the most homologous human germline sequences. Specifically, V_H and V_L CDR from P6.2 were engrafted to human IgV_H 1-2 and IgV_κ 7-3, from F11 to human IgV_H 1-18 and IgV_κ 2-8, and from mE17 to human IgV_H 7-81 and IgV_κ 7-3 frameworks. Their ability to bind IgE in solid phase and the FcεRIα bound on cell surface was screened with an ELISA (Fig. 1A) and a flow cytometry-based IgE binding assay employing human FcεRIα-expressing 3D10 cells loaded with myeloma IgE (Fig. 1B). The selected LARI clones were further confirmed with surface plasmon resonance (SPR) (Fig. 1C).

The founder clones P6.2, F11, and mE17 had affinities for human IgE with K_D of 2.0 × 10^{−6} M, 4.05 × 10^{−7} M and 3.16 × 10^{−8} M, respectively. The affinities of the selected LARI for IgE were in the 3.88 × 10^{−6} M–8.09 × 10^{−8} M range (K_D = 1.37 × 10^{−7} M for E14, 3.44 × 10^{−7} M for E17, 8.09 × 10^{−8} M for E23, 1.21 × 10^{−7} M for E59, and 3.88 × 10^{−6} M for S91) (Fig. 1C, and data not shown), indicating these LARI had an appropriately low affinity, even though the engraftment of mouse CDR into human Ig frameworks had slightly decreased their affinities as expected. Evaluation of the binding specificity of each LARI demonstrated that they specifically bound to human IgE, but not to human IgM, IgG, or IgA or murine, cynomolgus, or canine IgE (Fig. 1A), and that their ability to bind to cell surface FcεRI attached IgE had not been altered by humanization process (Fig. 1B).

The predicted *in vivo* *t*_{1/2} of the selected LARI were measured with MassBiologics' FcRn-based binding assay using a set of five recombinant human IgG₁ mAbs with known *in vivo* *t*_{1/2} to derive a standard calibration curve (7). LARI E14, E17, E23, and E59 displayed an *in vivo* *t*_{1/2} being 1091 h (45.5 d), 1041 h (43.4 d), 1181 h (49.2 d), and 1061 h (44.2 d), respectively (Fig. 1D), as compared with that of the five humanized Ab assay standards (14–35 d, ~25 d in average). The predicted *t*_{1/2} of S91 was 624 h (26 d), falling in the average range for humanized IgG₁ (Fig. 1D). These data revealed that the parental founder mE17-derived LARI E14, E17, E23, and E59 have an unusually long predicted *in vivo* *t*_{1/2}.

Epitope mapping of LARI

LARI were epitope mapped employing CLIP technology (6). Because the four LARI clones E14, E17, E23, and E59 were derived from the parental founder mE17, they shared the same

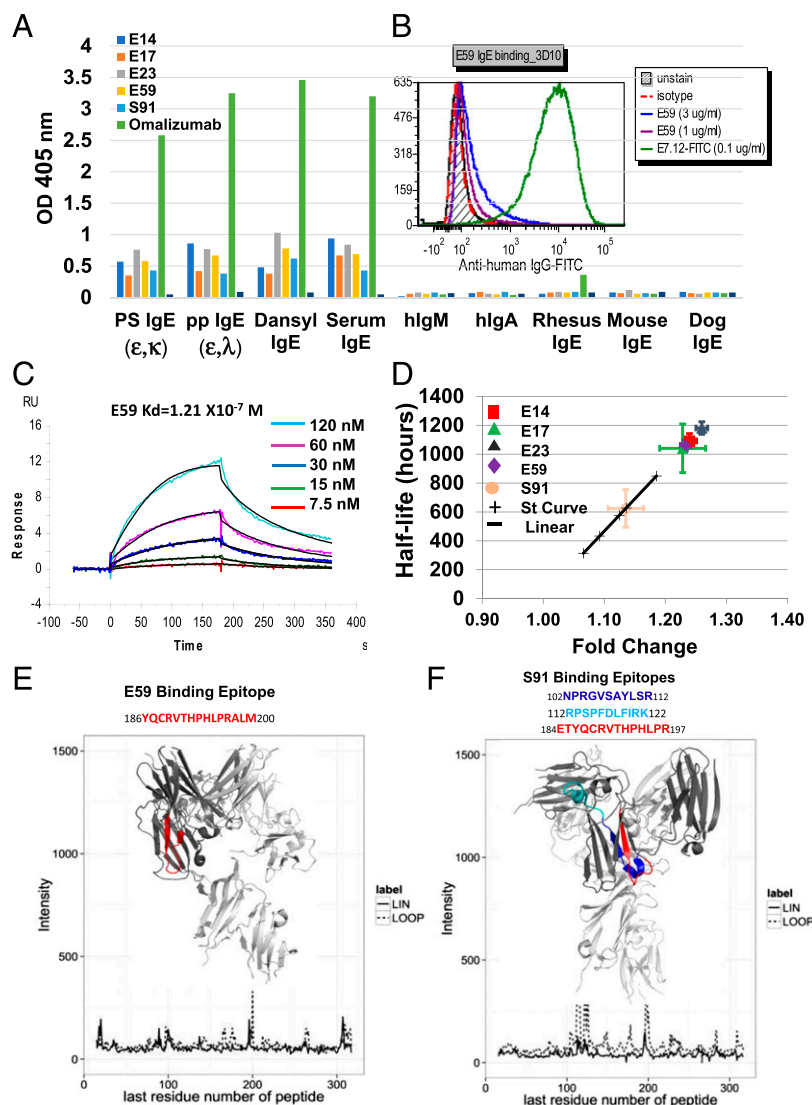


FIGURE 1. Characterization of the humanized LARI. **(A)** Specificity of LARI for human IgE determined with ELISA, using omalizumab as a comparison. **(B)** Binding of LARI E59 (1 and 3 μg/ml, respectively) to FcεRI-bound IgE on 3D10 cells. The FITC-E7.12 served as a staining comparison. **(C)** LARI E59 affinity for IgE measured with SPR using Biacore T200. **(D)** Predicted in vivo $t_{1/2}$ of LARI using FcRn binding assay. Five humanized mAbs with known in vivo $t_{1/2}$ were used as calibration standards to determine the predicted in vivo $t_{1/2}$ of LARI. **(E)** Epitope mapping of LARI E59. Inserted is the three-dimensional visualization of the E59 binding epitope colored in red. **(F)** Epitope mapping of LARI S91. The inserted three-dimensional visualization represents the linear epitope 102–112, 112–122, and 184–197 colored in blue, cyan, and red, respectively.

CDR and presumably bound to the same IgE epitope, albeit with slightly different affinity because of single mutation introduced for affinity fine-tune. Thus, we selected two LARI clones derived from mE17, LARI E59, and E17 for epitope mapping. As shown in Fig. 1E, E59 bound weakly to several linear peptides in the intensity profiles recorded with linear peptides (solid line), which were inconclusive in defining the binding epitope(s). When intensity profiles recorded with single loop peptides (Fig. 1E, dashed line), E59 resulted in one major peak at the same position corresponding to residues stretch $_{186}\text{YQCRVTHPHLPRALM}_{200}$, which was colored in red in three-dimensional visualization of IgE H chain (insert of Fig. 1E). LARI E17 displayed the identical pattern with E59, confirming the same epitope binding (data not shown).

The founder F11-derived S91 yielded binding intensity profiles with few peaks of equal intensities resulting from interaction with three series of overlapping peptides, $_{102}\text{NPRGVSAYLSR}_{112}$, $_{112}\text{RPSPFDLFIRK}_{122}$, and $_{184}\text{ETYQCRVTHPHLPR}_{197}$ (Fig. 1F). These results indicated recognition of discontinuous epitopes (e.g., conformational epitope), one of which partially overlaps with the epitope identified for E59. The three-dimensional visualization of the epitopes revealed that despite being separated in the primary structure, the peptide sections $_{102}\text{NPRGVSAYLSR}_{112}$ and $_{184}\text{ETYQCRVTHPHLPR}_{197}$ came together to form a conformational epitope as predicted in the three-dimensional modeling,

with peptide section $_{112}\text{RPSPFDLFIRK}_{122}$ being spatially separated (insert of Fig. 1F).

Under high and moderate stringency conditions, the founder P6.2-derived C6 did not bind any linear or single looped peptide present on the array. When tested under low stringency conditions, this mAb yielded an intensity profile with numerous peaks of high intensity (data not shown). Such data prevented the direct linear epitope definition, suggesting that C6 binds to a more complexed conformational epitope on IgE that could not be resolved with the applied epitope mapping approach. As the humanized p6.2 clone C6 displayed too low an affinity to be effective for allergy therapy (data not shown), we made no further attempt to map the epitopes of this clone.

LARI itself fails to trigger acute release of prestored or newly synthesized allergic mediators ex vivo

An inability of LARI to trigger acute allergic mediator release and subsequent allergic reactivity is paramount to the safety of low affinity anti-IgE based allergy therapy. Thus, we extensively evaluated the LARI's safety profiles using multiple assays and systems including basophils, CMC, lung and skin mast cells, LAD2 cells, and RBL-SX-38 cells.

In the BAT assay, the high affinity mAbs E4.15 and nonreceptor activator fMLF triggered strong basophil CD63 expression

indicative of anaphylactic degranulation within 30 min (Fig. 2A). In contrast, LARI E14, E17, E23, E59, and S91, at concentrations up to 50 $\mu\text{g/ml}$, failed to trigger basophil CD63 expression in any blood donors tested in the same time frame ($n = 32$, 24 nonallergic and 8 allergic donor, Fig. 2A) and did not trigger acute basophil histamine release ($n = 6$, 3 nonallergic and 3 allergic donors, Fig. 2B).

IL-3 is capable of priming basophils for enhanced basophil activation and CD63 expression upon allergen and/or anti-IgE stimulation (15). To test whether LARI would activate IL-3-primed basophils or synergize with IL-3 in basophil activation, BAT was performed under IL-3 priming conditions with cat

allergic donor basophils. As data summarized in Fig. 2C ($n = 5$), although IL-3 itself had little effect on CD63 induction, IL-3 at 20 ng/ml significantly enhanced CD63 expression by suboptimal concentration of PAE, Fel d1, and fMLF. However, IL-3 (2–100 ng/ml tested), either preincubated for 30 min or simultaneously added with LARI, did not promote higher than background basophil CD63 expression. Thus IL-3 did not synergize with LARI to drive basophil activation.

With mast cells, LARI at 20–100 $\mu\text{g/ml}$ did not trigger β -hex release from 1) the freshly human lung mast ($n = 3$, Fig. 2D); 2) cultured human skin mast cells ($n = 2$, Fig. 2E); or

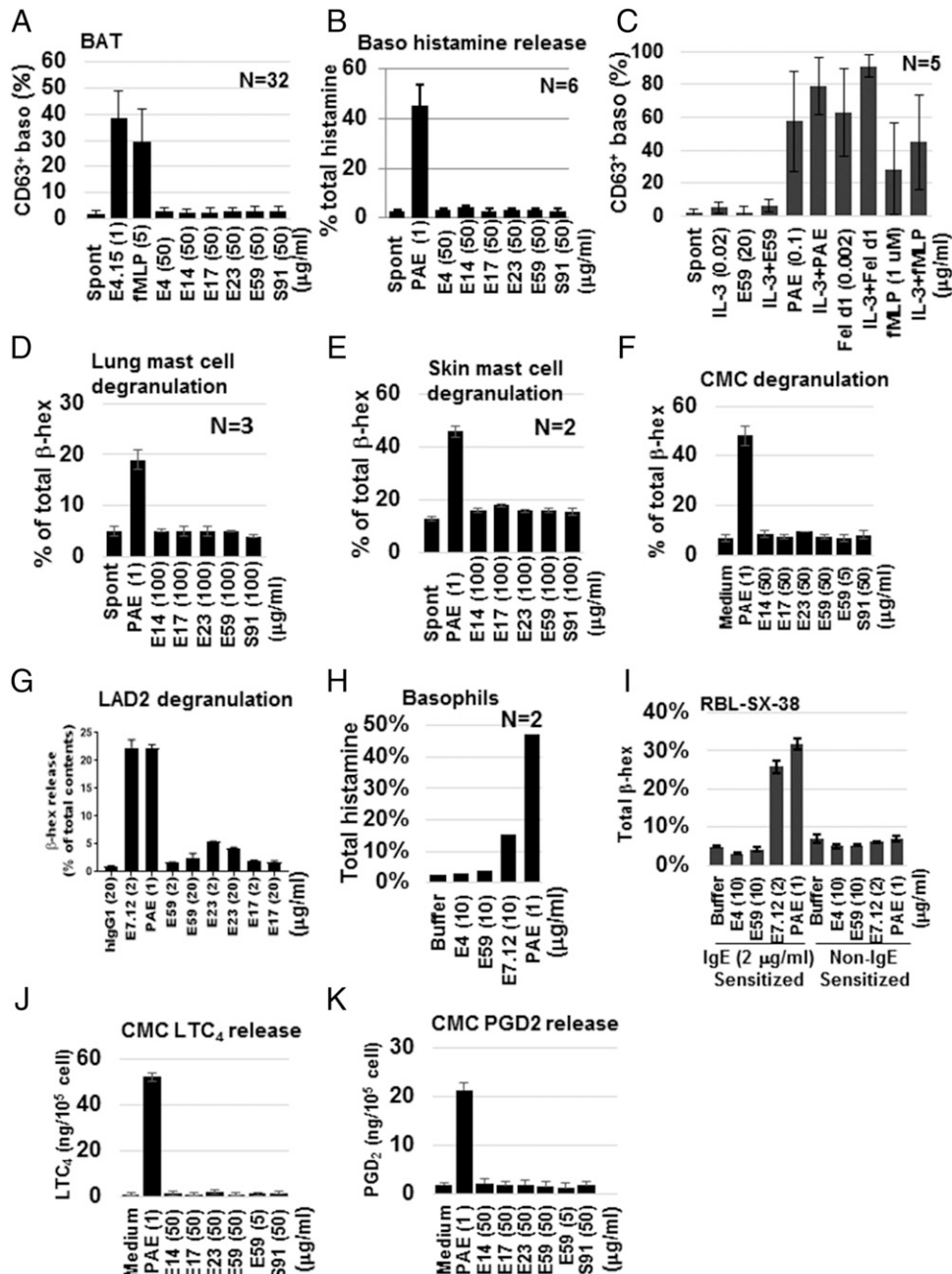


FIGURE 2. Safety profiles of LARI ex vivo. High and low concentrations of LARI was tested for their capacity of triggering basophil activation and mast cell degranulation using multiple allergic effector cells, including appropriate positive and negative controls for comparison as indicated in the corresponding panels. (A) BAT ($n = 32$). (B) Basophil histamine release ($n = 6$). (C) BAT with IL-3 priming ($n = 5$). (D) β -hex release of lung mast cells ($n = 3$). (E) β -hex release of skin mast cells ($n = 2$). (F) CMC β -hex release (representative of three experiments). (G) LAD2 β -hex release (representative of four experiments). (H) Basophil histamine release triggered by immobilized LARI and its controls ($n = 2$). (I) β -hex release of RBL-SX-38 cells triggered by immobilized LARI and its controls (representative of two experiments). (J) CMC LTC₄ production (representative of two experiments). (K) CMC PGD₂ production (representative of two experiments).

3) CD34⁺ hematopoietic stem cell-derived CMC (Fig. 2F). Among the five LARI tested, only E23 induced any, although minimal, increased β -hex release in LAD2 cells (Fig. 2G), a notable exception given that E23 has the highest affinity for IgE among five LARI. As the positive control for the assay, PAE triggered robust β -hex release.

To test if the cross-linking LARI as might occur with an anti-LARI Ab could trigger degranulation of allergic effector cells, LARI was directly coated onto the surface of ELISA plate, followed by incubation with the freshly enriched blood basophils ($n = 2$) or IgE-sensitized RBL-SX-38 cells (16) and mediator release measured. As shown in Fig. 2H and 2I, immobilized LARI on ELISA plate did not trigger histamine release from basophils, nor was β -hex released from RBL-SX-38 cells, whereas plates coated with the high affinity E7.12 or PAE induced robust mediator release.

To test the acute effects of LARI on rapidly synthesized lipid mediators, IgE-sensitized CMC were challenged with LARI for 1 h, followed by measurement of supernatant LTC₄ and PGD₂. LARI at 5 and 50 μ g/ml, respectively, did not drive higher than background levels of LTC₄ or PGD₂ secretion, whereas the positive control PAE drove robust LTC₄ (Fig. 2J) and PGD₂ secretion (Fig. 2K).

It has been reported with murine mast cells that low affinity interaction of the Fc ϵ RI-bound IgE with its corresponding ligand preferentially elicited a robust slowly synthesized cytokine/chemokine response (17). To examine the potential effects of LARI on slowly synthesized cytokine and chemokine production, a multiplex assay was used to simultaneously measure 38 cytokines/chemokines from dansyl-IgE-sensitized CMC that were stimulated with LARI at 5 and 50 μ g/ml, levels well above the therapeutic concentration. PAE and dansyl-BSA were the positive controls. Twelve (CCL22, IL-10, MCP-3, CX3CL1, FGF2, MIP-1 β , CXCL1, IL-8, VEGF, MCP-1, MIP-1 α , and Eotaxin) were detected in the presence of LARI and/or positive controls, but the levels were less than a 1-fold increase compared with the spontaneous secretion level in culture medium. In contrast, the other 25 cytokines/chemokines in the panel were undetectable (<3.2 pg/ml) at all three time points examined (data not shown). These results indicated that LARI did not appear to promote CMC to produce these slowly synthesized mediators.

LARI fails to trigger acute allergic reactions in vivo

To assess the safety of LARIs in vivo, hFc ϵ RI α Tg mice systemically sensitized with human myeloma IgE (PS IgE, 50 μ g/mouse, 2 μ g/g body weight) was used (4, 14). None of the tested LARI, at up to 100 μ g/ml, triggered local PCA reactivity (Fig. 3A, $n = 3$). As a positive control, PAE at 1 μ g/ml induced strong PCA reactivity (low right corner, Fig. 3A).

To test whether LARI could trigger systemic anaphylaxis in vivo, IgE-sensitized mice were challenged with 200 μ g of LARI E59 (8 μ g/g body weight) or 50 μ g of high affinity E4.15 (2 μ g per gram body weight) as a positive control. The fall in core body temperature indicative of systemic anaphylaxis was not seen in LARI E59-challenged animals but was observed in E4.15-challenged mice ($n = 4$, Fig. 3B).

It is predictable that some subjects given LARI may produce anti-drug Abs. For LARI, in addition to standard concerns, this raises the possibility that such anti-drug Abs, by cross-linking of LARI bound to IgE on Fc ϵ RI, could trigger allergic reactivity. To test this possibility, the IgE-sensitized hFc ϵ RI α Tg mice were treated systemically with LARI E59, followed by anti-human IgG mAb HB60, which served to cross-link LARI. Notably, no significant core body temperature change occurred upon the cross-linking

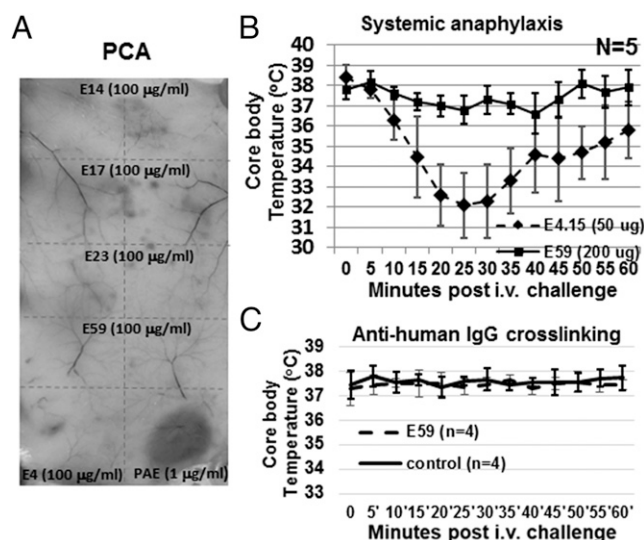


FIGURE 3. Safety profile of LARI E59 in vivo in hFc ϵ RI α Tg mice systemically sensitized with human IgE. (A) PCA. PAE and PBS were included as the positive and negative control respectively. (B) Systemic anaphylaxis. The high affinity anti-IgE mAb E4.15 was included as a positive control. (C) LARI cross-linking with anti-human IgG mAb HB60.

($n = 4$, Fig. 3C), indicating that LARI cross-linking by anti-human IgG mAb did not induce anaphylactic degranulation. Combined with ex vivo safety data in which immobilized LARI failed to activate basophils (Fig. 2H, 2I), this data further support the idea that cross-linked LARI will fail to elicit allergic reactivity.

LARI suppresses allergic basophil activation

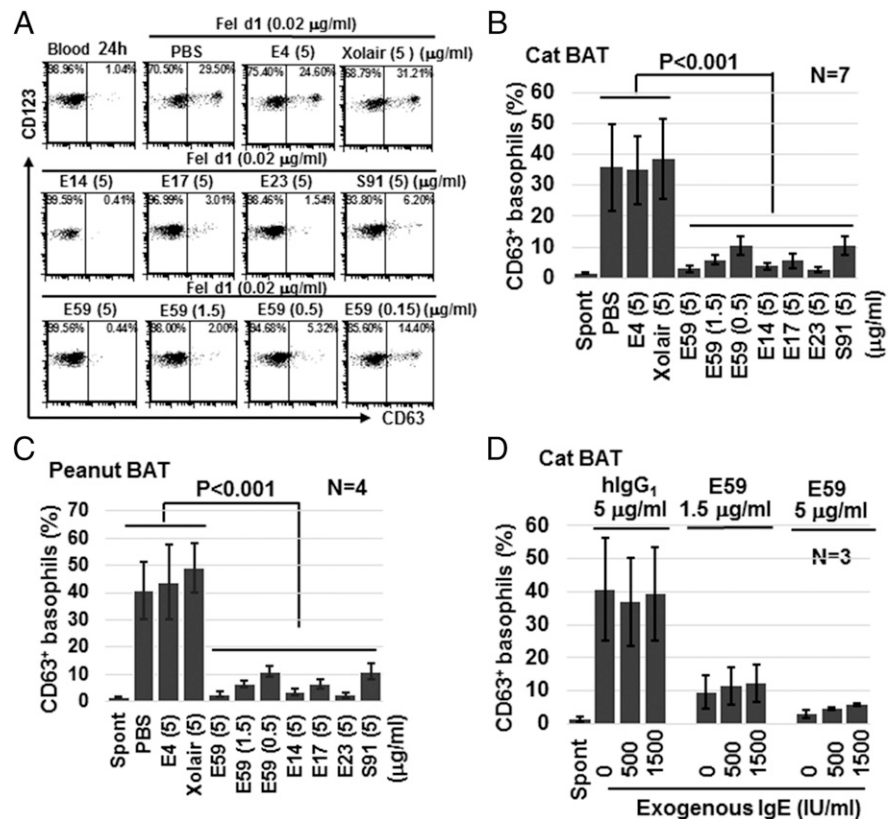
The therapeutic potential of LARI on basophil-mediated allergic reactivity was examined using a modified BAT assay as described (4) that includes a prolonged incubation time of 24–48 h so that there is time for full drug effect. Ex vivo allergic basophil activation from both cat and peanut allergic donors was profoundly suppressed by all five LARI tested (i.e., E14, E17, E23, E59, and S91) in a dose-dependent fashion between 0.5 and 5 μ g/ml, and maximal BAT inhibition (>90%) occurred at 5 μ g/ml, compared with E4 as an appropriate isotype control (Fig. 4A–C). Omalizumab (Xolair), a Food and Drug Administration–approved allergy therapeutic via neutralizing and depleting free serum IgE, did not block basophil activation in BAT as expected given its known binding profile and mechanism. In fact, Xolair slightly enhanced the Fel d1- and Ara h126-induced basophil activation (Fig. 4A–C). We ranked the LARI as E59 = E23 > E14 > E17 > S91 based on their efficacy for BAT inhibition.

To test whether LARI's effect would be altered by high levels of free IgE, as is the case for omalizumab, we examined the impact of addition of IgE on LARI's ability to inhibit the cat allergen driven BAT. Addition of 500 or 1500 IU/ml (equivalent to 1205 or 3615 ng/ml, respectively) of exogenous IgE to the allergic donor's blood samples did not alter the baseline Fel d1 induced BAT (Fig. 4D, $n = 3$). Notably, BAT inhibition by 1.5 or 5 μ g/ml LARI E59 was also unchanged by this additional IgE as compared with BAT with the donor serum alone (Fig. 4D). Thus, reasonably high serum IgE levels as commonly seen in patients are not predicted to be a significant limiting factor for the therapeutic efficacy of LARI.

LARI suppresses the immediate release of the prestored allergic mediator triggered by Fc ϵ RI cross-linking

The ability of LARI to inhibit immediate release of the prestored allergic mediators triggered by Fc ϵ RI cross-linking was tested

FIGURE 4. Ability of LARI to inhibit allergen-induced basophil activation. **(A)** Flow cytometry profile of cat allergen induced CD63 expression inhibited by various LARIs. Upper panel, Controls; middle panel, various LARIs; and lower panel, E59 dose-dependence. **(B)** Summary of the inhibitory effects of LARI on Fel d1-induced CD63 expression ($n = 7$). **(C)** Summary of the inhibitory effects of LARI on peanut allergen (Ara h1, 2, and 6)-induced CD63 expression ($n = 4$). **(D)** Summary of the inhibitory effects of LARI on cat allergen induced CD63 expression in the presence of exogenous IgE ($n = 3$). Statistical significance was tested with Student t test.



using cultured CMC, LAD2, and skin mast cells. The mast cells were first sensitized with myeloma IgE, NP-IgE, or biotinylated IgE, then treated with LARI or various controls for 48 h, followed by challenging with the FcεRI pathway cross-linker PAE or streptavidin, or the MRGPRX2 pathway agonist mastoparan (18), to determine the effect of LARI on inhibiting degranulation.

Treatment for 48 h with LARI E14, E17, E23, or E59, but not the isotype control E4, culture medium, or omalizumab, significantly blunted the CMC β-hex release when the cells were subsequently challenged with PAE and streptavidin (Fig. 5A, the gradient darkener filled bars). However, LARI treatment did not affect the response to mastoparan (Fig. 5A, patterned filled bars), indicating that LARI-inhibited FcεRI, but not the MRGPRX2, mediated degranulation in CMC. In LAD2 cells, LARI also significantly blunted LAD2-derived β-hex release in response to PAE challenge or streptavidin/biotin IgE cross-linking although to a lesser extent (Fig. 5B). The ability of LARI to suppress Ag-specific IgE-mediated degranulation was tested using cultured human skin mast cells sensitized with NP-IgE followed by a 48 h treatment. As shown in Fig. 5C, LARI significantly inhibited NP-BSA (and NP-LPS as well, data not shown) activated β-hex release at relatively higher concentration (10 µg/ml).

LARI causes slow loss of prestored allergic mediators from human mast cells

Although LARI, even at high concentration, did not trigger acute (within 60 min) release of allergic mediators (Fig. 2), we examined whether LARI could stimulate small amount of mediator release over a longer culture period, as we had seen an phenotype of mediator loss in LARI-treated cells (discussed below). IgE and biotinylated IgE-sensitized CMC were incubated with LARI, along with the corresponding controls, for 48 h, followed by assaying the histamine level in the culture supernatants (β-hex release was not tested, as the color changes in 48 h cultured

medium interferes with the β-hex assay). After 48 h culture, LARI, but not E4 or omalizumab, slightly but reproducibly increased the supernatant histamine level to ~5–10% of that seen with strong activation by PAE (Fig. 5D). Examination of the total β-hex content in the 48-h incubated CMC lysates revealed that LARI treatment, but not the corresponding controls, simultaneously decreased the total β-hex content in the cells (Fig. 5E). As with CMC, the total β-hex content in 48-h LARI-treated LAD2 cells, which are relatively insensitive to FcεRI cross-linking, mirrored the results with CMC but on a smaller scale, albeit not reaching statistical significance (Fig. 5F). Taken together, these results suggest that LARI promotes slow release of mediators while rendering the mast cells less responsive to strong FcεRI cross-linking.

LARI blunts lipid allergic mediator release from CMC

The effects of LARI on rapidly induced lipid allergic mediator synthesis were assessed using CMC in conjunction with the histamine and β-hex release experiments presented in Fig. 5. In 48 h culture supernatants, PAE, the positive control, induced robust LTC₄ and PGD₂ secretion (Fig. 6A, 6B respectively). LARI, but not the E4 or omalizumab, slightly enhanced the LTC₄ and PGD₂ compared with spontaneous production, however at a much lower level compared with PAE (Fig. 6A, 6B). Notably, E23 displayed a slightly stronger effect compared with other LARI.

When CMC were treated with LARI E14, E17, E23, or E59, PAE- or streptavidin (for biotin-IgE loaded cells)-driven LTC₄ and PGD₂ secretion was significantly blunted (Fig. 6C, 6D, the light and dark filled bars, respectively). LARI treatment did not affect the CMC response to mastoparan stimulation (Fig. 6C, 6D, patterned filled bars). As IgE or biotinylated IgE sensitized LAD2 cells did not produce LTC₄ and PGD₂ in response to PAE and/or streptavidin (data not shown), the effect of LARI on lipid mediator production in LAD2 cells was not tested.

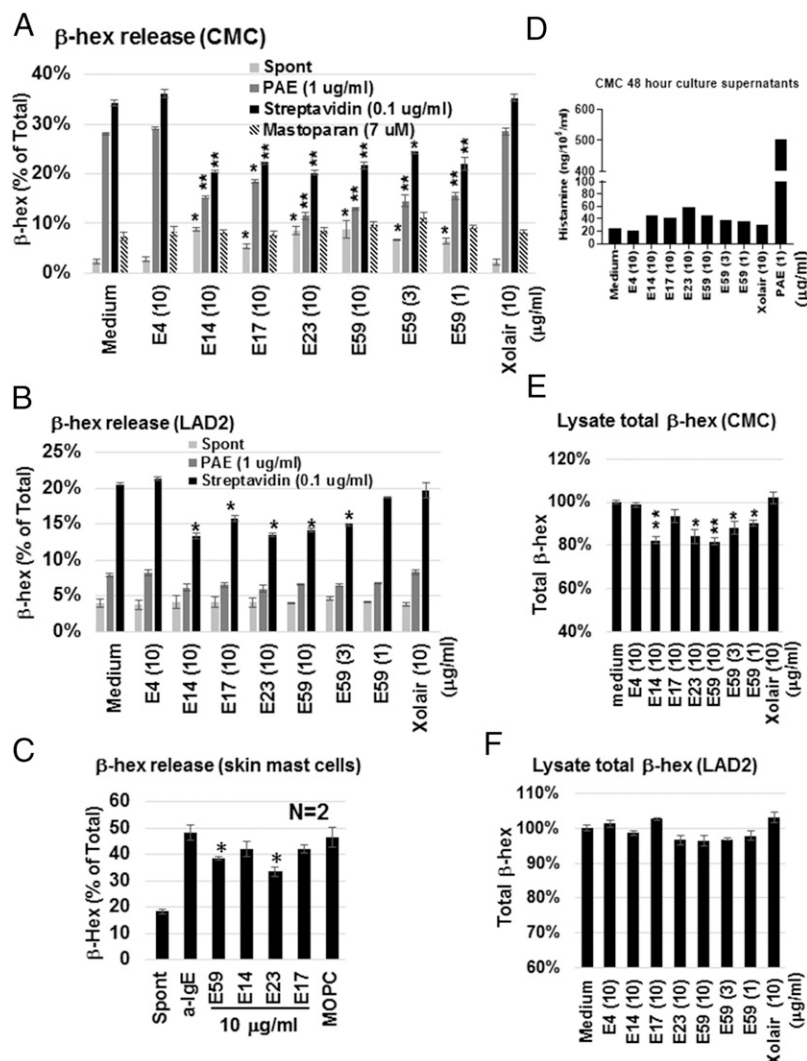


FIGURE 5. Effects of LARI on inhibition of pre-stored mediator release from 48 h cultured human mast cells. The 48 h cultured supernatants were assayed for histamine levels, whereas the harvested cells were challenged with buffer, PAE, streptavidin, and mastoparan to test for β -hex or lysed to determine mediator content. (A) β -hex release from the LARI treated CMC. (B) β -hex release from LARI treated LAD2. (C) β -hex release from LARI treated skin mast cells. (D) Histamine level in 48-h cultured CMC supernatants. (E) The total β -hex level in LARI treated CMC lysates. (F) The total β -hex level in LARI-treated LAD2 cell lysates. * p < 0.05; ** p < 0.01, Student t test.

LARI suppresses peanut IgE mediated PCA

Human peanut IgE-mediated PCA testing was used to evaluate the ability of LARI to block in vivo mast cell-mediated allergic cutaneous reactivity in hFc ϵ RI α Tg mice as described (4, 14). In the presence of a control human IgG isotype, a dose-dependent PCA reaction in the range of 0.5–0.125 μ g/ml of the IgE purified from the pooled peanut allergic plasma was established (left panel, Fig. 7A; and the black column, Fig. 7B–E). PCA at each sensitized IgE concentration was suppressed by E59, E14, E17, and E23 (Fig. 7A). The inhibition level for each LARI at each sensitizing dose of IgE as quantified by EBD extravasation is presented in Fig. 7B–E, where significant (p < 0.05) or very significant (p < 0.01) PCA inhibition was achieved in all LARI tested except for the IgE dose of 0.5 μ g/ml for E23 (Fig. 7E). LARI effectiveness in peanut IgE PCA inhibition was ranked as E59 > E23 > E14 > E17 > S91.

LARI blocks systemic anaphylaxis in vivo

The effects of LARI on systemic anaphylaxis was evaluated using systemic anaphylaxis in the human anti-dansyl IgE sensitized hFc ϵ RI α Tg mice (4, 13, 14). Dansyl-BSA challenge induced a profound drop in core body temperature and a high anaphylactic clinical scores (3.8 in average, Fig. 7F) in sensitized mice treated with a human IgG₁ isotype control. In contrast, mice treated with 50 μ g E59 (2 μ g per gram body weight) showed markedly blunted temperature changes and reduced clinical

scores (0.6 in average, Fig. 7F), indicating that E59 blocked dansyl-specific human IgE-mediated systemic anaphylaxis.

LARI downregulates surface IgE and Fc ϵ RI expression via internalization of IgE/Fc ϵ RI complex

We used the cultured CMC and LAD2 to examine LARI's ability to downregulate the surface IgE and Fc ϵ RI expression. ppIgE (human ϵ , λ)-sensitized CMC and LAD2 (with unbound free IgE washed away) were treated with medium as a blank, E4 (10 μ g/ml) as an isotype control, LARI E59 (10 μ g/ml) as the test agent, and E7.12 (1 μ g/ml) and PAE (1 μ g/ml) as the positive controls and then Fc ϵ RI α and IgE levels on the cell surface were measured. FITC-labeled anti-Fc ϵ RI α mAb AER-37, which binds to an epitope not masked by IgE binding, was used to measure the surface Fc ϵ RI level. As several labeled anti-human IgE Abs tested showed differing efficacy to detect the receptor-bound IgE that was interacting with LARI E59, E7.12, and/or PAE because of direct binding site competition and/or steric hindrance, we used a PE-labeled anti-Ig λ as the measurement for surface IgE level, because the sensitized ppIgE was Ig ϵ/λ . As shown in Fig. 8A, the CMC surface Fc ϵ RI and IgE levels at baseline were minimally, if at all, affected by the isotype control E4, but downregulated by the positive controls E7.12 and PAE. E59 (as well as E14 and E23, data not shown), which induced a moderate downregulation of both Fc ϵ RI α and IgE expression at day 2 and 5, with a more profound decrease of IgE compared with that of Fc ϵ RI at day 5

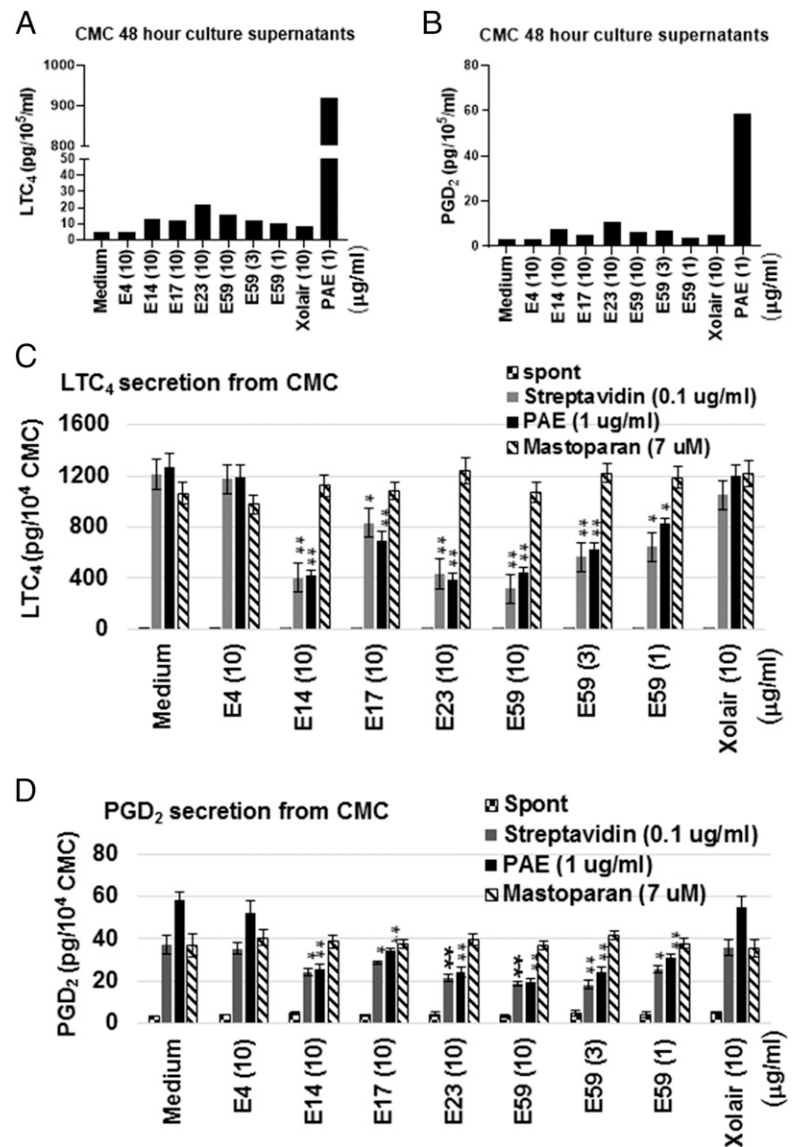


FIGURE 6. Effects of LARI on suppression of the newly synthesized lipid mediator release from 48-h cultured CMC. **(A)** LTC₄ in CMC supernatants. **(B)** PGD₂ in CMC supernatants. **(C)** LTC₄ secretion from LARI-treated CMC that subsequently challenged by buffer, streptavidin, PAE, and mastoparan. **(D)** PGD₂ secretion from LARI-treated CMC that subsequently challenged by buffer, streptavidin, PAE, and mastoparan. * $p < 0.05$; ** $p < 0.01$, Student t test.

(Fig. 8A). The unequal downregulation of FcεRIα and IgE expression by LARI, as well as by E7.12 and PAE, suggested that newly synthesized and/or recycled FcεRI being expressed at the surface was likely responsible for this difference, as the surface FcεRI, but not IgE, could be replaced in this experimental setting. Similar downregulation effect of FcεRI and IgE by LARI E59 was also confirmed with LAD2 cells (data not shown).

Confocal microscopy was used to examine the fate of the downregulated surface IgE/FcεRI using FITC-IgE-sensitized CMC treated with E59 and appropriate controls (Fig. 8B). FITC-IgE, along with FcεRI, was generally evenly distributed across the cell surface, without or rarely forming aggregates, and certainly not large aggregates, in the medium or E4 controls at 4 and 24 h. In contrast, E59-treated CMC formed more and larger aggregates that were located on both surface and intracellularly. There were less FcεRI involved in the E59 induced aggregates (light yellow color) at 4 h time point, but progressively larger aggregates with higher FcεRI content (bright/darker yellow color) appearing at 24 h. In a sharp contrast to E59, the positive controls E7.12- and/or PAE-treated CMC displayed a massive amount of colocalized IgE/FcεRI signals intracellularly, indicative of intense internalization of IgE/FcεRI complex at both 4 and 24 h time points (Fig. 8B). These data showed that surface IgE and

FcεRI internalization was driven by LARI E59, albeit in a lower level compared with high affinity anti-IgE Abs.

LARI induces a piecemeal degranulation-like phenotype in mast cells in vivo

The evidence that LARI failed to trigger acute mediator release but promoted a slow small level of mediator appearance in cultures (Figs. 5D, 6A, 6B) prompted us to hypothesize that LARI could stimulate a “leak” pathway whereby low levels of preformed mediators are released leading to partial granule mediator depletion through a mechanism resembling the “piecemeal degranulation” pattern seen in eosinophil degranulation (19). We therefore examined via electron microscopy skin mast cells from IgE-sensitized hFcεRIα Tg mouse that had received an intradermal injection of LARI E59 and compared this to injections with PBS or LARI E4 as negative controls or PAE as an anaphylactic degranulation positive control. As expected, PBS- (Fig. 9A) and LARI E4 (Fig. 9B)-treated skin mast cells contained mainly intact granules with electron-dense materials evenly distributed throughout (solid arrows, Fig. 9A, 9B). The granules of the LARI E59-treated skin mast cells displayed the various degree of mediator depletion appearing as partially empty granules (dotted arrows) but no granule fusion (Fig. 9D–F). Mast cells at the PAE

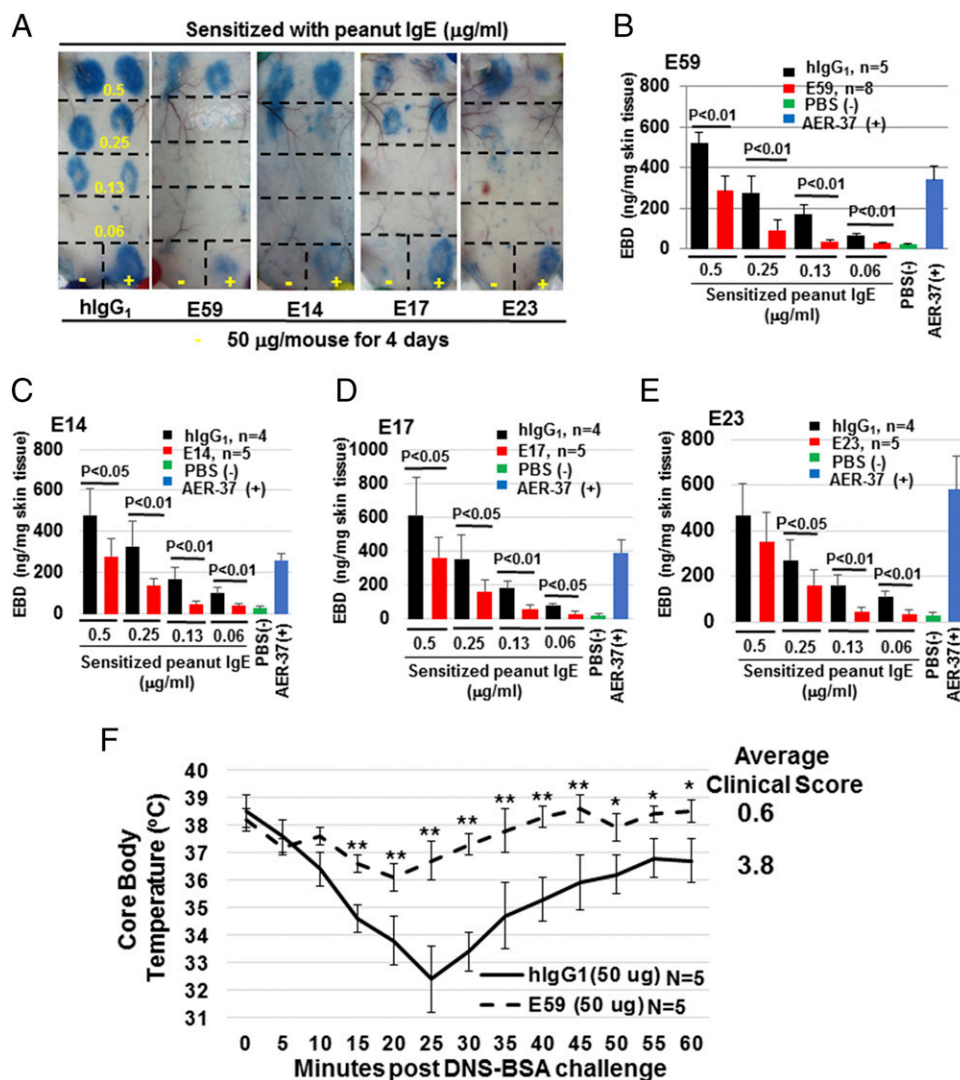


FIGURE 7. LARI suppresses IgE-mediated cutaneous and systemic allergic reactions in vivo in hFcεRIα Tg mice. **(A)** Effects of LARI on peanut allergic IgE-mediated PCA. (+) is the PCA positive control, whereas (–) is the negative PCA control. **(B–E)** Quantitative EBD assessment of peanut allergic IgE-mediated PCA by LARI, compared with isotype control hlgG₁. The statistical significance for each IgE sensitized dose is individually noted. * $p < 0.05$; ** $p < 0.01$, two-tailed ANOVA. **(F)** Effects of LARI E59 on attenuating the dansyl-specific IgE-mediated systemic anaphylaxis. The core body temperature changes, along with the anaphylactic clinical scores, in isotype control and E59 treated mice ($n = 5$) are plotted. * $p < 0.05$; ** $p < 0.01$, Student t test.

injected site showed typical compound anaphylactic degranulation, with fusion between most of the intracellular granules and fusion of the granules with the cell membrane, leading to the formation of irregular intracellular sacs with the electron-dense mediators completely depleted (Fig. 9C). The phenotypic changes in granules induced by E59 indeed resembled those of piecemeal degranulation reported in eosinophilic degranulation (19).

LARI induces partial FcεRI signaling antagonizing the full FcεRI activation signaling

The effects of LARI on early signal transduction were investigated with basophils from both cat allergic and normal healthy nonallergic donors using flow cytometry-based intracellular signaling staining (20). E59 itself weakly induced Syk phosphorylation (p-Syk) compared with that activated by the strong FcεRI signaling pathway stimulators PAE (for both allergic and nonallergic donors) and allergen Fel d1 (for cat allergic donor only, upper panel, Fig. 10A). Pretreatment of the basophils with E59 for 60 min moderately blunted subsequent PAE- and Fel d1-stimulated p-Syk (middle and lower panels, Fig. 10A). However, E59 itself

promoted an almost comparable level of p-Akt level to that driven by PAE and Fel d1 (upper panel, Fig. 10B), whereas E59 pretreatment blunted PAE-activated p-Akt and almost completely blocked Fel d1-induced p-Akt (middle and lower panels, Fig. 10B). E59 itself induced p-Erk but displayed a rather weak blunting effect on both PAE- and Fel d1-induced p-Erk under our experimental conditions with this cat allergic donor's basophils (Fig. 10C). Similarly, E59, which weakly activated p-p38MAPK by itself, also exerted a weakly blunting effect on p-p38MAPK by both PAE and Fel d1 (Fig. 10D). E59 induced a similar pattern of basophil signaling modification in other cat allergic and nonallergic donors ($n = 7$), although with slightly relative values between individuals (Data not shown). Taken together, these data demonstrated that LARI induced partial basophil FcεRI signaling that antagonized subsequent full FcεRI activation signaling.

We also employed Western blotting as an approach to confirm and extend the signaling effects of E59 on p-Erk in LAD2 cells sensitized with IgE. p-Erk, readily detected at a low level in baseline control buffer, was increased by E59 and E23 stimulation in an E59 dose-dependent fashion. Notably, LARI stimulated much stronger p-p42 Erk level compared with that of p-p44 Erk

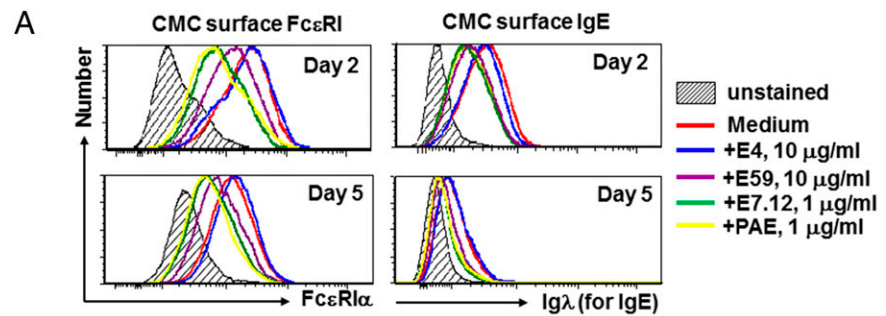
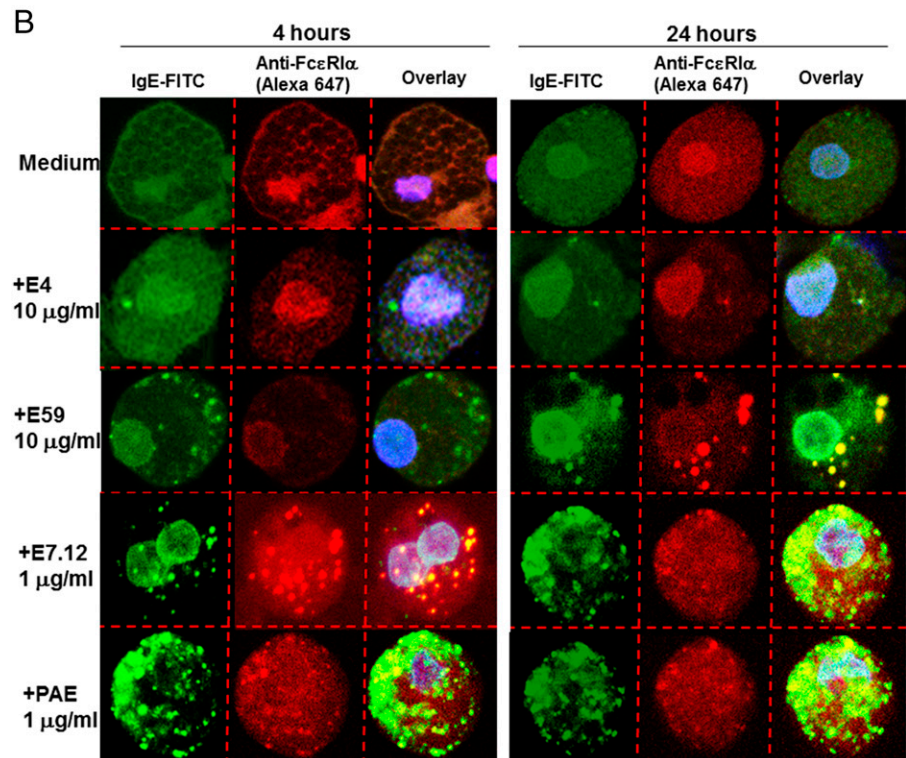


FIGURE 8. LARI downregulates CMC surface FcεRI and IgE expression via promoting IgE/FcεRI internalization. **(A)** CMC surface FcεRI and IgE expression levels in the absence or presence of E59 at 2 and 5 d (representative of two experiments). **(B)** IgE/FcεRI colocalization, aggregate formation, and intracellular distribution indicative of IgE/FcεRI complex internalization in the absence and presence of E59 for 4 and 24 h (representative of more than 20 cells per group analyzed). Original magnification $\times 100$.



(top panel, Fig. 10E). PAE itself induced the most p-Erk (both p42 and p44), whereas this PAE-driven p-Erk was inhibited by pretreatment of LAD2 with E59 for 30 min when assayed on a normalized basis adjusted to the total protein loaded (lower panel, Fig. 10E). Unfortunately, Syk, Akt, and p38MAPK phosphorylation in LAD2 cells were refractory even to strongest FcεRI cross-linking, (e.g., PAE stimulation, data not shown); the modulatory effect of LARI on p-Syk, p-Akt, and p-p38MAPK could not be investigated in these cells.

Discussion

Direct targeting the FcεRI-bound IgE on human basophils and mast cells, the primary effector cells of allergic response, with an anti-IgE mAb as an allergy therapeutic has been deemed unlikely because of the consensus expectation of its triggering anaphylactic reactivity. However, we have shown that murine anti-human IgE mAbs specifically designed with low affinity were capable of binding surface-bound IgE without triggering anaphylactic degranulation while simultaneously having potent antiallergic effects (4). That seminal discovery suggested the possibility of using the low-affinity IgE targeting approach as a novel allergy therapeutic. To move this concept forward to a practical allergy therapeutic level, we have, in this study, comprehensively characterized the biological and immunological features of humanized low affinity anti-IgE mAbs, LARI, tested their safety profiles, determined their

therapeutic ability, and explored the potential effects responsible for their mechanism of action. Our results clearly indicate that humanized low affinity anti-IgE mAbs can be designed in such a way that they fail to trigger anaphylactic degranulation even at high concentration but are able to profoundly blunt allergic reactivity.

Although a sufficiently low affinity for IgE is critical so that the LARI fail to trigger acute release of allergic mediators, this needs to be balanced by an appropriate affinity capable of IgE binding to drive efficient inhibition of allergic effector cell function. Clearly too low an affinity, although providing greater safety, will degrade the therapeutic value as seen with the p6.2 derived mAbs. Thus the balance between safety and efficacy needs to be carefully evaluated to define the most suitable candidates. Our studies determined that LARI with affinity for IgE in the K_D range of 10^{-7} M– 10^{-8} M had the optimal therapeutic index of safety/efficacy. LARI E59, with a K_D of 1.2×10^{-7} M, was chosen as the candidate for further development based on its excellent therapeutic index and long predicted in vivo $t_{1/2}$.

Two other key characteristics of LARI in general and LARI E59 in particular make this approach appealing. First is that cross-linking LARI in vivo or ex vivo failed to induce allergic reactivity. This is critically important, as some individuals will turn Abs to humanized mAbs. For LARI, in addition to the usual concern of decreased efficacy from drug neutralization, there was also the specific concern that such anti-drug Abs and their resulting

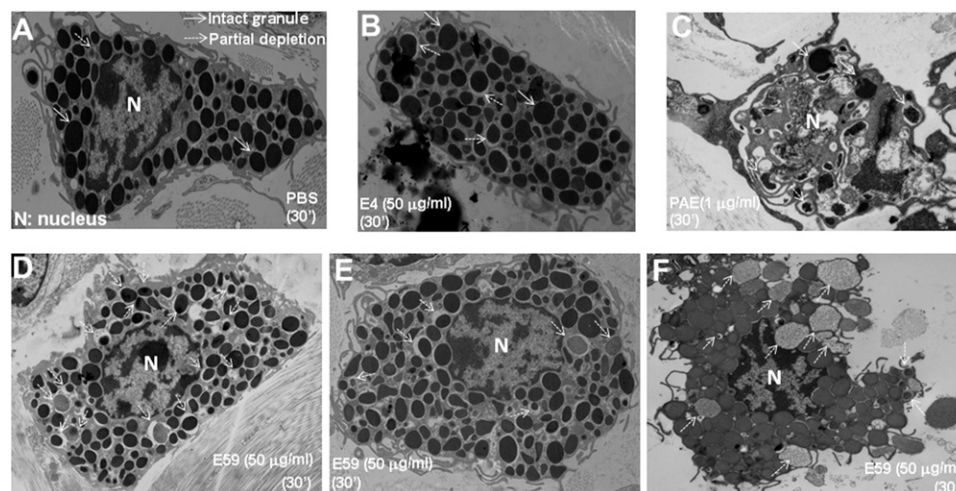


FIGURE 9. Ultrastructural view of LARI E59-treated hFcεRIα Tg mouse skin mast cell granules. Intact granules are designated with solid arrows, whereas the granules with partial depletion of the interiors are designated with dotted line arrows. (A) A representative mast cell from a PBS injected site as a negative-normal resting control. (B) A representative mast cell from LARI E4 injected site as an isotype control. (C) A The representative mast cell at the PAE-injected site as a positive control. (D–F) Ultrastructural views of skin mast cells at the LARI E59-injected site. Original magnification $\times 14,000$.

complexes might provide a full activation signal that would give rise to anaphylaxis. Second is that the high levels of free IgE did not alter the therapeutic efficacy of LARI. This is not surprising given the low affinity of LARI (e.g., E59 has an affinity ~ 500 times less or even lower than most therapeutic mAbs currently in use). We expect that LARI binds to and rapidly releases from IgE in a catalytic-like fashion in contrast to high affinity Abs that remain bound to their target Ag for prolonged periods. This lack of sensitivity to the levels of free IgE should permit a simplified dosing regimen in contrast to what is necessary for omalizumab (21).

The overall mechanism of action of LARI is unique compared with other allergy therapeutic biologics on the market or under development. LARI's weak binding to the FcεRI-bound IgE as a partial agonist appears to result in several downstream effects. Initially, LARI induces weak FcεRI signaling that antagonizes the strong activation driven allergic reactivity. Such a mechanism was observed with low affinity ligands interacting with IgE on murine mast cells (22). This occurs within minutes and acts at the most proximal aspects of FcεRI signaling. This is similar to what has been observed with low-dose allergen (23–25), or low-dose, high affinity anti-IgE and anti-FcεRI Ab (26)- induced desensitization.

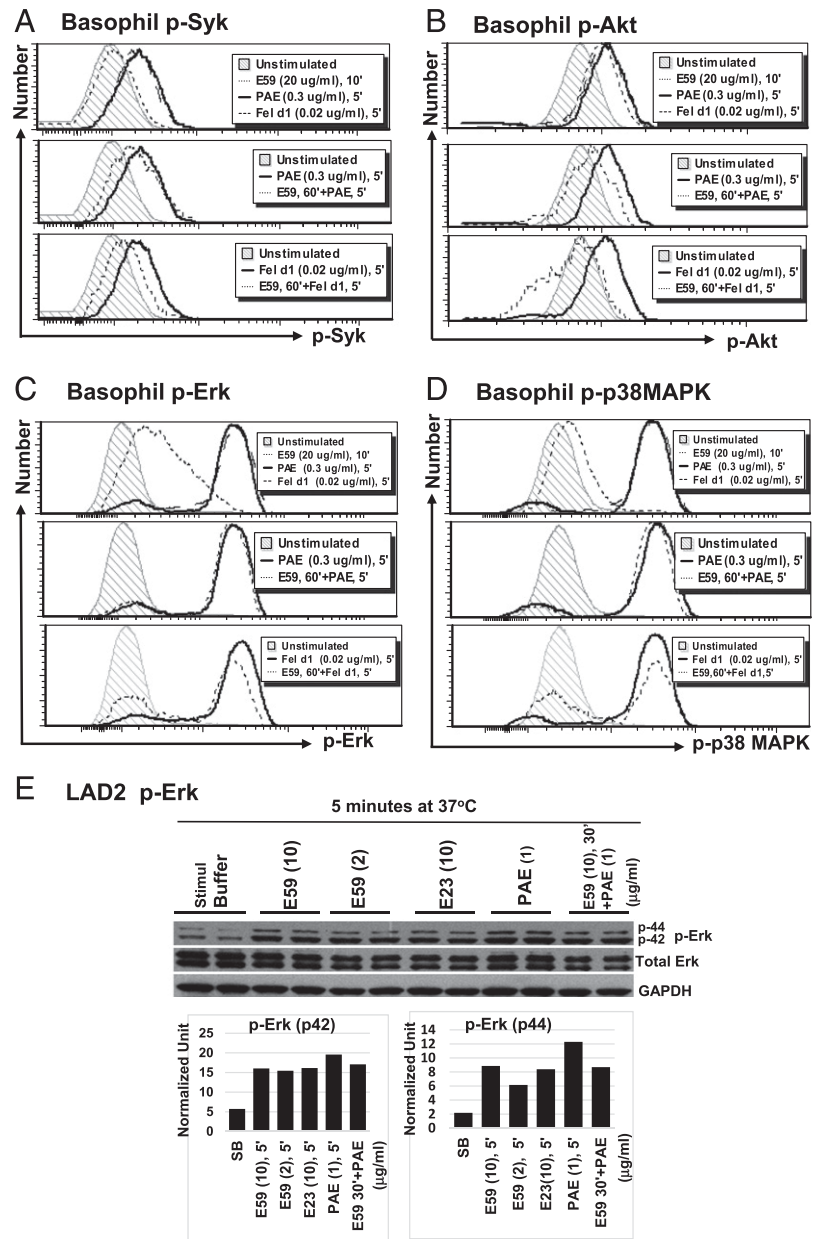
In the classic low-dose allergen desensitization protocol, the subthreshold allergen is insufficient to cross-link enough specific IgE Ab to trigger apparent degranulation. The resulting partial activation signaling renders the cells transiently desensitized. The partial activation signal–desensitization effect is rapidly lost following the internalization of the allergen-engaged, allergen-specific IgE/FcεRI complex (27–29). As the triggering threshold gradually increases, increased allergen dosing can be given, eventually achieving longer-term desensitization. Compared with low dose allergen desensitization, LARI rapidly induces allergic desensitization in an allergen nonspecific manner, functionally acting as a “low dose pan-allergen” but maintaining a persistent rather than transient effect because of its continuous presence. This allergen nonspecific desensitization feature will not only allow LARI to be a stand-alone therapy (e.g., in IgE food allergy), but when combined with allergen immunotherapy, its use could significantly shorten the time span and improve the safety of attempted allergen desensitization/tolerance induction protocols for foods and medications.

LARI-mediated internalization of the IgE/FcεRI complexes and subsequent downregulation of surface IgE and/or FcεRI level is likely the other mechanism by which LARI exerts its therapeutic effect. Although the internalized receptors may be destroyed, recycled, or simply replaced, any future FcεRIs expressed, upon binding IgE, should be immediately engaged by LARI, leading to continuous desensitization and next round of IgE/FcεRI internalization. As the therapeutic benefit of the loss of FcεRI expression is validated by studies with omalizumab, internalization mediated decrease of IgE/FcεRI expression also likely contributes to the therapeutic effects of LARI.

The partial granule depletion phenotype induced by E59 seen in skin mast cells is intriguing and supported by the slow increase in mediators in longer term culture supernatants accompanied by the loss of mediator levels in the cells. The ultrastructural appearance shares the similarity with the so called piecemeal degranulation phenotype seen in eosinophil degranulation (19). The concept of piecemeal degranulation was initially coined by Ann and Harold Dvorak to describe a novel basophil degranulation pattern distinctive from the classic anaphylactic degranulation under the electron microscopy (30, 31). It did not originally refer to the partial granule depletion pattern of basophils we have observed. The term of piecemeal degranulation was later widely adapted to describe the partial granule content depletion pattern seen in eosinophils. Activated eosinophils show this partial granule depletion pattern upon activation but not the “full granule content depletion,” or anaphylactic degranulation, pattern seen in activated basophil and/or mast cells (19). Therefore, the term of piecemeal degranulation relates different degranulation processes between for basophils and eosinophils and so not to be confused with either situation, we tentatively use the term “piecemeal degranulation-like” to refer the partial granule depletion pattern we observed E59 to induce in human skin mast cells.

The physiological and/or pathological importance of this piecemeal degranulation-like pattern and slow and low level of LARI-induced mediator release/depletion is unclear, as the granule content depletion has not been previously described in basophils or mast cells under experimental conditions. However, our studies with LARI offer some intriguing insight into this process. LARI E59 only induced a piecemeal degranulation-like picture; it did not trigger the typical anaphylactic degranulation either phenotypically

FIGURE 10. LARI-mediated signaling in basophils (A–D) and LAD2 cells (E). (A) Effects of E59 on basophil p-Syk. (B) Effects of E59 on basophil p-Akt. (C) Effects of E59 on p-Erk. (D) Effects of E59 on p-p38MAPK ($n = 7$). (E) Effects of E59 on LAD2 p-Erk with Western blotting. Data are representative of two experiments.



or functionally. It is reasonable to propose that this LARI triggered piecemeal degranulation-like picture would result from a leak of the allergic mediators over time. The observation that LARI induced a slow low-level release of the prestored histamine and β -hex and newly synthesized LTC₄ and PGD₂ into the mast cell culture supports the notion that LARI is capable of triggering or enhancing such a pathway. One can envisage that an ongoing mediator “leak” process would regulate the cellular content of allergic mediators and function as a mechanism for controlled mediator release as has been defined in piecemeal degranulation in eosinophils. If LARI is triggering or enhancing such a natural pathway, the longer term effects remain to being an open question.

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Disclosures

K.Z., H.Z., J.L., and A.S. are the employees of Sixel Inc. and K.Z. and A.S. hold shares in the company. The other authors have no financial conflicts of interest.

References

- Cruz, A. A., P. J. Cooper, C. A. Figueiredo, N. M. Alcantara-Neves, L. C. Rodrigues, and M. L. Barreto. 2017. Global issues in allergy and immunology: parasitic infections and allergy. *J. Allergy Clin. Immunol.* 140: 1217–1228.
- Zellweger, F., P. Gasser, D. Brigger, P. Buschor, M. Vogel, and A. Eggel. 2017. A novel bispecific DARPIn targeting FcγRIIB and FcεRI-bound IgE inhibits allergic responses. *Allergy* 72: 1174–1183.
- Jabs, F., M. Plum, N. S. Laursen, R. K. Jensen, B. Mølgaard, M. Miehe, M. Mandolesi, M. M. Rauber, W. Pfützner, T. Jakob, et al. 2018. Trapping IgE in a closed conformation by mimicking CD23 binding prevents and disrupts FcεRI interaction. *Nat. Commun.* 9: 7–17.

4. Zhang, K., J. Liu, T. Truong, E. Zukin, W. Chen, and A. Saxon. 2017. Blocking allergic reaction through targeting surface-bound IgE with low-affinity anti-IgE antibodies. *J. Immunol.* 198: 3823–3834.
5. Macy, E., M. Kemeny, and A. Saxon. 1988. Enhanced ELISA: how to measure less than 10 picograms of a specific protein (immunoglobulin) in less than 8 hours. *FASEB J.* 2: 3003–3009.
6. Timmerman, P., W. C. Puijk, and R. H. Meloen. 2007. Functional reconstruction and synthetic mimicry of a conformational epitope using CLIPS technology. *J. Mol. Recognit.* 20: 283–299.
7. Souders, C. A., S. C. Nelson, Y. Wang, A. R. Crowley, M. S. Klempner, and W. Thomas, Jr. 2015. A novel in vitro assay to predict neonatal Fc receptor-mediated human IgG half-life. *MAbs* 7: 912–921.
8. Yin, Y., Y. Bai, A. Olivera, A. Desai, and D. D. Metcalfe. 2017. An optimized protocol for the generation and functional analysis of human mast cells from CD34⁺ enriched cell populations. *J. Immunol. Methods* 448: 105–111.
9. Kepley, C. L., J. R. Pfeiffer, L. B. Schwartz, B. S. Wilson, and J. M. Oliver. 1998. The identification and characterization of umbilical cord blood-derived human basophils. *J. Leukoc. Biol.* 64: 474–483.
10. Kepley, C. L., and N. Cohen. 2003. Evidence for human mast cell nonreleaser phenotype. *J. Allergy Clin. Immunol.* 112: 457–459.
11. Schwartz, L. B., R. A. Lewis, D. Seldin, and K. F. Austen. 1981. Acid hydrolases and tryptase from secretory granules of dispersed human lung mast cells. *J. Immunol.* 126: 1290–1294.
12. Kirshenbaum, A. S., C. Akin, Y. Wu, M. Rottem, J. P. Goff, M. A. Beaven, V. K. Rao, and D. D. Metcalfe. 2003. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεpsilonRI or FcγgammaRI. [Published erratum appears in 2003 *Leuk. Res.* 27: 1171.] *Leuk. Res.* 27: 677–682.
13. Lyczak, J. B., K. Zhang, A. Saxon, and S. L. Morrison. 1996. Expression of novel secreted isoforms of human immunoglobulin E proteins. *J. Biol. Chem.* 271: 3428–3436.
14. Zhang, K., C. L. Kepley, T. Terada, D. Zhu, H. Perez, and A. Saxon. 2004. Inhibition of allergen-specific IgE reactivity by a human Ig Fcγgamma-Fcεpsilon bifunctional fusion protein. *J. Allergy Clin. Immunol.* 114: 321–327.
15. Gentinetta, T., T. Pecaric-Petkovic, D. Wan, F. H. Falcone, C. A. Dahinden, W. J. Pichler, and O. V. Hausmann. 2011. Individual IL-3 priming is crucial for consistent in vitro activation of donor basophils in patients with chronic urticaria. *J. Allergy Clin. Immunol.* 128: 1227–1234.e5.
16. Dibbern, D. A., Jr., G. W. Palmer, P. B. Williams, S. A. Bock, and S. C. Dreskin. 2003. RBL cells expressing human Fc epsilon RI are a sensitive tool for exploring functional IgE-allergen interactions: studies with sera from peanut-sensitive patients. *J. Immunol. Methods* 274: 37–45.
17. Suzuki, R., S. Leach, W. Liu, E. Ralston, J. Scheffel, W. Zhang, C. A. Lowell, and J. Rivera. 2014. Molecular editing of cellular responses by the high-affinity receptor for IgE. *Science* 343: 1021–1025.
18. McNeil, B. D., P. Pundir, S. Meeker, L. Han, B. J. Udem, M. Kulka, and X. Dong. 2015. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature* 519: 237–241.
19. Crivellato, E., B. Nico, F. Mallardi, C. A. Beltrami, and D. Ribatti. 2003. Piecemeal degranulation as a general secretory mechanism? *Anat. Rec. A Discov. Mol. Cell Evol. Biol.* 274: 778–784.
20. Witting Christensen, S. K., I. Kortekaas Krohn, J. Thuraiiah, T. Skjold, J. M. Schmid, and H. J. Hoffmann. 2014. Sequential allergen desensitization of basophils is non-specific and may involve p38 MAPK. *Allergy* 69: 1343–1349.
21. MacGlashan, D., Jr. 2009. Therapeutic efficacy of omalizumab. *J. Allergy Clin. Immunol.* 123: 114–115.
22. Torigoe, C., J. K. Inman, and H. Metzger. 1998. An unusual mechanism for ligand antagonism. *Science* 281: 568–572.
23. Mendoza, G. R., and K. Minagawa. 1982. Subthreshold and suboptimal desensitization of human basophils. II. Nonspecificity and irreversibility of desensitization. *Int. Arch. Allergy Appl. Immunol.* 69: 282–284.
24. Rotiroti, G., M. Shamji, S. R. Durham, and S. J. Till. 2012. Repeated low-dose intradermal allergen injection suppresses allergen-induced cutaneous late responses. *J. Allergy Clin. Immunol.* 130: 918–24.e1.
25. MacGlashan, D., Jr. 2012. Subthreshold desensitization of human basophils recapitulates the loss of Syk and FcεRI expression characterized by other methods of desensitization. *Clin. Exp. Allergy* 42: 1060–1070.
26. Khodoun, M. V., Z. Y. Kucuk, R. T. Strait, D. Krishnamurthy, K. Janek, I. Lewkowich, S. C. Morris, and F. D. Finkelman. 2013. Rapid polyclonal desensitization with antibodies to IgE and FcεRIα. *J. Allergy Clin. Immunol.* 131: 1555–1564.
27. MacGlashan, D., Jr., M. Mogowski, and L. M. Lichtenstein. 1983. Studies of antigen binding on human basophils. II. Continued expression of antigen-specific IgE during antigen-induced desensitization. *J. Immunol.* 130: 2337–2342.
28. Oka, T., E. J. Rios, M. Tsai, J. Kalesnikoff, and S. J. Galli. 2013. Rapid desensitization induces internalization of antigen-specific IgE on mouse mast cells. *J. Allergy Clin. Immunol.* 132: 922–932.e1–16.
29. Sancho-Serra, M. C., M. Simarro, and M. Castells. 2011. Rapid IgE desensitization is antigen specific and impairs early and late mast cell responses targeting FcεRI internalization. *Eur. J. Immunol.* 41: 1004–1013.
30. Dvorak, H. F., A. M. Dvorak, and W. H. Churchill. 1973. Immunologic rejection of diethylnitrosamine-induced hepatomas in strain 2 guinea pigs: participation of basophilic leukocytes and macrophage aggregates. *J. Exp. Med.* 137: 751–775.
31. Dvorak, H. F., M. C. Mihm, Jr., A. M. Dvorak, R. A. Johnson, E. J. Manseau, E. Morgan, and R. B. Colvin. 1974. Morphology of delayed type hypersensitivity reactions in man. I. Quantitative description of the inflammatory response. *Lab. Invest.* 31: 111–130.