Title

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Authors

Zhang, Ke
Liu, Jeffrey
Truong, Thao
et al.

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Ke Zhang[*,+], **Jeffrey Liu**[*], **Thao Truong**[+], **Elyssa Zukin**[+], **Wendy Chen**[+], **Andrew Saxon**[*,+]

[*]Sixal Inc, 570 Westwood plaza, Building 114, MC 722710, Los Angeles, California, USA

[+]Section of Clinical Immunology/Allergy, Division of Pulmonary, Critical Care and Clinical Immunology/Allergy, Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California, USA

Abstract

Allergic disorders now become a major worldwide public health issue but the effective treatment options remain limited. We report a novel approach to block allergic reactivity by targeting the surface-bound IgE of the allergic effector cells via low affinity anti-human IgE antibodies with dissociation constants in 10^{-6}–10^{-8} M range. We demonstrated that these low affinity anti-IgE mAbs bind to the cell surface-bound IgE without triggering anaphylactic degranulation even at high concentration, albeit they would weakly upregulate CD203c expression on basophils. This is in contrast to the high affinity anti-IgE mAbs which trigger anaphylactic degranulation at low concentration. Instead, the low affinity anti-IgE mAbs profoundly block human peanut and cat allergic IgE-mediated basophil CD63 induction indicative of anaphylactic degranulation; suppress peanut, cat and dansyl specific IgE-mediated passive cutaneous anaphylaxis, and attenuate dansyl IgE-mediated systemic anaphylaxis in human FcεRIα transgenic mouse model. Mechanistic studies reveal that the ability of allergic reaction blockade by the low affinity anti-IgE mAbs was correlated with their capacity to down-regulate the surface IgE and FcεRI level on human basophils and the human FcεRIα transgenic mouse bone marrow derived mast cells via driving internalization of the IgE/FcεRI complex. Our studies demonstrate that targeting surface-bound IgE with low affinity anti-IgE antibodies is capable of suppressing allergic reactivity while displaying an excellent safety profile, indicating that use of low affinity anti-IgE mAbs holds promise as a novel therapeutic approach for IgE mediated allergic diseases.

Introduction

IgE-mediated allergic diseases, including asthma and severe food allergy, have significantly increased in both prevalence and incidence over the past decades in the US and other developed countries to the point where they now have become a major worldwide public health issue (1, 2). Currently ~40% of the US population is sensitized to IgE of one or more common inhalant or food allergen, and half of those will be symptomatic at any one time (3, 4).

Address for correspondence: Dr. Ke Zhang. Sixal Inc, 570 Westwood plaza, Building 114, MC 722710, Los Angeles, California, USA. Tel: 1-310-228-0069; kzhang@ucla.edu.

Recognition of this epidemic of IgE-driven allergy disease and asthma has led to a corresponding interest in the development of novel immune based biologic therapies. Although major efforts are being undertaken to develop better therapeutic approaches for these allergic disorders, new effective and safe therapies for prevention/treatment of severe allergic disorders remain quite limited. Omalizumab (Xolair®)-based anti-IgE therapy that binds free but not FcεRI–bound IgE was the only approved biologic for allergy disease and asthma for nearly 13 years and has shown modest success in reducing allergic airway reactivity in a subset of asthmatics (5, 6) and recently has been approved for chronic urticaria. Very recently, anti-IL5 was approved for a subset of asthma (7) while a variety of other novel therapeutic approaches are in various stages of development. Thus the effective treatment of severe allergic disorders continues to be a major unmet medical need.

Direct targeting of the IgE bound to FcεRI on basophils/mast cells using anti-IgE Abs has not been considered a feasible therapeutic approach due to the expectation that anti-IgE Abs would very likely trigger immediate degranulation by crosslinking the surface IgE/FcεRI complexes when the administrated doses escalate to certain point. However, it was also discovered that some anti-IgE and anti-FcεRI Abs could induce allergic effector cell desensitization, but not allergic degranulation (8); the naturally occurred anti-IgE autoantibodies from normal and allergic subjects were able to inhibit allergen-induced basophil activation (9); and the anti-IgE and anti-FcεRIα autoantibodies in chronic urticaria patients could render basophils unresponsive to following anti-IgE stimulation (10). These findings suggest that anti-IgE Abs do not necessarily induce degranulation, instead, some of them could induce allergic desensitization, just as that of very low dose of allergen administration in the course of allergen immunotherapy. The exploit of those anti-IgE Abs with the allergic desensitization capacity as a novel allergy therapy has not been attempted previously. In this paper, we report a novel approach using low affinity anti-IgE (LAIGE) mAbs that target surface-bound IgE on allergic effector cells to suppress allergic reactions. This work demonstrates that LAIGE Abs have potential for therapeutic use for IgE driven allergic disorders while showing a good safety profile.

Materials and Methods

Antibodies and Reagents

FITC- and PE-labelled anti-human CD123 (Clone 6H6), CD63 (clone H5C6), PerCP-HLA-Dr (Clone L243), PE- and Alexa 647-labeled or unlabeled anti-human FcεRIα (clone AER-37), PE-CD203c and APC-CD203c (clone NP4D6), anti-mouse CD117 (c-kit) (clone 2B8) were purchased from Biolegend (San Diego, CA). Polyclonal anti-human IgE Ab (PAE) derived from goat (Ab9159) was purchased from Abcam (Cambridge, MA). AP-labeled anti-human IgM, IgG, IgA and IgE, and anti-mouse IgG were obtained from KPL (Gaithersburg, MD), recombinant mouse IL-3 from Peprotech (Rocky Hill, NJ) and the natural cat allergen Fel d1, peanut allergen Ara h1, Ara h2 and Ara h6 from Indoor biotechnologies (Charlottesville, VA). The pre-screened peanut and cat allergic plasmas were purchased from PlasmaLab international, Inc (Everett, WA). Anti-human IgE mAbs were produced with standard hybridoma technology (27), and purified with protein A and protein L (GenScript USA, Piscataway, NJ) affinity chromatography.

Surface plasmon resonance studies

The affinity determination of the anti-IgE mAbs was performed on a Biacore T2000 instrument (Biacore AB, Uppsala, Sweden). Human myeloma IgE (ppIgE and PSIgE) were immobilized on the CM5 sensor chips by amine coupling. The purified anti-IgE mAbs 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 50μl/minute for binding analysis. Binding results were expressed in resonance units. Kinetic studies were analyzed with BIAevaluation Software Version 4.1, and the calculated affinity results fitted well from the two channels coupled with ppIgE and PSIgE.

Allergic subject population—A total of 23 (15 male, 8 female) healthy and 13 previously clinically diagnosed peanut (5 male and 2 female) and cat (3 male and 3 female) allergic subjects were recruited for this study. Allergic subjects' allergic status was confirmed by basophil activation test (BAT). Usage of human blood for BAT from allergic subjects was approved by the UCLA Institutional Review Boards committee.

Purification of peanut and cat allergic IgE

Pooled peanut and cat allergic donor plasmas were diluted 1:1 with PBS, and passed through an anti-human IgE mAb (clone CIA-E4.15) (11) coupled sepharose 4B resin at the flow rate of 1 mL/min. The affinity column was washed with PBS and the bound IgE fraction was eluted with elution buffer (10 mM glycine-HCl, PH 2.5). The eluted fraction was combined and dialyzed against PBS overnight, following by ELISA determination of the total IgE concentration.

Multiplex luminex assay—The human ppIgE sensitized human FcεRIα transgenic (hFcεRIα Tg) mouse serum samples collected at 30-minute post anti-IgE mAb challenge were used to measure the allergic mediators release with multiplex luminex assay (Mouse Cytokine/Chemokine Magnetic Bead Panel) for mouse mast cell protease-1 (mMCP-1), Eotaxin, IL-4, IL-5, IL-10 and IL-13. This assay service was provided by Westcoast Biosciences (San Diego, CA).

Basophil Activation Test

To determine the triggering capacity of allergen or anti-IgE Abs, BAT was performed using heparinized whole blood (100 μl/test) within 4 hours of blood draw, with the stimulating time at 15 minutes 37°C for peanut or cat allergens, and 30 minutes for anti-IgE mAbs E4.15, E7.12, C6, E5.1, p6.2 and E2.18. For therapeutic effect determination using BAT based approach, the allergic (peanut and cat) subjects' heparinized whole blood (200 μl/test) was incubated with mouse anti-human IgE mAbs or $mIgG_1$ isotype control in the ranges of 0.01–0.1 μg/mL for the high affinity anti-IgE mAb E4.15, E7.12, C6, E5.1, and the range of 0.25–4 μg/mL for low affinity anti-IgE mAb p6.2 and E2.18. The tested blood was incubated with anti-IgE mAbs at room temperature (22–24°C) with slow agitation (100 RPM) for 24 or 48 hours prior to basophil activation by the mixed purified Ara h allergens (Ara h1, h2, and h6 combined) or cat allergen Fel d1, respectively, for 15 minutes at 37°C with shaking. The activation was stopped by EDTA at final concentration of 10 μM. The allergen and anti-IgE Ab stimulated blood samples were stained with CD123-FITC, CD63-PE, HLA-DR-

PerCP and CD203c-APC, followed by erythrocyte lysis and flow cytometry acquisition and analysis (12–14). The cut-off value of the unstimulated blood basophil CD63 expression was always set as <3% as the background, and CD203c expression level <10% since unstimulated basophils constitutively express low level CD203c. Histamine release of basophils was performed with the ELISA kit from Immnuo-biological Laboratories Inc (IBL-America, Minneapolis, MN) followed the instruction manual.

Human IL-4 production from basophils

PBMCs of non-allergic donors (~5×10^8 cells from each donor) were used for enrichment of basophils with human basophil enrichment kit from STEMCELL technologies (Cambridge, MA) followed the kit instruction. The basophil purity varied from 54%–78% measured by flow cytometry staining with CD123-FITC/FcεRIα-PE. The enriched basophils were cultured at the density of 5×10^5 cell/mL in complete RPMI 1640 stimulated with isotype control (mIgG1 at 20 μg/mL), LAIGE p6.2 at 0.2, 2 and 20 μg/mL, and PAE at 0.1 μg/mL, respectively, for 1, 2 and 4 hours. IL-4 levels in the culture supernatants were measured with human IL-4 ELISA kit from MyBioSource (San Diego, CA).

Determination of the allergic therapeutic index with BAT-based method—The dose of the anti-IgE mAbs sufficient to block 50% of Ara h allergen induced CD63 expression with BAT was arbitrarily defined as the "BAT effective therapeutic dose", whereas the lowest concentration of the anti-IgE mAbs capable of inducing >5% CD63 expression was arbitrarily defined as the "BAT triggering dose". The BAT-based therapeutic index was calculated as the triggering dose dividing by the effectively therapeutic dose. The larger the therapeutic index, the safer the anti-IgE mAb is predicted to be as therapy for IgE mediated allergic diseases.

Passive Cutaneous Anaphylaxis (PCA)

The previously-established cat, peanut and dansyl-specific IgE mediated PCA assays were used to assess the ability of LAIGE mAbs to suppress cutaneous IgE mediated allergic reactivity in hFcεRIα Tg mice (15). The hFcεRI Tg mice (16) were sensitized with the purified allergic IgE and the recombinant dansyl specific IgE (17) at 2, 1, 0.5 and 0.125 μg/mL through intradermal injection on the back skin for 2 hours, followed by injecting the mouse i.p. with the testing LAIGE mAbs (2 μg/gram body weight), with the same amount of mIgG$_1$ as an isotype control. Two and four days later, the animals were challenged i.v. with Fel d1 (10 μg), Ara h1, 2 and 6 combined (10 μg), or Dansyl-BSA (100 μg) mixed with 100 μl 2% Evans Blue Dye (EBD) dissolved in saline. The mice were euthanized 30 minutes post allergen challenge for assessing PCA reaction by photography. The mouse skin was dried overnight, and the injected skin areas were cut out, weighted, and EBD extracted with formamide at 55°C overnight. The extravasated EBD was either quantified with a spectrophotometer at the wavelength of 620 nm (18), or 650 nm with a conventional microtiter ELISA reader. The quantitative results from both methods fit well. EBD quantity from each PCA spot was normalized as per milligram skin tissue. Using hFcεRIα Tg mice were approved by UCLA animal research committee.

Systemic Anaphylaxis Assay

The hFcεRIα Tg mice were sensitized with a total of 40 μg (20 μg twice at 16-hour interval) of recombinant dansyl-specific IgE (17) overnight (~24 hours), followed by injection with p6.2 (50 μg/mouse) or mIgG$_1$ isotype as control. Four days later, the mice were intravenously challenged with Dansyl-BSA (100 μg/mouse) to induce systemic anaphylaxis. Core body temperature changes and the anaphylaxis clinical index (scores) were measured as indicators of systemic allergic reactivity. The anaphylaxis clinical score was defined as 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, pilar erecti, diarrhea, and reduced activity or standing still with an increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth; 4, symptoms as in no. 3 with loss of consciousness, tremors, and/or convulsion; 5, death (19). The core body temperature changes were monitored every five minutes for the first hour after Dansyl-BSA challenge. The temperature changes were plotted for comparison and statistical analysis to determine the effect of p6.2 and control mIgG$_1$ on systemic anaphylaxis (20).

Bone marrow derived mast cells (BMMC) from hFcεRIα Tg mouse

The bone marrow was flushed out from the femurs and tibias of the hFcεRIα Tg mouse with 0.22 μm filtered flushing medium (DMEM supplemented with 10% fetal bovine serum, 2mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin, 5×10^{-5} M β-mercaptoethanol). The collected bone marrow cells were cultured in two T75 flasks with 10 mL culture medium (flushing medium plus 10 ng/mL recombinant mouse IL-3) each. Three days later, the suspended cells from each flask were transferred and expanded into two flasks with fresh culture medium. One week later, the suspended cells from each flask were expanded into 2–3 flasks, maintaining the cell density in ~1×10^6 cells/mL. Half of the culture medium was replaced with fresh medium every 3–4 days, and the suspended cells were transferred to new flasks until no adhere cells were observed. Six weeks later, the suspended cells were stained with anti-mouse CD117-FITC and anti-human hFcεRIα-PE to assess the maturity of the cultured BMMC.

Confocal microscopy

BMMCs were centrifuged at 1500 RPM for 3 minutes, and the supernatant was carefully aspirated, and the cell pellets were washed with PBSX2, followed by blocking with 10% of normal mouse serum and incubated for 30 minutes on ice, then added diluted FITC-labeled myeloma IgE (PS IgE) at 5 μg/mL for sensitization for 2 hours. After washing, the FITC-IgE sensitized BMMCs (1×10^6 cell/mL) were incubated with the control mIgG$_1$ and p6.2 at 2 μg/mL respectively for 24 or 48 hours at 37°C. The BMMCs were fixed with 0.5 mL of 2% formaldehyde (Polysciences, Ultrapure, E. M. Grade 10% solution. Dilute to 2% in PBS) for 30 minutes. After completing the above staining procedure, the tube(s) were kept at room temperature (dark) for 20 minutes. The cells were washed with PBS for 2 times following by adding 50 μl of permeabilization medium, along with the Alexa 647-labelled anti-hFcεRIα for incubation at room temperature for 30 min. The washed cells were then fixed with 0.5 mL of 2% formaldehyde, and span to the poly-lysine coated glass slide using cytospin at 500 rpm for 5 min. The cells on the slides were stained with a drop of prolong gold anti-Fade

reagent with DAPI (Life technologies, Eugene, OR). The slides were examined with Leica SP2-1P-FCS confocal microscope.

Statistics—Data are expressed as mean ± SEM. Unless indicated in the figure legend, Student's T test was used to determine the statistical significance for the paired data. A statistically significant difference was defined as $P < 0.05$, which was indicated with one asterisk while a significant difference of $P < 0.01$ was indicated with double asterisks in the appropriate figures.

Results

Comparison of basophil CD63 expression induced by allergens, high affinity and low affinity anti-IgE mAbs

Anti-IgE Abs are expected to engage the cell surface-bound IgE in a fashion similar to allergen- mediated crosslinking of the surface FcεRIs on basophils and mast cells, leading to anaphylactic degranulation and resulting in allergic reaction. However, some anti-IgE Abs and autoantibodies, rather than triggering allergic degranulation, induce allergic effector cells desensitization, and blocked allergen-induced basophil activation through currently unknown mechanism (8, 9). These studies suggested that not all anti-IgE Abs would trigger allergic degranulation, instead, some anti-IgE Abs would render allergic effector cells desensitized. Such anti-IgE Abs with the ability to desensitize allergic effector cells might have the potential to function as a novel therapeutic for IgE mediated allergic diseases. To investigate the reason why various anti-IgE Abs display differential ability for allergic triggering, we first defined the ability of an array of anti-human IgE mAbs to induce basophil activation employing BAT with peanut and cat allergic subjects' blood challenging with relevant allergens.

BAT profiles of peanut (n=3) and cat (n=2) allergic subject's blood were shown in Figure 1, where basophil surface CD63 expression, a marker of basophil activation indicative of anaphylactic degranulation (12–14), was induced by the peanut allergen Ara h2 (Figure 1A–1C) and cat allergen Fel d1 (Figure 1D, 1E) in the dose range of 0.002–0.2 μg/mL. A high affinity anti-IgE mAb E4.15, with dissociation constant (Kd) at 9.56×10^{-10}M, also triggered significant basophil CD63 expression in the dose range between 0.2–20 μg/mL (the middle row, Figure 1A–E). A LAIGE mAb p6.2 (Kd=2.54×10^{-6}M), which binds to a conformational epitope on IgE CH2-CH4 region with low affinity (supplemental Figure S1), did not induce CD63 expression at 0.2–20 μg/mL (the low row, Figure 1A–E) or even up to 100 μg/mL (Figure 3A). A quantitative comparison of CD63 expression induced by all the stimuli at various concentrations from these allergic subjects is summarized in Figure 1F. These data indicate that both allergens and the high affinity anti-IgE E4.15 are capable of activating basophils with the activation potency of E4.15 being 2–3 logs lower than that of Ara h2 or Fel d1 allergens to achieve similar level of basophil activation. In contrast, the LAIGE p6.2 did not induce basophil CD63 expression even at high concentrations in these peanut and cat allergic donors, indicating that LAIGE p6.2 did not trigger basophil anaphylactic degranulation from both peanut and cat allergic subjects.

Comparison of basophil CD203c expression promoted by allergens, high affinity anti-IgE and LAIGE mAbs

Although not directly linked with anaphylactic degranulation (12, 21, 22), CD203c upregulation on basophils is also frequently used as an alternative basophil activation marker (28, 31). To test whether LAIGE p6.2 promoted CD203c expression, we simultaneously assessed CD203c and CD63 expression on basophils induced by cat allergen Fel d1, polyclonal anti-IgE Ab (PAE), high affinity anti-IgE mAb E4.15 and LAIGE p6.2. In cat allergic donors (n=3), the allergen Fel d1 and E4.15 induced robust basophil CD203c and CD63 expression, with apparent CD203c upregulation and CD63 induction even at lower concentration of Fel d1 (e.g., 0.002 μg/mL) (upper row, Figure 2A) or E4.15 (e.g., 0.2 μg/mL) (middle row, Figure 2A). Both CD203c and CD63 expression was progressive to higher levels with the increased Fel d1 (e.g., 0.02–0.2 μg/mL) or E4.15 concentration (e.g., 2–20 μg/mL) (Figure 2A and 2E). However, a substantial population of the CD203c positive basophils remained CD63 negative in all the Fel d1 concentration tested, indicating that not all the CD203c positive cells would co-express the anaphylactic degranulation marker CD63 upon optimal Fel d1 stimulation. In normal healthy donors (n=5), PAE and E4.15 also promoted apparent basophil CD203c and CD63 expression in a similar fashion as that in cat allergic donors (upper and middle row of Figure 2B and 2F). These results collectively demonstrated that only a portion of the CD203c positive basophils undergo anaphylactic degranulation induced by strong stimuli such as allergen, PAE or high affinity anti-IgE mAb. Therefore CD203c expression as a basophil activation marker would always have higher sensitivity compared with that of CD63 expression (Figure 2E, 2F), but not necessarily reflect the anaphylactic degranulation status.

LAIGE p6.2, however, weakly upregulated CD203c expression, compared with that promoted by Fel d1, PAE or E4.15, from both cat allergic and normal healthy donors, in a p6.2 dose-dependent fashion under the relatively high concentration (e.g., 2–20 μg/mL) (Figure 2C, 2D), but the LAIGE promoted CD203c positive cells did not co-express CD63 (the lower rows of Figure 2A and 2B). LAIGE induced CD203c expression appeared to be rate-limited as even higher LAIGE doses (up to 100 μg/mL) would not further increase CD203c expression to the level driven by low concentration of allergen or high affinity anti-IgE mAbs (data not shown). Histamine release level of LAIGE p6.2 treated blood of both cat allergic and normal healthy donors showed no difference from the spontaneous release level (Figure 2G, 2H). As a positive control, allergen Fel d1, PAE and E4.15 mAb triggered apparent histamine release (Figure 2G, 2H). In addition, LAIGE p6.2 at concentration of 0.2, 2 and 20 μg/mL did not stimulate IL-4 secretion for 1, 2, and 4 hours incubation (n=3) (Figure 3B). These results demonstrated that allergen or high affinity anti-IgE triggered basophil anaphylactic degranulation, whereas LAIGE p6.2 neither induce basophil anaphylactic degranulation nor IL-4 secretion, albeit a weak CD203c upregulation was promoted by higher concentration.

Determination of the relationship between anti-IgE mAb affinity and their capacity to induce basophil CD63 expression

To further test the hypothesis that anti-IgE Abs would display differential basophil activation capability based on their affinity to IgE, we surveyed an array of the anti-human IgE mAbs

for their ability to activate basophils, while measuring their corresponding binding affinity to IgE by surface plasmon resonance (23). The kinetic CD63 expression induced by six anti-IgE mAbs with different affinities to IgE is presented in Figure 3A, whereby the relationship between IgE affinity and basophil activation can be directly assessed. The high affinity anti-IgE mAbs E4.15, E7.12, C6 and E5.1, with Kd in 10^{-9}M to 10^{-10}M range, all induced robust CD63 expression at the concentration < 2 μg/mL. In contrast, the LAIGE mAb p6.2 and E2.18, with Kd in 10^{-6}M to 10^{-8}M range, did not induce CD63 expression at the concentrations < 40 μg/mL, and only sometimes induced low level CD63 expression at concentrations >60 μg/mL with E2.18 (Figure 3A, and supplemental Figure S2A). This comparative study revealed that the anti-IgE Ab affinity was tightly correlated with their ability to trigger basophil anaphylactic degranulation, with high, but not low, affinity anti-IgE mAbs being capable of robustly triggering basophil activation and anaphylactic degranulation.

Determination of allergic triggering capacity of LAIGE in hFcεRIα Tg mice

As both LAIGE p6.2 and E2.18 failed to trigger apparent basophil CD63 expression (Figure 1, 2, 3 and supplemental Figure S2A), to evaluate safety profile of LAIGE as a potential allergy therapeutic, we further examined whether LAIGE would trigger degranulation of allergic effector cells employing other available experimental assays, including IgE-mediated PCA reflecting mast cell degranulation, and systemic anaphylaxis reflecting both basophil and mast cell function combined, to rigorously evaluate the ability of LAIGE to trigger allergic reactions with higher dose, using p6.2 as a prototype of LAIGE.

For PCA, the hFcεRIα Tg mice were systemically sensitized with human myeloma IgE (PSIgE), followed by local challenge with increasing amounts of p6.2 (shown in yellow, Figure 4A). As a positive control, PAE at 1 μg/mL induced a strong skin reaction (low left corner, Figure 4A). However, p6.2 did not trigger PCA reactivity at doses up to 60 μg/mL and weak reactivity was seen at doses of 80–100 μg/mL (Figure 4A, 4B).

To test whether p6.2 induced *in vivo* systemic anaphylaxis, hFcεRIα Tg mice systemically sensitized with human PSIgE (50 μg/mouse) were challenged with 50 μg and 250 μg of p6.2, or 50 μg E4.15 (equivalent 2 μg/gram body weight) as a positive control. The expected fall in core body temperature was seen in the mice challenged with E4.15 but not in those challenged with 50 μg (data not shown) and 250 μg of p6.2 (Figure 4D). Correspondingly, a positive anaphylactic clinical score (38) was only observed in E4.15-, but not in p6.2-, challenged mice (Figure 4C). In a separate experiment to measure allergic mediator release with multiplex luminex assay in serum samples following p6.2 challenge, mMCP-1 release level was minimal compared with the positive control challenged with E4.15 (Figure 4E). The serum IL-4, IL-5, IL-10 and IL-13 levels were also not substantial different between p6.2 and mIgG$_1$ challenged mice (Figure 4F–4I). However, p6.2 appeared to induce eotaxin secretion to the level comparable to that of E4.15 challenge (Figure 4J). Collectively, these data indicated that p6.2 did not show anaphylactic activity at doses <60 μg/mL, and might display weak degranulation activity with the doses >60 μg/mL, and might selectively affect certain chemokine secretion at high concentration.

LAIGE blocks peanut and cat allergen-induced basophil anaphylactic degranulation

A modified BAT assay capable of detecting basophil CD63 induction with prolonged incubation time (24 or 48 hours) was used to test the effectiveness of LAIGE to block allergen-induced basophil anaphylactic degranulation. Although compromised, the basophil CD63 induction of allergic blood by either peanut or cat allergens was largely preserved for up to 48 hours if the blood was incubated in ambient temperature (22–24°C) with slow agitation at 100 RPM. Such a modified BAT assay enables us to evaluate the therapeutic effects of LAIGE (or other agents with anti-allergy potential) *ex vivo* in a BAT based assay format because of the 24–48 hours incubation window. As peanut allergen or Fel d1 induced basophil CD63 expression did not apparently affect by incubation with p6.2 for up to 8 hours (data not shown), the whole blood from peanut or cat allergic subjects was incubated for 24 or 48 hours with various amounts of p6.2 or E2.18, with $mIgG_1$ as an isotype control, following by challenging with the corresponding allergens for basophil CD63 induction. Because 48-hour incubation of blood would frequently have $CD63^{low}$ background stain (Figure 5), we only focused on assessing the $CD63^{high}$ expression (gated in circle in Figure 5) in this modified BAT. Peanut allergen (Ara h1, h2, and h6 combined)-induced basophil $CD63^{high}$ expression was inhibited in a dose-dependent manner by incubation with p6.2 for 48 hours, but not $mIgG_1$, in 0.5 – 4 μg/mL range (Figure 5A), with ~90% inhibition achieved at 2–4 μg/mL in all five peanut allergic bloods tested (Figure 5B). Similarly, Fel d1-induced basophil $CD63^{high}$ expression from cat allergic donors' blood was also significantly blocked in a similar scale to that in peanut allergic donors, also in a dose-dependent fashion (Figure 5C, 5D). Fel d1-induced $CD63^{high}$ expression was also blocked by E2.18 in a dose-dependent manner (supplemental Figure S2B). These data demonstrate that LAIGE p6.2 and E2.18 at relatively lower doses could effectively block both peanut and cat allergic IgE mediated CD63 expression indicative of anaphylactic degranulation.

LAIGE exhibits a higher safety profile than that of high affinity anti-IgE mAbs

To quantitatively assess the therapeutic potential and safety profile of LAIGE mAbs in IgE mediated allergies, we used a BAT-based therapeutic index to evaluate the safety of the LAIGE based allergy therapy, with the high affinity anti-IgE mAbs as a comparison. The therapeutic index of the peanut BAT based approach was calculated as the "BAT triggering dose" dividing by the "BAT effective therapeutic dose" (Figure 6). The BAT triggering dose was arbitrarily defined as the lowest anti-IgE mAb concentration capable of inducing >5% basophil CD63 expression, whereas the effective therapeutic dose was arbitrarily defined as the lowest anti-IgE mAb dose sufficient to block >50% of peanut allergen triggered CD63 expression. Thus the higher the therapeutic index, the safer the therapy. As expected, the LAIGE mAb E2.18 and p6.2 that exhibited a much larger therapeutic index compared to the high affinity anti-IgE mAb E4.15, E7.12, C5 and E5.1 that displayed much smaller therapeutic index (Figure 6), indicating that the LAIGE mAbs as an allergy therapeutic are much safer compared with that of the high affinity anti-IgE mAbs.

LAIGE blocks IgE-mediated PCA

We used the previously established PCA assay (15) to test whether LAIGE p6.2 was able to block mast cell-mediated allergic reactivity. Although PCA was a short-term assay, we found

that the passively skin-sensitized IgE would last for up to 4 days for reliable PCA reaction (Data not shown). The hFcɛRIα Tg mice were skin-sensitized with serially diluted peanut, cat and/or dansyl-specific IgE (from 0.125 – 2 μg/mL) for 2 hours, treated i.p. for two days and four days, respectively, with LAIGE p6.2 or control mIgG$_1$, followed by systemic i.v. challenge with corresponding allergens at day 4 for assessing PCA reaction by quantifying the extravasated EBD in PCA spots from the LAIGE treated and controlled mice, respectively. P6.2, at 2 μg/gram body weight, did not apparently affect PCA reaction after p6.2 treatment for two days (data not shown), but significantly blocked peanut allergic IgE- (Figure 7A & 7B), cat allergic IgE- (Figure 7C & 7D), and dansyl-specific IgE- (36) (Figure 7E & 7F) mediated PCA reactivity after 4-day treatment. Cat allergic IgE-mediated PCA was also strongly blocked by E2.18 after 4-day treatment (supplemental Figure S2C). Collectively, these data demonstrated that p6.2 exhibited a slower onset (>2 days) for skin mast cell desensitization, and the desensitized status was maintained at least up to 4 days after LAIGE administrated.

LAIGE p6.2 attenuates dansyl IgE-mediated systemic anaphylaxis

To test the effect of LAIGE on systemic anaphylaxis, the hFcɛRIα Tg mice were systemically sensitized with the recombinant dansyl-specific human IgE, followed by treatment with p6.2 or a mIgG$_1$ isotype control. Four days later the mice were intravenously challenged with the modal allergen dansyl-BSA and the animals' core body temperature changes and systemic anaphylaxis clinical index were recorded as measures of systemic anaphylaxis (19, 20). Dansyl-BSA induced a significant drop in core body temperature (Figure 8A), and a high anaphylactic clinical score (Figure 8B), in the mIgG$_1$ isotype control mice. In contrast, the markedly reduced clinical scores and temperature changes reflecting systemic anaphylaxis were observed in p6.2 (2 μg per gram body weight) treated group (Figure 8), clearly indicating that p6.2 attenuated dansyl-specific IgE mediated systemic anaphylaxis.

P6.2 down-regulates basophil and mast cell surface IgE and FcɛRI expression

To gain insight into the mechanism by which LAIGE mAbs suppress allergic reactivity, we examined whether the LAIGE p6.2 altered surface IgE and FcɛRI expression on allergic effector cells. To this end, basophils (CD123$^+$/HLA-Dr$^-$) from blood PBMCs (which were *in vivo* sensitized with IgE) were treated with p6.2 or mIgG$_1$ isotype control, and the surface IgE/FcɛRI levels were determined. Figure 9 is a representative experiment showing that the IgE and FcɛRI levels, but not that of CD123 (IL-3Rα), were decreased by treatment of p6.2 in a dose-dependent manner in the dose range of 1–5 μg/mL (Figure 9A).

To define the effects of p6.2 on mast cell surface IgE and FcɛRI expression, the hFcɛRIα Tg mouse BMMCs were sensitized with human myeloma IgE, incubated with p6.2 or the mIgG$_1$ isotype control for 4 days, and then the surface level of IgE and FcɛRI was assessed by staining with FITC labeled anti-human IgE mAb E4.15 (which does not compete with p6.2 for IgE binding) and PE-anti-human FcɛRIα. C-kit (CD117) served as an internal staining control. Figure 9B shows that both surface IgE and FcɛRIs were markedly decreased by p6.2 in a dose-dependent manner. These data documented that both basophil and mast cell surface IgE and FcɛRI were down-regulated by p6.2 treatment.

P6.2 directly mediates internalization of surface IgE

Since surface IgE and FcεRI were down-regulated by p6.2, we used confocal microscopy to define whether p6.2 drove internalization of surface IgE. FITC-IgE sensitized hFcεRIα$^+$ BMMCs were treated with, a) mIgG$_1$ isotype control; b) 2 μg/mL of p6.2 for 24 and 48 hours; and c) PAE as a positive control. Following mIgG$_1$ treatment, IgE remained stably bound on the surface at 24 (data not shown) and 48 hours, being detected as bright rings on the periphery of BMMCs with minimal internalization (minimal green signals inside the cells, left panel of Figure 10A). The bright surface rings became markedly dimmer in BMMCs treated with p6.2 for 48 hours; instead, numerous fine green signals appeared inside the cells (middle panel, Figure 10A). Confocal sections from top to bottom of the p6.2 treated BMMCs demonstrated that the fine green signals were located intracellularly, being distributed throughout all 8 sections selected (Figure 10B, enlarged from the highlighted area of the middle panel of Figure 10A). The p6.2-induced internalization of surface IgE exhibited a strikingly different pattern compared with that triggered by PAE. In the latter case, large aggregates and a capping and polarized distribution of surface IgE were seen (right panel, Figure 10A). These data revealed that surface IgE has undergone internalization triggered by p6.2 treatment.

P6.2 triggered IgE Internalization is co-localized with FcεRI

Confocal microscopic analysis was employed to define whether surface IgE was internalized with FcεRI as the mechanism responsible for p6.2 driven FcεRI down-regulation. The hFcεRIα$^+$ BMMCs were first sensitized with FITC-IgE (showed green), treated for 24 or 48 hours with 2 μg/mL of p6.2 or an mIgG$_1$ isotype control, following by intracellular staining with Alexa fluro-647 labeled anti-human FcεRIα (showed red). The mIgG$_1$-treated BMMCs displayed bright surface as well as confined intracellular compartment FcεRI staining, including the areas surrounding the nucleus (Figure 10C, panel 1 and 7), while IgE was mainly on the surface with little appearing intracellularly (Figure 10C, panel 2 and 8). FcεRI was primarily co-localized with IgE on the surface areas (shown as yellow on "merge" in Figure 10C, panel 3 and 9). without apparent intracellular co-localized signals. In contrast, a weak surface IgE signal was seen on the p6.2 treated BMMCs, accompanied by a significantly increased intracellular IgE signal (Figure 10C, panel 2, 5, 8, 11), indicating that the surface IgE had undergone internalization. The intracellular IgE in the p6.2-treated BMMCs was mainly co-localized with FcεRI (Figure 10C, panel 6, 12), suggesting that p6.2 drove internalization of IgE-FcεRI complexes. Taken together, these data provide evidence for a mechanism by which p6.2 mediates down-regulation of both surface IgE and FcεRI on BMMCs.

Discussion

While direct targeting of the FcεRI-bound IgE on basophils/mast cells with an anti-IgE Ab as an allergy therapeutic had generally not been considered feasible as anti-IgE Abs were expected to crosslink surface IgE/FcεRI complexes and lead to allergic reactivity, Finkelman and his colleagues discovered that some anti-IgE and anti-FcεRI Abs could briefly induce allergic effector cell desensitization, but not allergic degranulation (8). In addition, naturally occurred anti-IgE autoantibodies were found to inhibit allergen-induced basophil activation

(9), and the anti-IgE and anti-FcɛRIα autoantibodies in chronic urticaria patients have been found to render basophils unresponsive to following anti-IgE stimulation (10). These studies support the concept that not all anti-IgE Abs are created equal in terms of their capacity of triggering allergic degranulation, instead, some anti-IgE Abs would play a role in allergic desensitization, albeit the mechanism (s) by which these anti-IgE Abs render allergic desensitization was not known currently. Thus anti-IgE Abs with the property of desensitizing allergic effector cells would have the potential as a novel allergic therapeutic.

In this study we find that LAIGE p6.2 and E2.18 are able to profoundly block allergic responses without triggering anaphylactic degranulation in both static and dynamic models of allergic reactivity. In contrast to allergens and/or the high affinity anti-IgE Abs that crosslink the surface IgE/FcɛRI and induce anaphylactic degranulation, LAIGE mAbs fail to trigger anaphylactic degranulation and cytokine production of allergic effector cells even at high concentrations. This is presumably due to its weak interaction with, and/or fast dissociation from the FcɛRI bound IgE such that the stable FcɛRI crosslinking required for transduction of the anaphylactic degranulation are not formed during the interaction of LAIGE with the surface-bound IgE. This feature of LAIGE mAbs interaction with surface-bound IgE provides the basis for its safety as a novel approach to suppress allergic reactions in contrast with the high affinity anti-IgE Ab that will generally trigger allergic anaphylactic degranulation immediately even in low dose. Instead, the weak and/or transient binding of p6.2 to the FcɛRI bound IgE promotes internalization of the surface IgE-FcɛRI complex, leading to rapid down-regulation of the surface IgE/FcɛRI that correlated with LAIGE's allergic blockade effects. Therefore we speculate that LAIGE-mediated down-regulation of the surface IgE-FcɛRI might be one of mechanisms responsible for LAIGE's allergic desensitization activity.

In contrast to the classic desensitization achieved through extremely low dose of allergens, such as with the traditional allergen immunotherapy, and/or anti-IgE Abs that induced rapid but short-lived desensitization within a matter of minutes (8, 24, 25), LAIGE induced allergic desensitization displayed quite distinct patterns and kinetics. First, onset of the LAIGE induced desensitization appeared taking much longer time, e.g., >8 hours for human BAT, and >2 days for skin mast cell PCA and systemic anaphylaxis in hFcɛRIα Tg mouse model. Second, the LAIGE-induced desensitization status appeared long-lasting, for at least more than 24 hours for basophils (as demonstrated in BAT), and > 2 days for skin mast cells (as demonstrated in PCA and systemic anaphylaxis), thus LAIGE-induced desensitization status was directly translated into the allergic blockade effect in all the experimental models tested. As the limit of the relatively short-term nature of human BAT, hFcɛRIα Tg mouse based PCA and passive systemic anaphylaxis that used in this study, the even longer-term of therapeutic effects could not be tested with these experimental models. Third, while allergen (s) and/or high affinity anti-IgE Abs induced both basophil CD203c and CD63, LAIGE only selectively promoted weak CD203c but not CD63 expression even with high concentration. The latter was particularly interesting because basophil CD63 expression was directly associated with anaphylactic degranulation pathway, whereas CD203c expression had been implicated in the piecemeal degranulation pathway (12, 21, 22). Given that LAIGE selectively promoted CD203c expression, and CD203c expression was not associated with preformed mediator (e.g., histamine) release and cytokine IL-4 production from basophil

(22, and Figure 2G, 2H, 3B), it is tempting to hypothesize that selectively activation of piecemeal degranulation pathway might be another mechanism involved in LAIGE-mediated allergic desensitization. Although the piecemeal degranulation pathway in basophil/mast cell is less understood, it was demonstrated that such a piecemeal degranulation pathway is likely a general mediator secretory pathway regulating physiological function of eosinophils (26).

It is also anticipated that the weak association of the LAIGE mAb with soluble IgE in the extracellular compartment *in vivo* will allow the LAIGE mAb released from the anti-IgE/IgE complex to function as an active drug. Thus the binding of LAIGE mAb to serum IgE, rather than "neutralizing" its therapeutic effects, is expected to provide a reservoir of active drug based on the reversible equilibrium of the low affinity interaction with serum IgE. Such a dynamic process is predicted to extend the pharmacologic effects of the LAIGE mAbs and allow longer dosing intervals. Additionally, it should provide another layer of safety by buffering the anti-IgE with soluble IgE so that upon administration, immediate direct contact of the administered anti-IgE with surface-bound IgE would not occur. Therefore the level of the preexisting IgE in serum would not likely be a limiting effect for p6.2's therapeutic effect as it is for omalizumab instead serum IgE will actually enhance/extend the action of the LAIGE based therapy.

Historically, low affinity protein-protein binding, or weak protein-protein interaction, was considered physiologically irrelevant. However, emerging evidence suggests that weak protein-protein interaction, which is not necessarily of low specificity (in fact, low affinity interaction often exhibits high specificity), is fundamentally important for promoting the rapid on/off switching of signal transduction, reversible cell-cell contacts, transient assembly/disassembly of signaling complexes, and dynamic regulation of enzymes' activity for broad substrate specificity (27–30). For example, the interactive affinity between lymphocytes' surface molecules is known to be very low with Kd in the 2×10^{-4} M to 10^{-6} M range. Such low affinity is favorable for reversible cell–cell adhesion processes and yet sufficient to transduce biologically important signaling with high specificity (31). By comparing the high- and low-affinity stimuli to elicit cellular responses of mast cells via the FcεRI receptor, it was found that while similar receptor phosphorylation was achieved, the receptor cluster size, mobility, distribution, and the effector responses including mediators release were markedly different between the high- and low affinity stimuli through preferentially activating separate signal pathways (32).

In the field of Ab-based biologics, specifically designing low affinity mAbs as a therapeutic approach has not been previously considered. Our studies demonstrating that LAIGE mAbs have potent anti-allergic efficacy in inhibiting allergic effector cells underscore the concept that low affinity Ab based approaches may have important overlooked therapeutic potential for allergy. As IgE/FcεRI provides the initial trigger for the allergic signaling cascade, it is not surprising that the effects of down-regulation of the surface IgE and FcεRI would translate into major attenuation of the downstream signaling cascade and ultimately the allergic effector response. Based on this feature, we anticipate that the LAIGE therapy would have broader application in treating and preventing allergic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviation

LAIGE	Low Affinity Anti-IgE
PAE	Polyclonal anti-IgE antibody
BAT	Basophil Activation Test
hFcεRIα Tg	human FcεRIα transgenic
PCA	Passive Cutaneous Anaphylaxis
mMCP-1	mouse mast cell protease-1
EBD	Evans Blue Dye
BMMC	Bone marrow derived mast cells

References

1. Follenweider LM, Lambertino A. 2013; Epidemiology of asthma in the United States. Nurs Clin North Am. 48:1–10. [PubMed: 23465442]

2. Berin MC, Sampson HA. 2013; Food allergy: an enigmatic epidemic. Trends Immunol. 34:390–397. [PubMed: 23648309]

3. Arbes SJ Jr, Gergen PJ, Elliott L, Zeldin DC. 2005; Prevalences of positive skin test responses to 10 common allergens in the US population: results from the third National Health and Nutrition Examination Survey. J Allergy Clin Immunol. 116:377–383. [PubMed: 16083793]

4. Moorman JE, Rudd RA, Johnson CA, King M, Minor P, Bailey C, Scalia MR, Akinbami LJ. 2007; National Surveillance for Asthma – United States, 1980–2004. MMWR. 56:1–54.

5. MacGlashen DW Jr, Bochner BS, Adelman DC, Jardieu PM, Togias A, McKenzie-White J, Sterbinsky SA, Hamilton RG, Lichtenstein L. 1997; Down-regulation of FcεRI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. J Immunol. 158:1438–1445. [PubMed: 9013989]

6. Corren J, Diaz-Sanchez D, Saxon A, Deniz Y, Reimann J, Sinclair D, Davancaze T, Adelman D. 2004; Effects of omalizumab, a humanized monoclonal anti-IgE antibody, on nasal reactivity to allergen and local IgE synthesis. Ann Allergy Asthma Immunol. 93:243–248. [PubMed: 15478383]

7. Tan LD, Bratt JM, Godor D, Louie S, Kenyon NJ. 2016; Benralizumab: a unique IL-5 inhibitor for severe asthma. J Asthma Allergy. 9:71–81. [PubMed: 27110133]

8. Khodoun MV, Kucuk ZY, Strait RT, Krishnamurthy D, Janek K, Lewkowich I, Morris SC, Finkelman FD. 2013; Rapid polyclonal desensitization with antibodies to IgE and FcεRIα. J Allergy Clin Immunol. 131:1555–1564. [PubMed: 23632296]

9. Chan YC, Ramadani F, Santos AF, Pillai P, Ohm-Laursen L, Harper CE, Fang C, Dodev TS, Wu SY, Ying S, Corrigan CJ, Gould HJ. 2014; "Auto-anti-IgE": naturally occurring IgG anti-IgE antibodies may inhibit allergen-induced basophil activation. J Allergy Clin Immunol. 134:1394–1401. [PubMed: 25112697]

10. Kaplan AP. 2004; Chronic Urticaria: pathogenesis and treatment. J Allergy Clin Immunol. 114:465–474. [PubMed: 15356542]

11. Macy E, Kemeny M, Saxon A. 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. [PubMed: 3263291]

12. MacGlashan D Jr. 2010; Expression of CD203c and CD63 in human basophils: relationship to differential regulation of piecemeal and anaphylactic degranulation processes. Clin Exp Allergy. 40:1365–1377. [PubMed: 20633031]

13. Monneret G, Gutowski MC, Bienvenu J. 1999; Detection of allergen-induced basophil activation by expression of CD63 antigen using a tricolour flow cytometric method. Clin Exp Immunol. 115:393–396. [PubMed: 10193408]

14. McGowan EC, Saini S. 2013; Update on the performance and application of basophil activation tests. Curr Allergy Asthma Rep. 13:101–109. [PubMed: 23188565]

15. Zhang K, Kepley CL, Terada T, Zhu D, Perez H, Saxon A. 2004; Inhibition of allergen specific IgE reactivity by a human Ig Fcg1-Fce bifunctional fusion protein. J Allergy Clin Immunol. 114:321–327. [PubMed: 15316510]

16. Dombrowicz D, Brini AT, Flamand V, Hicks E, Snouwaert JN, Kinet JP, Koller BH. 1996; Anaphylaxis mediated through a humanized high affinity IgE receptor. J Immunol. 157:1645–1651. [PubMed: 8759751]

17. Lyczak JB, Zhang K, Saxon A, Morrison SL. 1996; Expression of novel secreted isoforms of human immunoglobulin E proteins. J Biol Chem. 271:3428–3436. [PubMed: 8631944]

18. Radu M, Chernoff J. 2013; An in vivo Assay to Test Blood Vessel Permeability. JoVE. 73:50062.

19. Li XM, Zhang TF, Huang CK, Srivastava K, Teper AA, Zhang L, Schofield BH, Sampson HA. 2001; Food Allergy Herbal Formula-1 (FAHF-1) blocks peanut-induced anaphylaxis in a murine model. J Allergy Clin Immunol. 108:639–646. [PubMed: 11590394]

20. Li W, Zhang Z, Saxon A, Zhang K. 2012; Prevention of oral food allergy sensitization via skin application of food allergen in a mouse model. Allergy. 67:622–629. [PubMed: 22339388]

21. MacGlashan D Jr. Marked differences in the signaling requirements for expression of CD203c and CD11b versus CD63 expression and histamine release in human basophils. Int Arch Allergy Immunol. 2012; 159:243–252. [PubMed: 22722613]

22. Monneret G, Boumiza R, S Gravel S, Cossette C, Bienvenu J, Rokach J, Powell WS. 2005; Effects of prostaglandin D(2) and 5-lipoxygenase products on the expression of CD203c and CD11b by basophils. J Pharmacol Exp Ther. 312:627–634. [PubMed: 15388786]

23. Vaynberg J, Qin J. 2006; Weak protein-protein interactions as probed by NMR spectroscopy. Trends Biotechnol. 24:22–27. [PubMed: 16216358]

24. Kepley CL. 2005; Antigen-induced reduction in mast cell and basophil functional responses due to reduced syk protein levels. Int Arch Allergy Immunol. 138:29–39. [PubMed: 16088210]

25. MacGlashan D, Miura K. 2004; Loss of syk kinase during IgE-mediated stimulation of human basophils. J Allergy Clin Immunol. 114:1317–1324. [PubMed: 15577829]

26. Crivellato E, Nico B, Mallardi F, Beltrami CA, Ribatti D. 2003; Piecemeal degranulation as a general secretory mechanism? The anatomical record part A. 274A:778–784.

27. Perkins JR, Diboun I, Dessailly BH, Lees JG, Orengo C. 2010; Transient protein-protein interactions: structural, functional, and network properties. Structure. 18:1233–1243. [PubMed: 20947012]

28. Westermarck J, Ivaska J, Corthals GL. 2013; Identification of protein interactions involved in cellular signaling. Mol Cell Proteomics. 12:1752–1763. [PubMed: 23481661]

29. Vaynberg J, Fukuda T, Chen K, Vinogradova O, Velyvis A, Tu Y, Ng L, Wu C, Qin J. 2005; Structure of an ultraweak protein-protein complex and its crucial role in regulation of cell morphology and motility. Mol Cell. 17:513–523. [PubMed: 15721255]

30. Velyvis A, Vaynberg J, Yang Y, Vinogradova O, Zhang Y, Wu C, Qin J. 2003; Structural and functional insights into PINCH LIM4 domain-mediated integrin signaling. Nat Struct Biol. 10:558–564. [PubMed: 12794636]

31. van der Merwe PA, Davis SJ. 2003; Molecular interactions mediating T cell antigen recognition. Annu Rev Immunol. 21:659–684. [PubMed: 12615890]

32. Suzuki R, Leach S, Liu W, Ralston E, Scheffel J, Zhang W, Lowell CA, Rivera J. 2014; Molecular editing of cellular responses by the high-affinity receptor for IgE. Science. 343:1021–1025. [PubMed: 24505132]
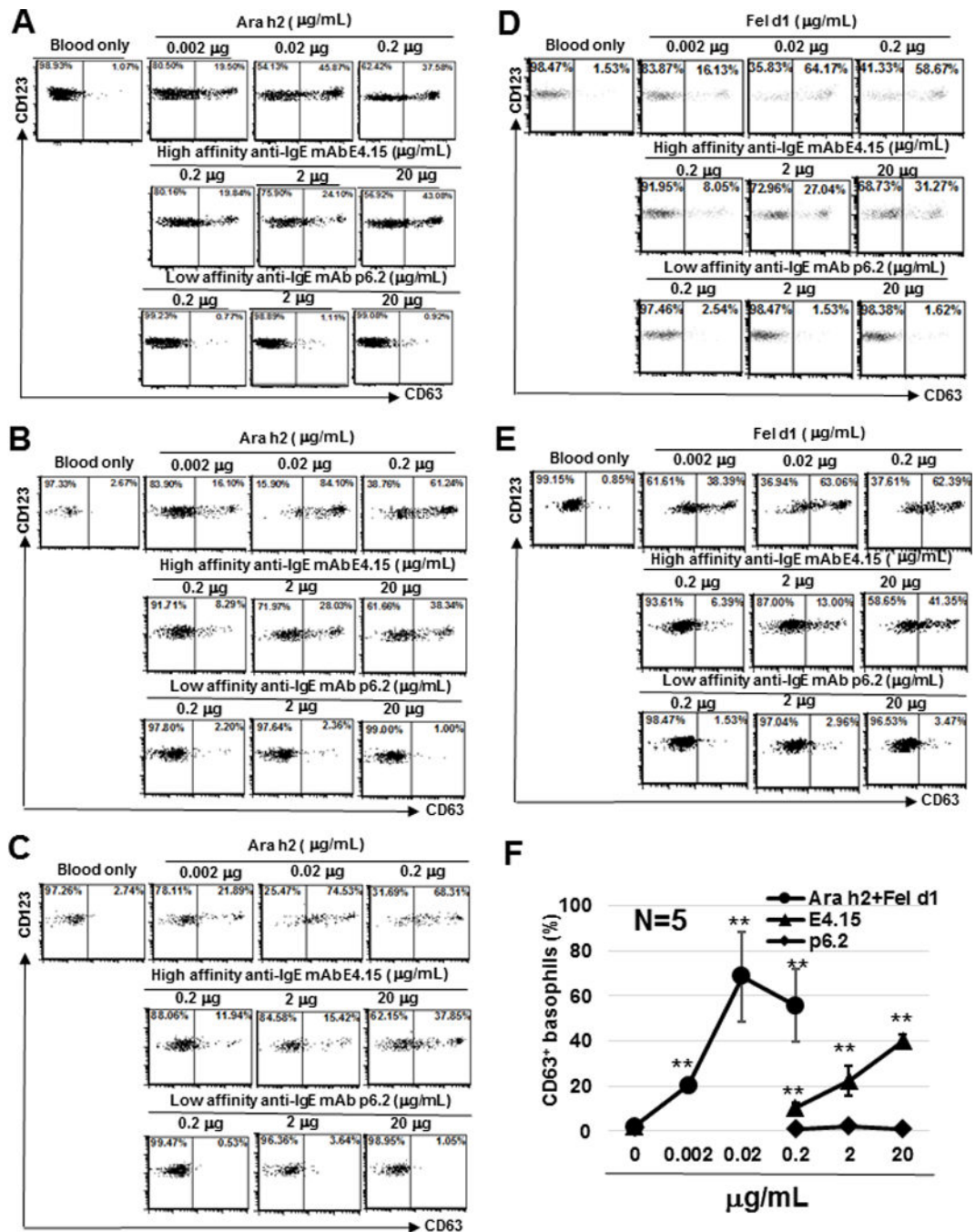
Figure 1.
Basophil CD63 expression profiles of the allergic subjects induced by allergens and anti-IgE Abs. Three-color FACS analysis was employed, and the $CD123^+/HLA-Dr^-$ population was gated as basophils for measurement of CD63 expression. (**A–C**) The dose-response basophil CD63 expression of three peanut allergic subjects induced by Ara h2, E4.15, and LAIGE p6.2. (**D** and **E**) The dose-response basophil CD63 expression profiles of two cat allergic subjects. (**F**) Summary of the kinetic dose-response curves of CD63 expression to allergens (Ara h2+Fel d1), E4.15 and p6.2 (n=5).
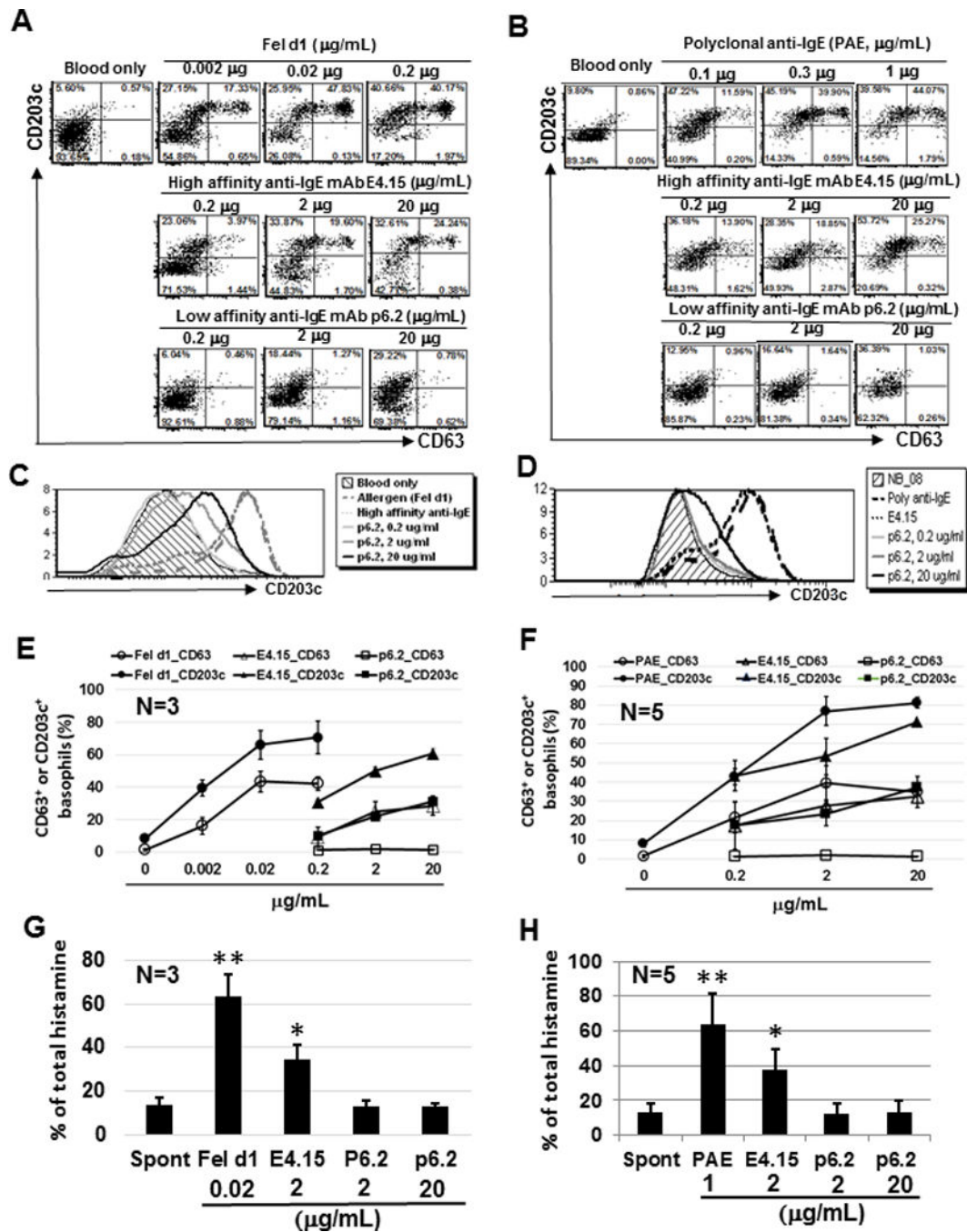
Figure 2.
Basophil CD63 and CD203c expression profiles of the cat allergic and normal healthy subjects induced by Fel d1 and anti-IgE Abs. Four-color FACS analysis was employed to simultaneously determine the basophil CD63 and CD203c expression. The CD123^{+}/HLA-Dr^{−} population was gated as basophils. (**A**) The dose-response basophil CD63/CD203c expression of the cat allergic subjects (n=3) induced by Fel d1, E4.15, and LAIGE p6.2. (**B**) The dose-response basophil CD63/CD203c expression of the normal healthy subjects (n=5) induced by PAE, E4.15, and LAIGE p6.2. (**C and D**) Dose-dependent CD203c upregulation

promoted by LAIGE p6.2 in both cat allergic and normal healthy subjects, respectively (solid lines). The dot lines represent the maximal CD203c upregulation driven by Fel d 1, PAE and E4.15 for comparison. (**E** and **F**) Summary of the kinetic dose-response curves of CD63 (open symbols) and CD203c (filled symbols) expression driven by Fel d1, E4.15 and LAIGE p6.2. (**G** and **H**) Histamine release levels from cat allergic (n=3) and normal subjects (n=5).
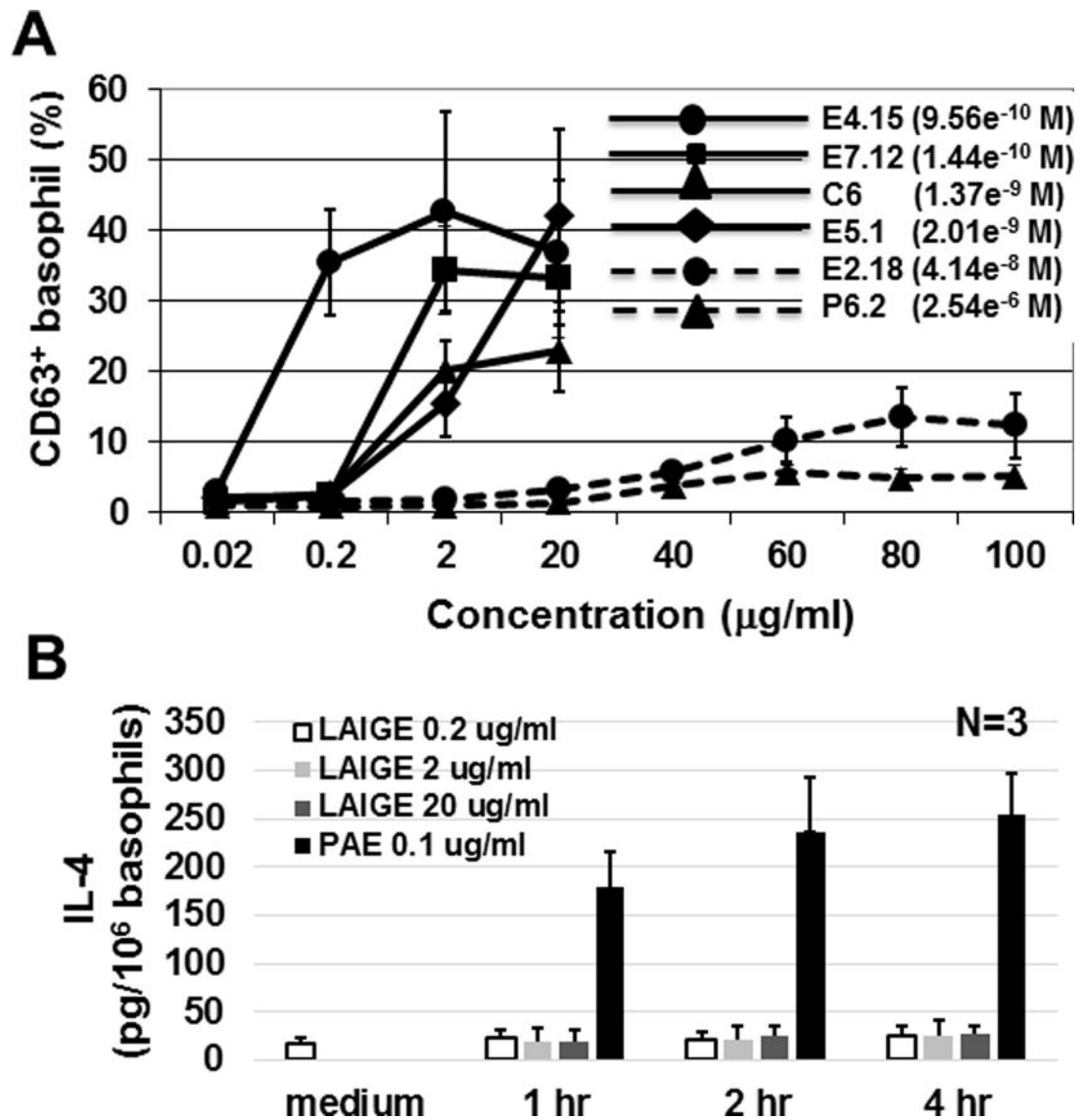
Figure 3.
Basophil degranulation-triggering capacity of anti-IgE mAbs. (**A**) Dose-response BAT of anti-IgE mAbs with differential affinities to IgE (n=3). (**B**) Basophil IL-4 production induced by LAIGE p6.2, with PAE as a positive control (n=3).
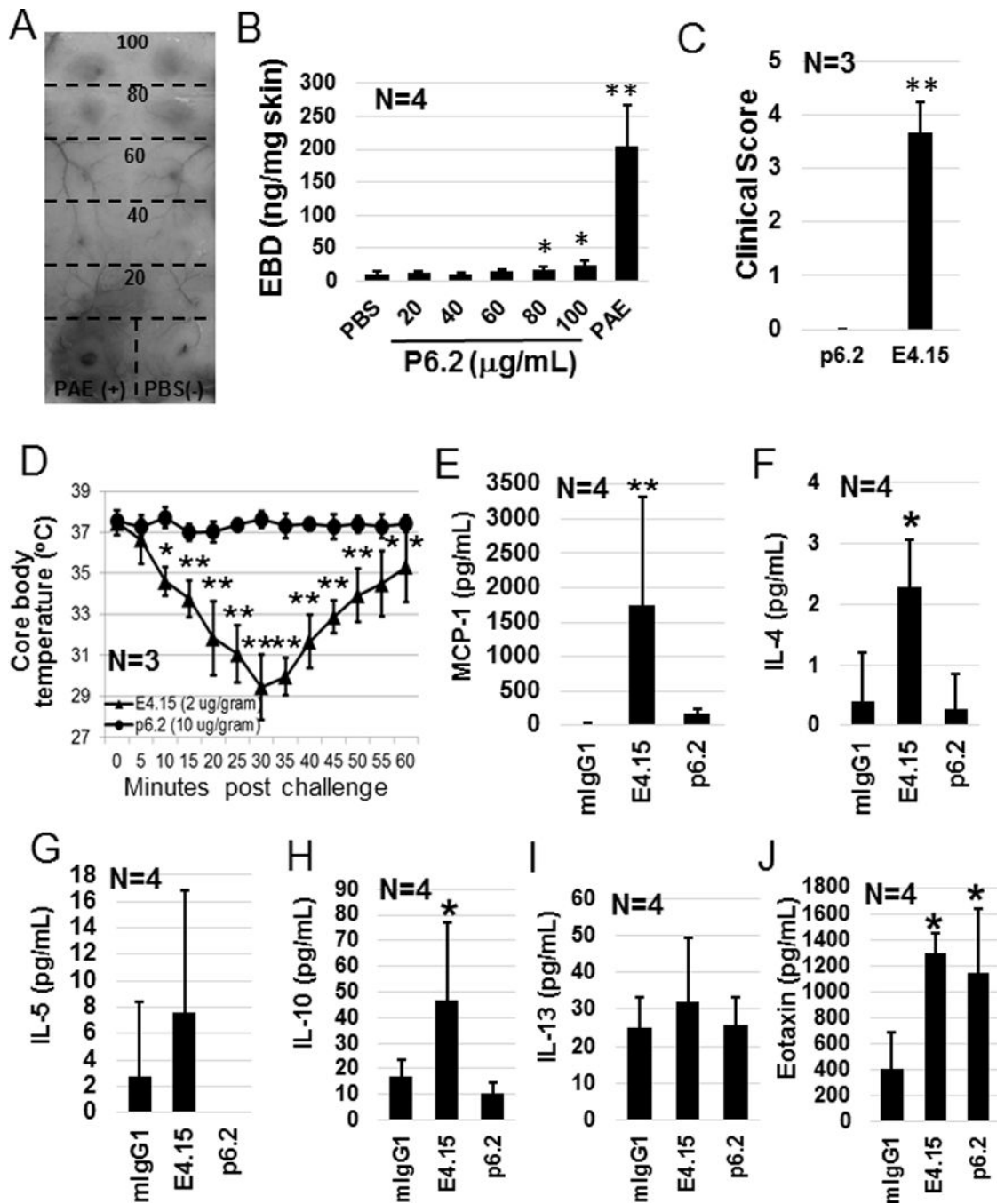
Figure 4.
Safety profiles of LAIGE p6.2 tested with hFcεRIα Tg mouse model. (**A**) PCA. The various concentrations of p6.2 as labelled were used to locally challenge IgE systemically sensitized mice. PAE was included as a positive, and PBS as negative, control. (**B**) Quantitative assessment of p6.2 induced PCA using EBD extraction (n=4). (**C**) Systemic anaphylaxis clinical scores from the p6.2 and E4.15 challenged mice (n=3). (**D**) Systemic anaphylaxis core body temperature changes from p6.2 (250 μg) and E4.15 (50 μg) challenged mice

(n=3). (**E–J**) Comparison of allergic mediator, cytokine and chemokine release triggered by E4.15 and p6.2 challenged mice (n=4).
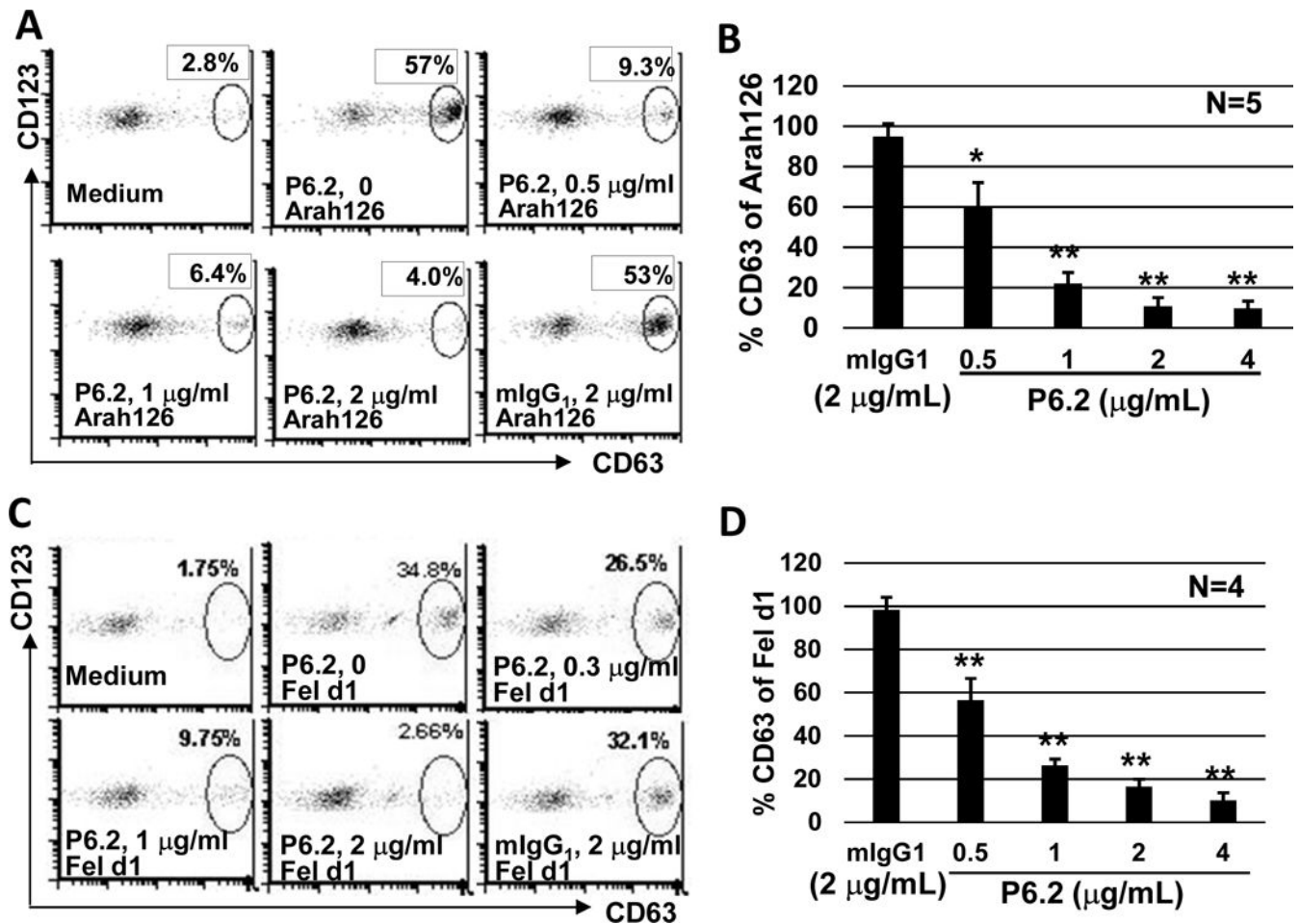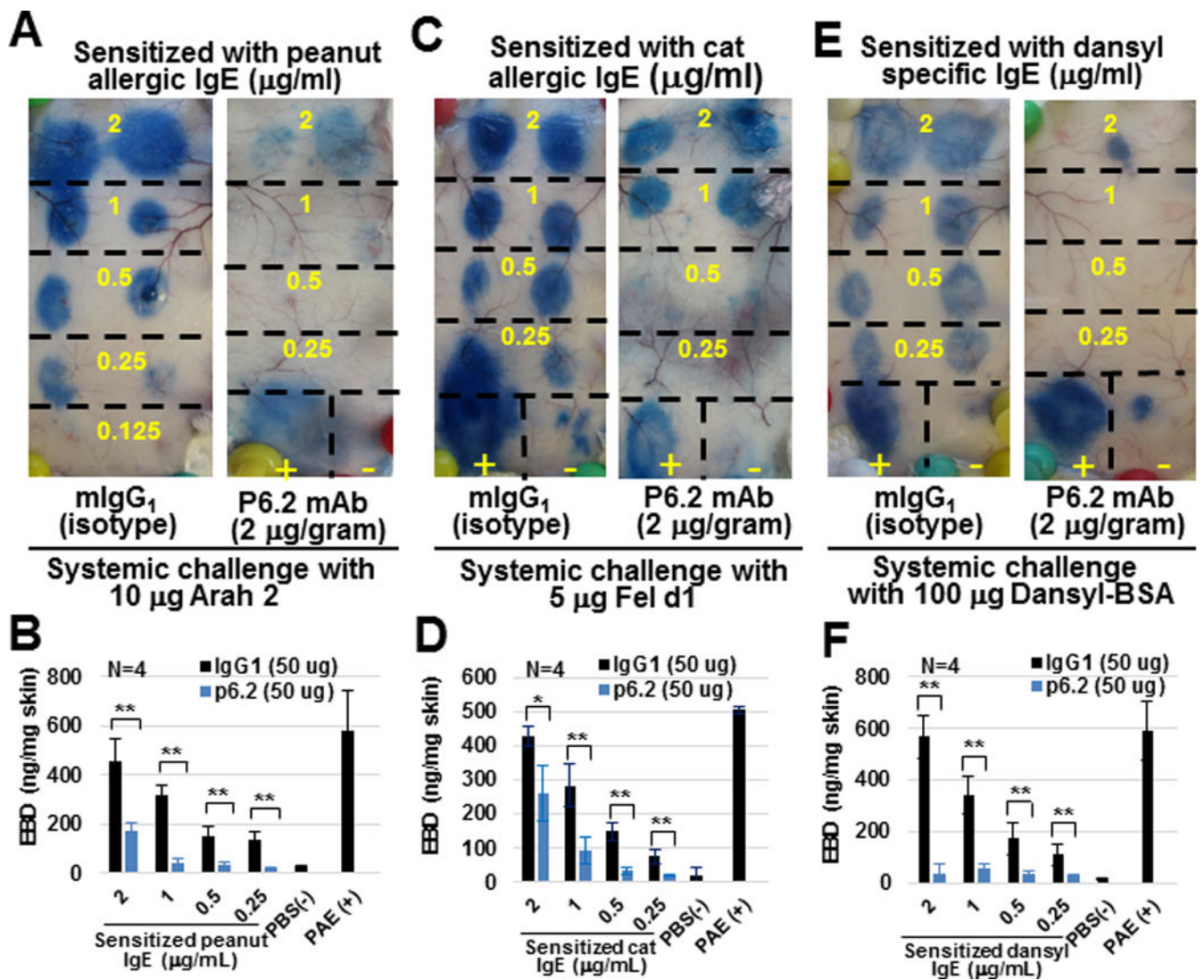
Figure 5.
LAIGE p6.2 blocks allergen-induced basophil CD63 expression. (**A**) FACS profile of the peanut allergen induced CD63^{high} expression inhibited by p6.2. (**B**) Summary of the inhibitory effects of p6.2 on peanut allergen induced CD63^{high} expression (n=5). (**C**) FACS profile of the cat allergen Fel d1 induced CD63^{high} expression inhibited by p6.2. (**D**) Summary of the inhibitory effects of p6.2 on cat allergen Fel d1 induced CD63^{high} expression (n=4).
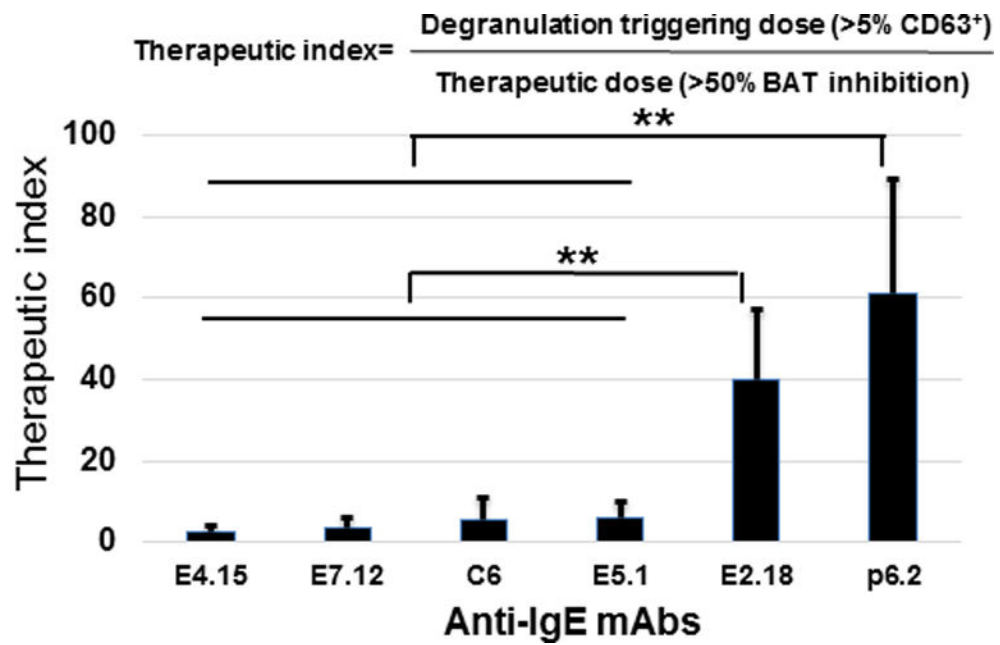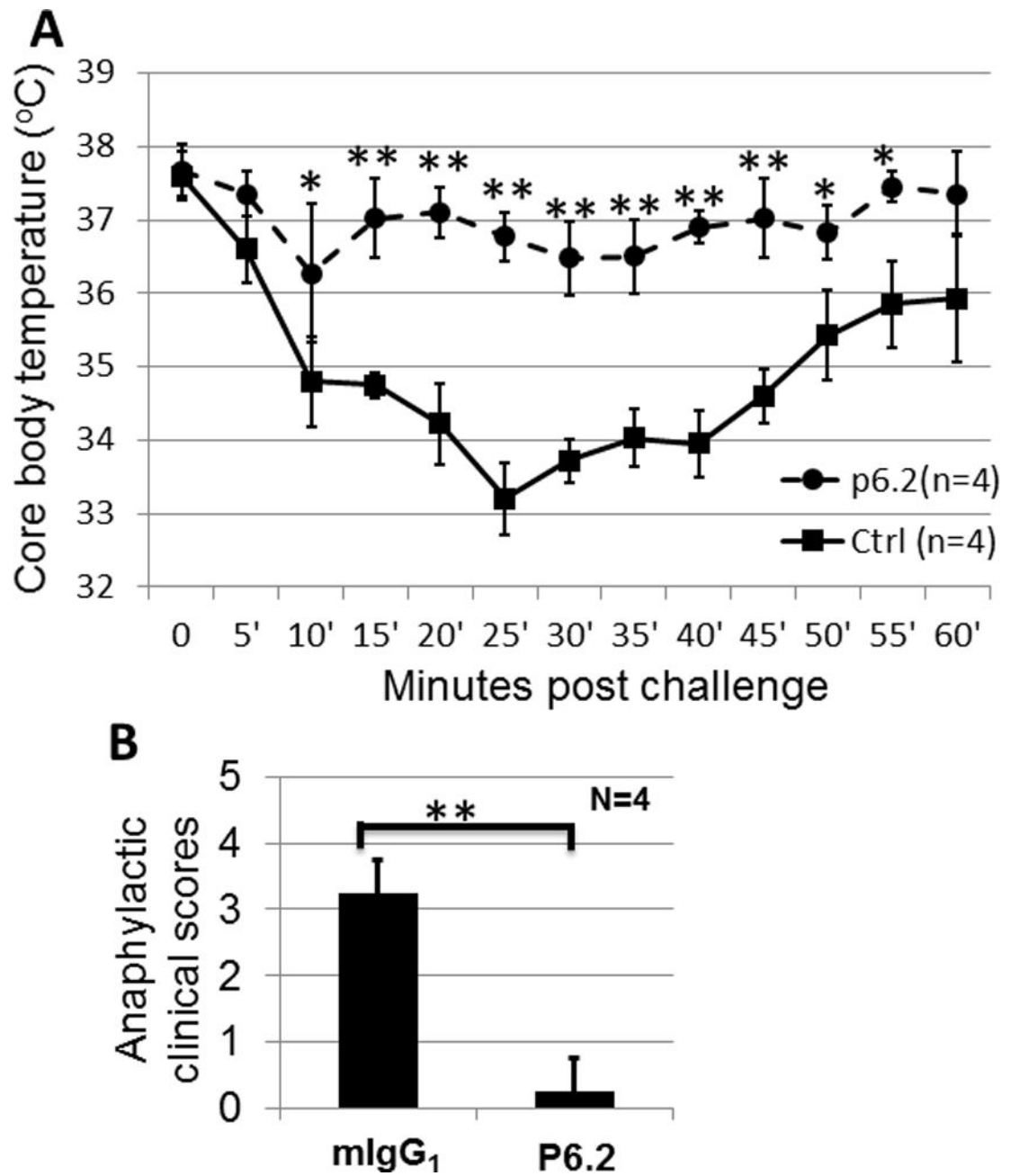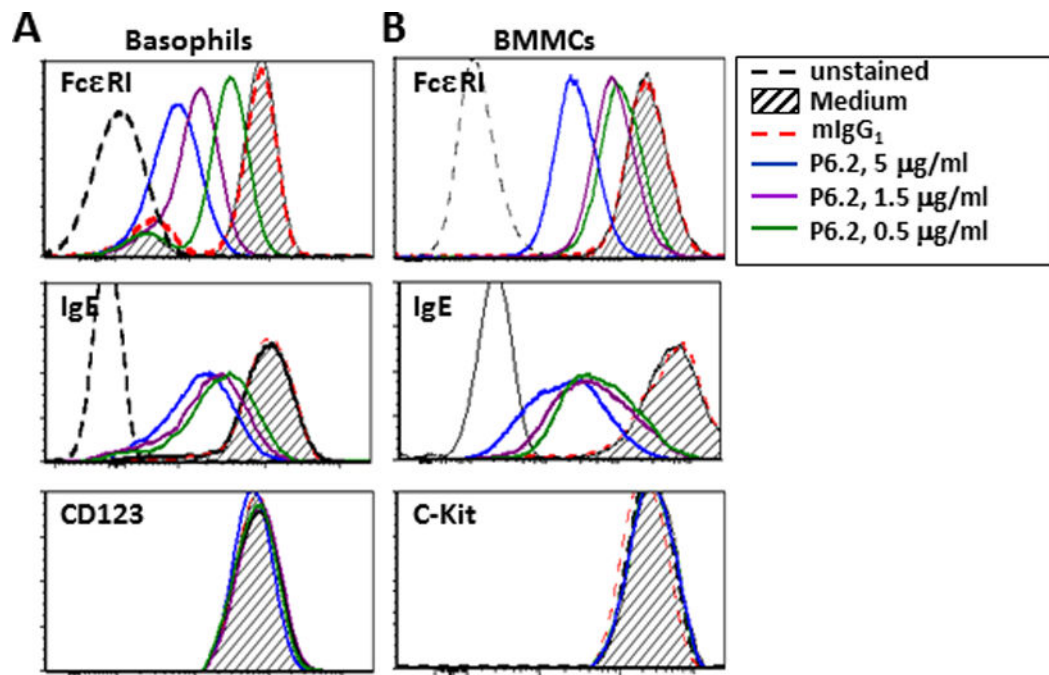
Figure 6.
The therapeutic index of high and low affinity anti-IgE mAbs. The therapeutic index of the peanut BAT based approach was calculated as "degranulation triggering dose" dividing by the "therapeutic dose". The BAT triggering dose was defined as the lowest anti-IgE mAb concentration capable of inducing >5% basophil CD63 expression, whereas the effective therapeutic dose was arbitrarily defined as the lowest anti-IgE mAb dose sufficient to inhibit >50% of peanut allergen triggered CD63 expression (n=3).

Figure 7.
LAIGE p6.2 blocks IgE-mediated PCA in hFcεRIα Tg mice. (**A**) p6.2 inhibited peanut allergic IgE-mediated PCA. (**B**) Quantitative EBD assessment from the peanut allergic IgE-mediated PCA spots. (**C & D**) p6.2 inhibited cat allergic IgE-mediated PCA. (**E & F**) p6.2 inhibited dansyl specific IgE-mediated PCA. The plus (+) was the PAE positive control, whereas the minus (-) was PBS negative control. N=4 mice for each group.
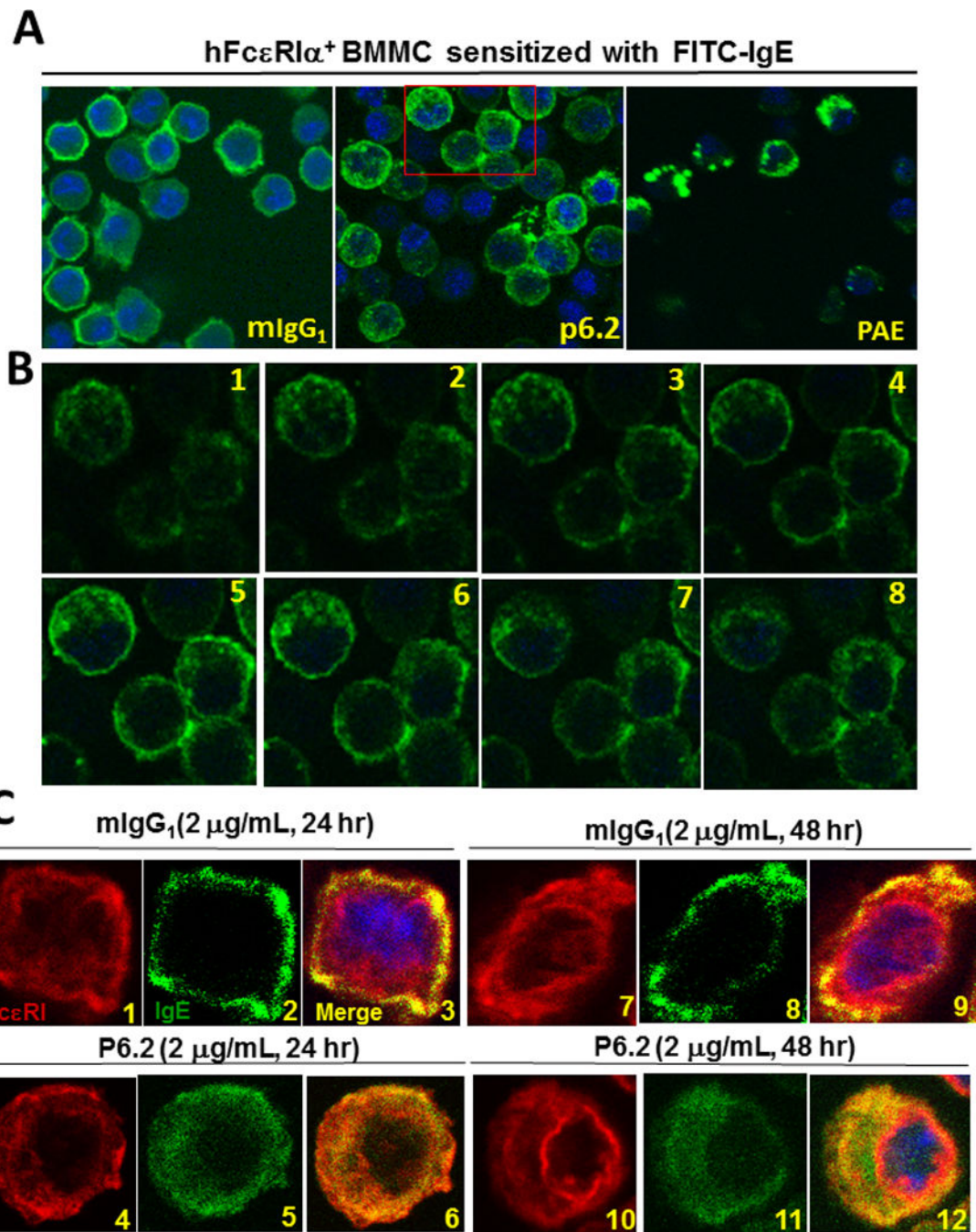
Figure 8.
P6.2 attenuates dansyl IgE-mediated systemic anaphylaxis. (A). The rectal temperature changes of the dansyl IgE sensitized hFcεRIα Tg mice in control (mIgG$_1$) and p6.2 treated mice (n=4). (**B**) The anaphylactic clinical scores in above two groups of mice.

Figure 9.
Down-regulation of surface IgE and FcεRI on basophils and BMMCs by p6.2. (**A**) Effects of p6.2 on expression of the basophil surface FcεRI and IgE levels determined by flow cytometry. CD123 staining was included as an internal staining control. The data shown is the representative of three experiments. (**B**) Effects of p6.2 on the BMMC surface FcεRI and IgE expression. The c-kit staining was included as an internal staining control. Shown is the representative data from two experiments.

Figure 10.
p6.2 triggers the surface IgE internalization and co-localization with FcεRI. (**A**) Effect of
p6.2 on promoting surface IgE internalization. (**B**) Internalized FITC-IgE in the confocal
sections. The highlighted area of the middle panel of Figure 10A was subjected to confocal
section analysis. The data are the representative of two experiments with similar results. (**C**)
Co-localization of IgE with FcεRI in the cytoplasmic compartment of BMMCs. The FcεRI
was stained as red, IgE as green and the co-localization of red and green merge as yellow,
whereas the nucleus was stained as blue. The BMMCs were treated for 24 (panel 1–6) and

48 (panel 7–12) hours respectively. The data are the representative of >20 cells from each condition analyzed.