

Suppression of IgE B Cells and IgE Binding to FcεRI by Gene Therapy with Single-Chain Anti-IgE¹

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IgE plays a pivotal role in allergic reactions and asthma through its ability to bind to the mast cell FcR for IgE (FcεRI). Current therapies to suppress such reactions include passive treatment with neutralizing Abs to IgE that block its binding to FcεRI. In theory, induction of immune tolerance in the B lymphocytes that carry IgE Ag receptors and give rise to IgE-secreting cells should provide longer term efficacy. However, recent data have suggested that such memory cells may lack cell surface IgE. Using a gene therapy approach, we show that a recombinant single-chain neutralizing anti-IgE could not only neutralize circulating IgE, but also reduce IgE⁺ B cell numbers and H chain transcripts. Therapeutic anti-IgE stimulated a calcium response in primary B cells or in a B cell line expressing membrane IgE and suppressed IgE secretion in vitro, suggesting that active signaling through membrane IgE likely promoted tolerance. Interestingly, upon subsequent challenge of anti-IgE-treated mice with an IgE cross-linking reagent capable of inducing activation of IgE-decorated mast cells, an anaphylaxis reaction was induced, apparently via a FcγRIII pathway involving recognition of anti-IgE Ab itself. These studies have important implications for the optimal design of safe and effective anti-IgE therapies and suggest that the IgE memory B cells may be targeted by such genetic Ab therapies. *The Journal of Immunology*, 2009, 182: 8110–8117.

Immunoglobulin E plays critical roles in allergic diseases including asthma, atopic dermatitis, and anaphylactic reactions (1). IgE binds to FcεRI α-chain expressed on mast cells and basophils (2). In people with Ag-specific IgE, contact with allergen induces IgE aggregation on mast cells, triggering their activation through the FcεRI signaling machinery. Activated mast cells release chemical mediators such as histamine that can induce life-threatening anaphylaxis. Two approaches can be considered to protect patients from allergic reactions. One is simply to remove IgE from the body. Alternatively, one could attempt to block the binding of IgE to mast cells, an approach that is currently used clinically with the anti-human IgE Ab omalizumab (3, 4).

In this study, we wished to investigate whether it was possible to not only block the interaction of IgE with FcRs, but also to suppress new IgE responses by providing a tolerogenic stimulus to developing or preexisting IgE memory B cells. In mammalian IgE H chain (*IgHε*) loci, there exist exons encoding not only secretory but also membrane forms of ε H chain. Although it has been suggested that, in the mouse, the memory B cells that give rise to IgE responses express surface IgG1 rather than membrane IgE (mIgE)³ (5), other studies point to a biologically active role of the mIgE as an Ag receptor (6–8). For example, germline truncations of the ε-chain membrane form result in reduced IgE titers, particularly in

the secondary response (7). Moreover, exons encoding the membrane form of IgE are conserved over evolution. Previous studies have indicated it should be possible to suppress new IgE production by targeting membrane IgE on B cells (6, 8). We show here additional functional evidence that mIgE is expressed on the surface of some activated B cells and can transmit biologically relevant signals. Importantly, we show that mIgE can transmit tolerogenic signals to B cells upon binding of a single-chain anti-IgE delivered by a gene therapy approach.

Materials and Methods

Mice

Eight- to 12-wk-old BALB/c mice were used in most experiments. C.129S2-*FcεRI*^{tm1Knt/J} (*FcεRI*^{−/−}) mice were purchased from The Jackson Laboratory. Mice were bred and maintained in The Scripps Research Institute Animal Resources facility according to the Institutional Animal Care and Use guidelines.

Cells

293F cells were purchased from Invitrogen. Anti-IgE hybridoma cells R1E4 (9) and EM95 (10) were provided by Drs. D. Conrad (Virginia Commonwealth University, Richmond, VA) and F. Finkelman (University of Cincinnati, Cincinnati, OH). Anti-FcR hybridoma cells 2.4G2 (11) were purchased from American Type Culture Collection.

Development R1E4 single-chain variable fragment (scFv)

R1E4 variable region sequence was obtained using a 5' RACE kit (Ambion) according to the manufacturer's protocol. The following oligonucleotides were used for H chain and L chain 5' RACE, respectively: 5'-AATAGCCCTTGACCAAGGCATCC-3' and 5'-CCAGTTGCTAAC TGTTCCTGGA-3'.

H chain and L chain genes were PCR amplified, respectively (T34 and T31 for L chain and T35 and T33 for H chain), and R1E4 scFv (pUb-R1E4) was generated with nested PCR. To generate a membrane-tethered single chain Ab, we subcloned this fragment into *SpeI*- and *XmaI*-digested pUb-187.1 plasmid (12). This strategy fuses the single chain Fv with rat-IgG1 hinge, CH2 and CH3 domains, followed by a C-terminal MHC class I transmembrane/intracellular domain. The primers were as follows: T34, 5'-ctattaattacagtgctgtgcaGACATTGTCTTGACCCAGTCT-3'; T31, 5'-ACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCCCGTTTCAATTCCAGCTTGGTGC-3'; T35, 5'-GCGGTTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGCAGGTGACTCTGAAAGAGTCT-3'; and T33, 5'-cctcccggtgttctggggctgtgtttcagcTGAGGAGACTGTGACCATGACTC-3'.

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³ Abbreviations used in this paper: mIgE, membrane IgE; scFv, single-chain variable fragment; qPCR, quantitative PCR; TNP, 2,4,6-trinitrophenyl.

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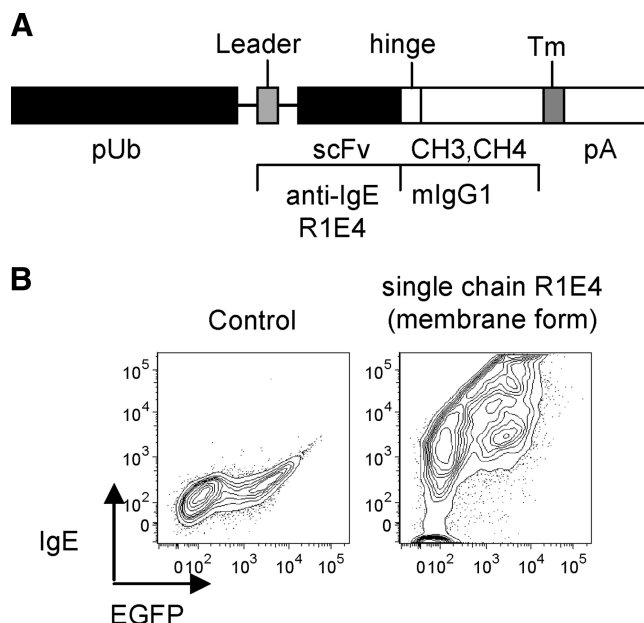


FIGURE 1. Generation and characterization of recombinant neutralizing anti-IgE single-chain Ab construct. **A**, The gene construct encoding rR1E4 (anti-IgE) showing intron/exon structure and selected features. Introns are depicted as thin lines. R1E4 scFv express under the control of human ubiquitin promoter. The R1E4 scFv was fused to hinge and Fc-encoding exons of the mouse IgG1 H chain. For membrane-bound anti-IgE expression, H-2K^b MHC class I transmembrane-coding sequences were included downstream. **B**, Control or recombinant plasmids expressing the membrane form of single-chain R1E4 were transiently cotransfected with enhanced GFP (EGFP) plasmid into 293 cells and the ability of the cells to bind to IgE was analyzed by flow cytometry.

To decrease the antigenicity of R1E4, rat-IgG1 was replaced with mouse IgG1 and several amino acid reversion mutations were introduced to L chain framework regions (supplemental Fig. 1⁴). To generate a secreted form in the final construct (pR1E4), the MHC transmembrane and cytoplasmic domains were excised. The specificity of recombinant single-chain membrane R1E4 to IgE was confirmed by the transient transfection to 293F cells and flow cytometry analysis of IgE binding (Fig. 1B). All of the plasmids used in *in vivo* injection were purified with an EndoFree Plasmid Maxi kit (Qiagen). For recombinant R1E4 (rR1E4), pR1E4 Ab-coding sequences were inserted into the pIRES-Zeocin hrGFP plasmid (pIRES-pR1E4). pIRES-pR1E4 (3 μ g) was transfected into 293F cells with Lipofectamine2000 (Invitrogen) and stable cells were established. From this supernatant, rR1E4 was purified with a rProtein A column (GE Healthcare).

Quantitative PCR (qPCR)

Total RNA was purified from 2 to 3 million spleen cells of control or pR1E4-treated mice using a RNeasy Plus kit (Qiagen). Reverse transcription was performed with a Quantitect Reverse Transcription Kit (Qiagen) per the manufacturer's protocol. *IgH* mRNA was quantitated using SYBR GreenER qPCR Supermix (Invitrogen) with 7900HT (Applied Biosystems) and normalized with *CD19* mRNA. Oligonucleotide primers used for IgE detection were 5'-acactcggagatgccagatc-3' and 5'-ggagcaccgttttgatacagtc-3' and for *CD19* detection, 5'-aggtcattgcaagtcagcagtg-3' and 5'-ggcgctcacttgaagaatctctg-3'.

Flow cytometry analysis

Erythrocyte-depleted cells were suspended in ice-cold staining buffer (HBSS buffer including 0.5 mM EDTA, 0.05 mM sodium azide, and 0.5% BSA) with appropriately titrated Abs. The following Abs were used: CD45R/B220 (RA3-6B2; BD Biosciences) (Pacific Blue), Fc ϵ RI α (MAR-1; eBioscience) (PE), IgE (23G3; eBioscience, or EM95) (FITC, PE, Alexa Fluor 647), CD49b (HMA2; BD Biosciences) (PE, allophycocyanin; biotin), c-kit (2B8; eBioscience) (allophycocyanin; biotin), CD4

(GK1.5; BD Biosciences) (PerCP-Cy5.5), CD8 (53-6.7; BD Biosciences) (PerCP-Cy5.5), and streptavidin-PE-Cy7 (eBioscience). For intracellular IgE staining, cells were incubated during surface staining with unlabeled anti-IgE (EM95); after fixation and permeabilization using a Cytofix/Cytoperm kit (BD Biosciences), cells were stained with labeled EM95 conjugate. These Abs were purchased from eBioscience or BD Biosciences as indicated. Propidium iodide (Invitrogen) was included in some experiments to exclude dead cells. To calculate the total Fc ϵ RI expression level on basophils based on IgE-binding capacity, Fc γ Rs were preblocked with 2.4G2 for 10 min and the cells further incubated with purified IgE (IgELa) at 10 μ g/ml for 30 min. Cells were then washed twice with FACS buffer and bound IgE was quantitated with anti-IgE conjugate. Data collection was done on an LSR II flow cytometer (BD Biosciences) and was analyzed using FlowJo software (Tree Star).

Hydrodynamic injection

Thirty micrograms of purified plasmid (pR1E4 or pUb control plasmid) was dissolved in 1.8 ml of TransIT-EE Delivery Solution (Mirus Bio) and injected via a tail vein. In the experiments depicted in Fig. 2, 10 μ g of a second plasmid driving human placental secreted alkaline phosphatase (pLIVE-SEAP; Mirus Bio) was coinjected, allowing one to monitor the efficiency of transfection by enzyme activity appearing in blood. All of the injected mice (five of five control and eight of eight pR1E4 treated) analyzed on day 13 after plasmid injection were alkaline phosphatase positive (data not shown).

IgE-eliciting immunizations

OVA (Sigma-Aldrich) was prepared with alum (Imject; Pierce) at a ratio of 10 μ g of protein:100 μ g of alum/mouse and was given *i.p.* Goat anti-mouse IgD (0.2 ml; eBioscience) was injected *i.p.*

IgE ELISA

An IgE ELISA quantitation kit was purchased from Bethyl and used according to the kit instructions. In experiments involving pR1E4, purified EM95 (anti-IgE) was coated on Nunc Maxisorp plates and bound serum IgE was detected with HRP-conjugated goat anti-mouse IgE (Bethyl). The presence of rR1E4 did not interfere with the total IgE concentration measurement because R1E4 and EM95 see nonoverlapping epitopes; R1E4 blocks IgE binding to Fc ϵ RI, whereas EM95 cannot. For quantitation of free IgE, rR1E4 was coated on the plate and bound IgE was detected with HRP-conjugated goat anti-mouse IgE. Color was developed with Ultra-TMB (Pierce).

Cell culture

Splenocytes (2×10^5 /ml) were cultured at 37°C/5% CO₂ in a final volume of 2.5 ml with 25 ng/ml IL-4, 1 μ g/ml anti-CD40 (1C10; eBioscience) with or without 20 μ g/ml rR1E4 in Advanced-RPMI 1640 medium supplemented with 5% FCS, 1 $\times$ penicillin-streptomycin-glutamine (Life Technologies), 2 mM GlutaMAX-I (Life Technologies), and 55 μ M 2-ME for 4 days. For Ca²⁺ mobilization analysis, splenocytes were cultured with 25 μ g/ml IL-4 and 10 μ g/ml anti-CD40. Culture supernatants were then tested for IgE concentration by ELISA; recovered cells were washed two times with FACS buffer before flow cytometry or Ca²⁺ mobilization analysis.

Calcium response

Cultured splenocytes were loaded with Fluo-4 (Invitrogen) per the manufacturer's instructions. Calcium mobilization was induced by addition of 20 ng/ml mAbs at a cell concentration of 10^6 /ml in volume of 0.5 ml. In some experiments, cells were preincubated with 10 μ g/ml anti-mouse IgG1 Ab for 30 min before challenge and fluorescence analysis. Analysis was conducted using the FL1 channel of an LSR-II flow cytometer and analyzed with FlowJo software.

Anaphylaxis

One hundred micrograms of EM95 was given per mouse retro-orbitally after anesthesia; rectal temperature was monitored with a RET-3 probe (Physitemp Instruments) for 60 min. In some experiments, 0.5 mg of 2.4G2 (anti-Fc γ R/III) Ab was given to each mouse 24 h before challenge. In the case of rR1E4 injection, 10 μ g of 2,4,6-trinitrophenyl (TNP)-BSA and 100 μ g of IgE anti-TNP (IgELa) were given per mouse and 50 μ g of rR1E4 was given retro-orbitally; rectal temperature was monitored for 60 min.

⁴ The online version of this article contains supplemental material.

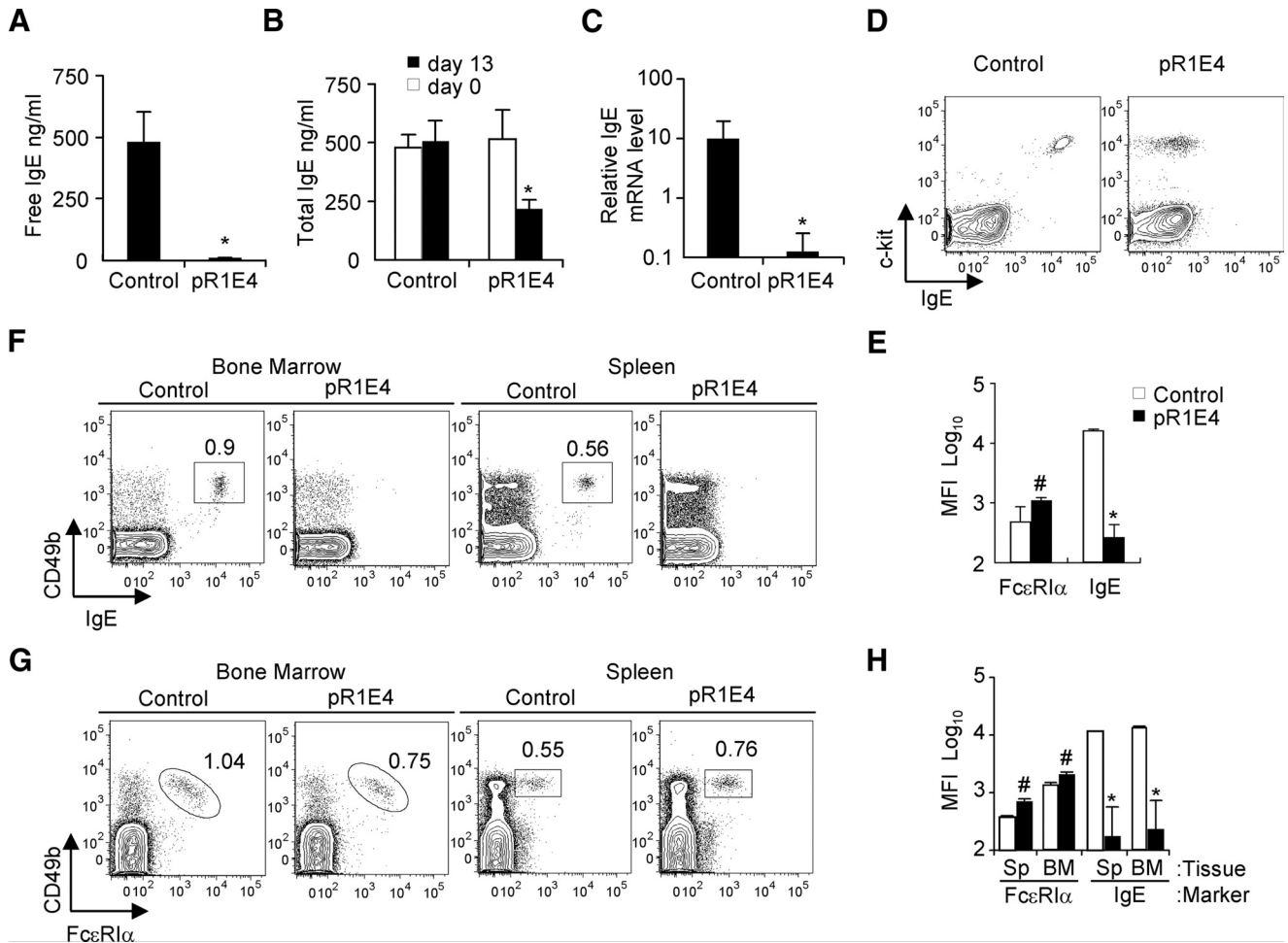


FIGURE 2. Effect of in vivo expression of secreted form of chimeric single-chain anti-IgE on markers of IgE expression. Two-month-old BALB/c mice were given pR1E4 or control plasmid i.v. and analyzed 13 days later. **A**, Free serum IgE level measured on day 13 after plasmid treatment. **B**, Total serum IgE concentration before treatment (□) and 13 days after treatment (■). **C**, qPCR comparison of relative *IgHε* mRNA levels in the spleen of control or pR1E4-treated mice at day 13 of treatment. **D** and **E**, Flow cytometry analysis of IgE bound to peritoneal mast cells in control and pR1E4-treated mice. Peritoneal mast cells were defined as c-kit⁺FcεRI⁺CD4[−]CD8[−]B220[−] cells. Mean fluorescence intensity (MFI) of binding by FcεRIα and anti-IgE Abs was monitored. **F** and **G**, Analysis of IgE bound to basophils in the spleen and bone marrow of control or treated individual mice. Basophils were identified as CD49b⁺FcεRI⁺CD4[−]CD8[−]B220[−]. **H**, Levels of surface IgE and FcεRI on basophils in the spleen (Sp) and bone marrow (BM) on day 13 after plasmid treatment. Results are means ± SD of eight mice receiving pR1E4 compared with five mice receiving empty vector. Statistical significance of differences between pR1E4-treated and control-treated mice was calculated using Student's *t* test: *, *p* < 0.001 and #, *p* < 0.2.

Results

Development of recombinant single-chain anti-IgE

In contrast to passive anti-IgE administration, anti-IgE delivery via gene therapy has the potential to provide permanent therapy of IgE-mediated disease. To study the effects of anti-IgE gene therapy in the mouse (an intensively studied model for human allergy, asthma, and anaphylaxis (13–15)), we generated a chimeric single-chain anti-IgE gene based on neutralizing rat anti-mouse IgE mAb R1E4 (9). R1E4, like omalizumab (Genentech) in the human system, is unable to activate mast cells via IgE bound to FcεRI, but binds free IgE. R1E4 Ig H and L chain variable region codons were cloned, sequenced, and joined together with a short linker sequence, yielding a *scFv* gene (Fig. 1A). The *scFv* gene was placed upstream of mouse *IgG1* hinge and membrane proximal codons. Plasmids driving plasma membrane expression under the control of the human ubiquitin promoter (16) were prepared and validated (Fig. 1B). A modified plasmid, called pR1E4, encoding a secreted version of the rR1E4 chimeric protein (rR1E4) was generated. The biological effects of in vivo expression of plasmids encoding membrane or secreted proteins was tested by the hydrodynamic (naked

DNA) injection method, which leads to transient expression in the liver. Because tolerance of cognate B cells to protein Ags can be induced either by membrane expression on the liver (17) or by soluble protein (18–20), we also compared in vivo efficacy of plasmids encoding membrane and secreted forms of recombinant anti-IgE.

Neutralizing serum IgE in vivo

pR1E4 plasmid given to BALB/c mice led to single-chain anti-IgE secretion lasting at least 30 days that diminished at later times (data not shown). At day 13 after pR1E4 injection, levels of free IgE were reduced to ~1% of those of control mice (Fig. 2A). There was also a marked decline in the levels of total IgE, which includes both free IgE and IgE-rR1E4 complexes (Fig. 2B). Plasmid encoding membrane-bound single-chain anti-IgE was also effective in reducing IgE levels, although the effect was less long-lasting (supplemental Fig. 2). For unknown reasons, plasmids encoding membrane-bound single-chain constructs were apparently somewhat toxic to expressing liver cells, even if they lacked specificity for IgE (data not shown). Therefore, additional experiments focused

Table I. Effects of pRIE4 treatment on basophil and mast cell numbers in selected tissues^a

Cell Type	Tissue Source	Treatment	Frequency (%)	Absolute Number ($\times 10^4$)
Mast cells	Peritoneal cavity	Control ^b	0.26 $\pm$ 0.05	1.8 $\pm$ 0.5
		pRIE4 ^c	0.47 $\pm$ 0.08	2.5 $\pm$ 0.5
Basophils	Spleen	Control ^b	0.10 $\pm$ 0.02	9.8 $\pm$ 2.0
		pRIE4 ^c	0.22 $\pm$ 0.05	20.9 $\pm$ 0.5 ^d
	Bone marrow	Control ^b	0.31 $\pm$ 0.06	8.4 $\pm$ 1.9
		pRIE4 ^c	0.24 $\pm$ 0.04	8.2 $\pm$ 1.4

^a Mast cells and basophils were identified using flow cytometry 13 days after treatment, as described in Fig. 2.

^b Control plasmid injection ($n = 5$ mice).

^c Experimental plasmid injection ($n = 8$ mice).

^d Statistically significant difference from control, $p < 0.05$, by two-tailed Student's t test.

on the effects of the soluble form. Importantly, RNA analysis of the spleens of mice treated with pRIE4 revealed a >99% reduction in the levels of *IgHε* mRNA (Fig. 2C). Consistent with the reduced IgE levels in pRIE4-treated mice, c-kit⁺ peritoneal mast cells and CD49b⁺ basophils in the bone marrow and spleens of anti-IgE-treated mice lacked detectable surface IgE, indicating that their FcεRs had lost bound IgE (Fig. 2, D–H). Basophils were still present in these tissues as indicated by FcεRIα/CD49b double staining (Fig. 2G). In treated mice, FcεRI expression levels in basophils appeared higher than in control plasmid-treated mice, however, total IgE-binding capacity of basophils was somewhat reduced (supplemental Fig. 3, A and B). This discrepancy was probably because anti-FcεRI mAb MAR-1 binds more tightly to FcεRI lacking bound IgE

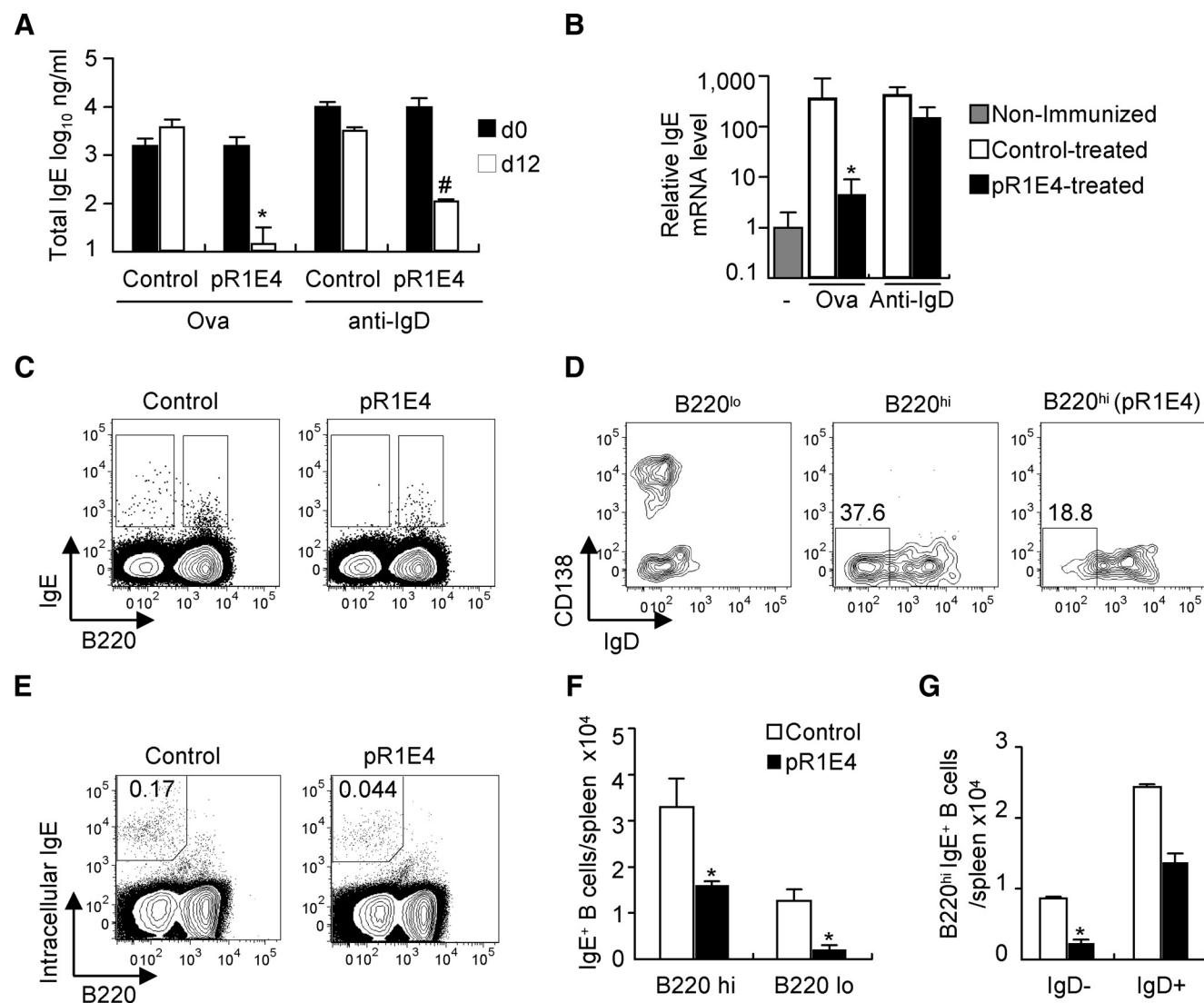


FIGURE 3. Effects of pRIE4 plasmid treatment on ongoing IgE responses induced by OVA/alum immunization or goat anti-mouse IgD treatment. Two-month-old BALB/c mice injected i.p. 10 days previously (day -10) with either 10 μ g of OVA/alum or 200 μ l of goat anti-mouse IgD serum were treated with control or pRIE4 plasmids i.v. on day 0 and analyzed on days 0 and 12 for suppression of the IgE response. **A**, Total serum IgE levels on day 0 (■) and day 12 (□). **B**, Splenic *IgHε* mRNA levels assessed by qPCR. **C–G**, Flow cytometry analysis of IgE⁺ B cells in spleens of mice injected with anti-IgD and subsequently treated with control or pRIE4 plasmid. **C**, Analysis of the frequencies of B220^{high} and B220^{low}IgE⁺ B cells among CD4⁺CD8⁺c-kit⁺CD49b⁺ gated viable spleen cells. **D**, Comparison of CD138 and IgD expression of B220^{low} and B220^{high} populations in control plasmid and pRIE4-treated mice, as gated in **C**. **Left**, B220^{low} cells from control plasmid-treated mouse; **center**, B220^{high} cells from control plasmid-treated mouse; and **right**, B220^{high} cells of pRIE4-treated mouse. **E**, Analysis of treated and control anti-IgD-injected mice for the presence of cells that expressed high levels of intracellular IgE. **F**, Summary of pRIE4-induced reductions in IgE⁺B220^{high} and IgE⁺B220^{low} subsets using the analysis in **C**. **G**, Quantitation of pRIE4-induced reduction in surface B220^{high}IgE⁺IgD⁺ B cells as in **D**. Significance was calculated using Student's t test. *, $p < 0.01$ and #, $p < 0.05$. $n = 4$ –6 mice/group.

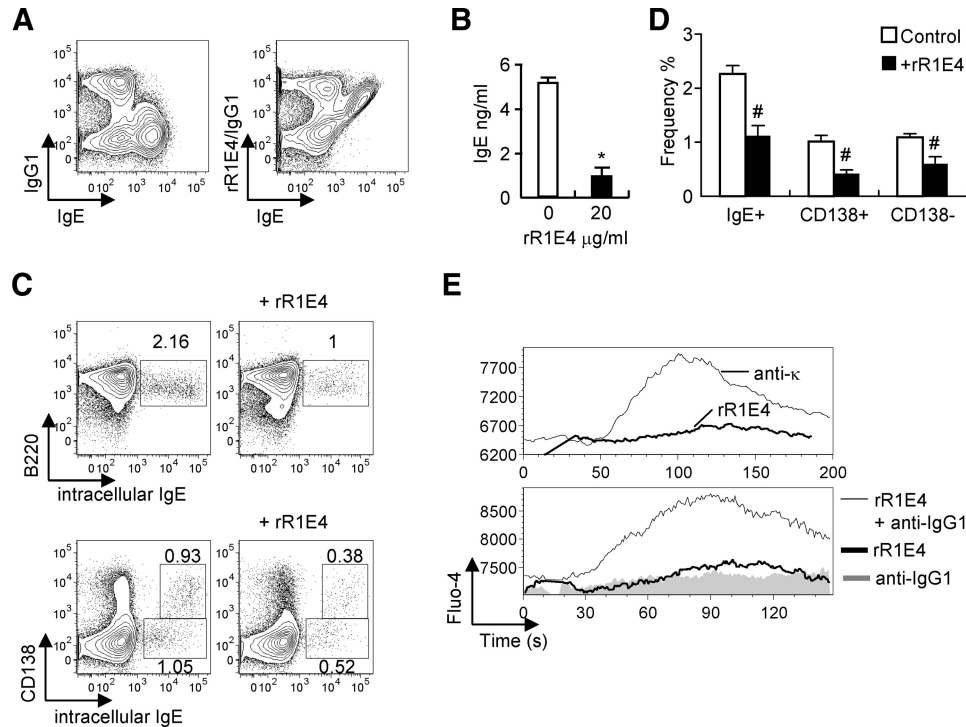


FIGURE 4. Ability of rR1E4 anti-IgE protein to affect the biological responses of IgE⁺ B cells generated in tissue culture. *A*, BALB/c spleen cells were induced to undergo IgE class switch by culture at 2×10^5 cells/ml for 4 days with IL-4 (25 ng/ml) and anti-CD40 (10 $\mu\text{g/ml}$); binding by rR1E4 was examined. *Right panel* shows staining with rR1E4 (10 $\mu\text{g/ml}$) followed by FITC anti-mouse IgG1 and Alexa Fluor 647 anti-IgE (EM95). *B–D*, Spleen cells (2×10^5 cells/ml) were stimulated for 4 days with IL-4 (25 ng/ml) and anti-CD40 (1 $\mu\text{g/ml}$) with or without 20 μg of rR1E4. *B*, IgE levels in culture supernatants on day 4 as measured by ELISA. *C*, Analysis of IgE⁺ B cell and IgE⁺ plasma cell frequencies at day 4 of culture, as determined by staining for intracellular IgE, B220, and CD138. *D*, Summary of rR1E4-induced changes in IgE subpopulations as shown in *C*. *E*, rR1E4-induced calcium flux in fluo-4-loaded B cells recovered at day 4 of culture. *Top panel*, B cells were treated with either anti-Ig κ or rR1E4 alone. *Lower panel*, Cells were precultured with anti-IgG1 alone for 30 min (to obscure the response of IgG1 cells) and then stimulated with either anti-IgG1 or rR1E4, which is supercross-linked by free anti-IgG1. Results presented in *B* and *D* represent the means and SDs of four separate experiments. *, $p < 10^{-4}$ and #, $p < 0.05$.

than to Fc ϵ RI carrying IgE (supplemental Fig. 3C). Reduced levels of bioactive IgE in pR1E4-treated mice did not lower the percentages or absolute numbers of mast cells and basophils (Table I). Overall, these data support the conclusions that 1) gene therapy with recombinant single-chain anti-IgE is feasible and can effectively neutralize circulating IgE; 2) reduced levels of bioactive IgE have surprisingly minimal negative effects on mast cell and basophil survival, at least at the time point tested, and 3) recombinant single-chain anti-IgE treatment could actively suppress new IgE production by B cells.

Recombinant anti-IgE treatment of preimmunized mice

We next determined whether pR1E4 could be used to treat and suppress ongoing, established IgE responses. Basal serum IgE levels in mice were quite low (~ 500 ng/ml in BALB/c mice and under 100 ng/ml in C57BL/6 mice). To evaluate the anti-IgE treatment, we used two different model responses: OVA immunization with alum adjuvant (OVA/alum), which is commonly used to elicit an Ag-specific IgE response, and anti-IgD treatment, a nonspecific B cell activator which elicits a stronger, but polyclonal IgE response (21, 22).

Ten days after receiving IgE-eliciting stimulus, mice were treated with pR1E4 or control plasmids and then analyzed 12 days later, i.e., 22 days after immunization (Fig. 3). OVA/alum immunization increased total serum IgE levels manyfold above preimmune levels, a response that was essentially fully suppressed by treatment with pR1E4 (Fig. 3A). *IgH ϵ* mRNA qPCR analysis showed that pR1E4 treatment reduced *IgH ϵ* mRNA levels in the spleen by 99% (Fig. 3B). pR1E4 treatment also reduced the levels

of the low-affinity IgE receptor CD23 (23) on follicular B cells by $\sim 50\%$ (supplemental Fig. 4, A and B). We conclude that in mice with ongoing IgE responses, treatment with the soluble recombinant anti-IgE-producing plasmid pR1E4 could significantly ($p = 0.0023$) suppress levels of serum IgE, new IgE synthesis, and B cell-expressed CD23.

Treatment of mice after anti-IgD injection served as a more stringent test of the ability to tolerize ongoing responses because IgE responses were >10 -fold further enhanced over the OVA/alum-induced response. Anti-IgD injection induced ~ 10 $\mu\text{g/ml}$ IgE in sera in control mice, which could be markedly (99%) suppressed by subsequent in vivo expression of pR1E4 (Fig. 3A). However, *IgH ϵ* mRNA qPCR analysis revealed that mice given pR1E4 after anti-IgD treatment still had high levels 12 days later, suggesting that IgE mRNA-producing cells remained in the spleen (Fig. 3B).

To identify the source of the IgE mRNA, we analyzed splenocytes from anti-IgD-treated mice for the presence of mIgE⁺ B cells (Fig. 3, C–G). In control plasmid-treated mice, B220^{high} and B220^{low} IgE⁺ B cells were detected (Fig. 3C). B220^{high} B cells were mostly IgD⁺CD138⁺ cells and possibly represented background staining of naive B cells (Fig. 3D, center). B220^{low} B cells were mostly IgD⁺CD138⁺ or CD138⁺ cells, indicating that they were IgE preplasma cells or B220^{low} memory B cells (Fig. 3D, left). In pR1E4-treated mice, B220^{high} IgE⁺ B cells were detected, but most B220^{low} IgE⁺ B cells were lost. The remaining B220^{high} IgE⁺ B cells in pR1E4-treated mice were IgD⁺CD138⁺ (Fig. 3D), suggesting that pR1E4 blocked development of IgE memory B cells and preplasma cells. The frequency of the

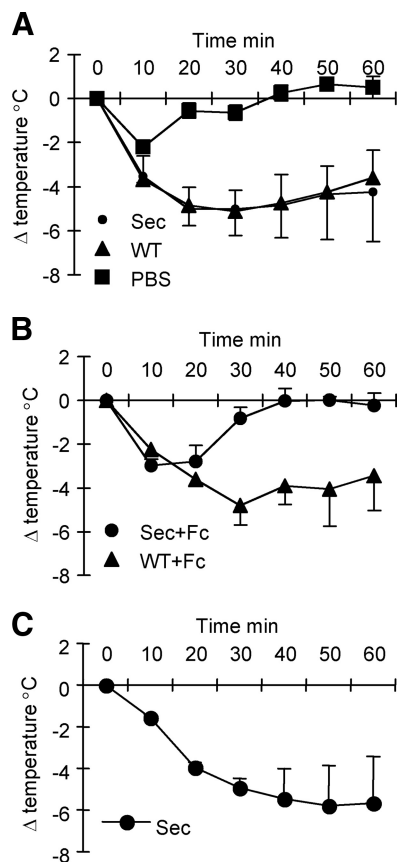


FIGURE 5. Systemic anaphylaxis reactions in rR1E4-treated mice. *A*, Untreated BALB/c (wild-type (WT)) mice or pR1E4-treated (Sec) mice were challenged with 100 μ g of activating anti-IgE mAb EM95 and rectal temperatures were measured over 60 min. *B*, Mice received 0.5 mg of Fc-blocking mAb 2.4G2 twenty-four hours before challenge with 100 μ g of anti-IgE EM95. Untreated BALB/c (WT + Fc), pR1E4 treated (Sec + Fc). *C*, *Fc ϵ RI α* ^{-/-} mice received 10 μ g of TNP-BSA and 100 μ g of anti-TNP IgE (IgELa) 1 h before challenge with 50 μ g of rR1E4. Shown are means and SDs of the results obtained with the following numbers of mice per group. All groups included three mice, except pR1E4-treated mice in *A* which included four mice.

B220^{low}IgE⁺ fraction declined in pR1E4-treated mice ($p = 0.0024$; Fig. 3*F*). Intracellular staining for IgE (preblocked for surface staining) was conducted, revealing that plasma cells remained in the spleens of pR1E4-treated mice (Fig. 3*E*). Total numbers of B220^{low}/intracellular IgE⁺ cells were on average lower in pR1E4-treated mice compared with control, but not to a statistically significant extent. We conclude that in anti-IgD- stimulated mice pR1E4 could neutralize circulating IgE and suppress the numbers of mIgE⁺ B cells, but could not suppress fully developed IgE plasma cells.

Anti-IgE can block IgE secretion in vitro

To determine mechanistically how soluble rR1E4 affects IgE B cells, we assessed the effects of R1E4 mAb on cultured naive B cells induced to switch to IgE by the addition of IL-4 and anti-CD40. Although the binding of R1E4 has been mapped to the ϵ CH3 domain (9), it was not clear whether this epitope would be accessible on mIgE⁺ B cells because on B cells mIg is oriented differently than in solution and is associated with Ig α / β signal transducers. We found that mIgE⁺ B cells could be clearly identified with both R1E4 IgG and rR1E4 Ab (Fig. 4*A*). Moreover, inclusion of rR1E4 both inhibited the secretion of IgE in the su-

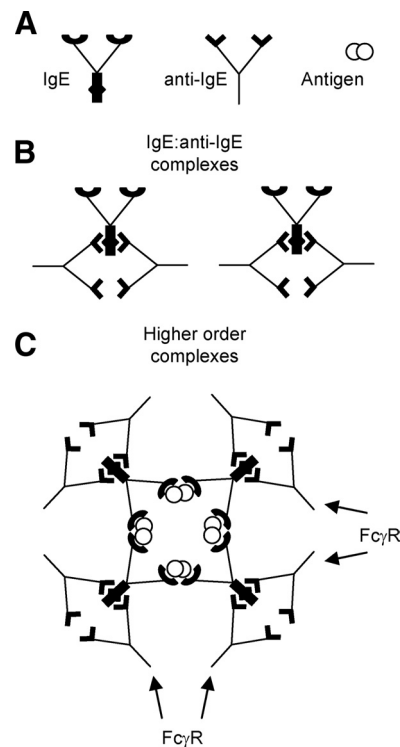


FIGURE 6. Schematic of how IgE-anti-IgE complexes may promote Fc γ R activation. *A*, Depicted are IgE, anti-IgE, and a bivalent Ag. *B*, IgG anti-IgE Abs such as Xolair carry Fc portions able to interact with Fc γ Rs. Upon interaction with IgE, anti-IgE may form small complexes that block binding of IgE to Fc ϵ RI and that are of too low valency to bind strongly to Fc γ Rs. *C*, Higher order complexes of IgE:anti-IgE formed by cross-linking with an additional ligand, such as the IgE's cognate Ag (allergen) shown here, permits multipoint binding to Fc γ Rs.

pernatant and also reduced the frequency of CD138⁺ and CD138⁻IgE⁺ B cells emerging in the cultures (Fig. 4, *B–D*). Ca²⁺ signaling analysis indicated that rR1E4 Ab could induce Ca²⁺ mobilization in mIgE⁺ B cells upon supercross-linking by a second Ab (rat anti-mouse IgG1; Fig. 4*E*). These data indicate that anti-IgE can block the development of IgE plasma cells in vitro and may in part explain the in vivo effects of this treatment.

Anaphylaxis reaction

The ability of in vivo pR1E4 treatment to suppress allergic responses was tested by challenging mice on day 13 after treatment with an activating anti-IgE, EM95, which has the ability to induce mast cell degranulation and anaphylactic manifestations. Surprisingly, and notwithstanding their suppressed IgE levels, pR1E4-treated mice challenged in this way showed a systemic anaphylaxis reaction as indicated by rapidly lowered body temperature (Fig. 5*A*). Recently, an alternative pathway for anaphylaxis has been elucidated in which IgG1-immune complexes activate Fc γ RIII on basophils, releasing platelet-activating factor (15, 24–27). To test the possibility that this pathway was triggered by clustering of anti-IgE Abs upon challenge, pR1E4-treated or control mice were given Fc γ RIII/III-blocking mAb 2.4G2 (28) 1 day before challenge. Indeed, anaphylaxis was blocked in pR1E4-treated but not in control mice receiving 2.4G2 (Fig. 5*B*). To test whether mouse IgG1 R1E4 could induce anaphylaxis directly in a Fc ϵ RI-independent manner, we preinjected TNP-BSA and IgE anti-TNP into *Fc ϵ RI α* ^{-/-} mice and challenged them 1 h later with rR1E4. As shown in Fig. 5*C*, mice that received rR1E4 manifested an anaphylaxis reaction, indicating that rR1E4 itself triggered the reaction through Fc γ Rs. Collectively, these data indicate that pR1E4

treatment suppresses Fc ϵ RI-mediated anaphylaxis, but permits and in fact promotes Fc γ RIII-mediated anaphylaxis.

Discussion

Although it has been known that anti-IgE Abs that block IgE-Fc ϵ RI interactions prevent acute allergic reactions, it has not been clear that such Abs have strong effects on mIgE⁺ B cells nor that a gene therapy approach to their application would be possible. Our data support earlier work of Haba and Nisonoff (6) who demonstrated that high doses of syngeneic anti-IgE could reduce IgE Ab-forming responses when given just before immunization. We show here that expression of an anti-IgE single-chain fusion construct can drive prolonged expression of a presumably dimeric anti-IgE protein in vivo and that this recombinant anti-IgE can block IgE binding to mast cells and basophils and suppress new IgE production. Because rR1E4 Ab was able to bind to mIgE⁺ B cells in vitro and to trigger an altered in vitro response of these cells, we conclude that it can trigger signals in mIgE⁺ B cells. Omalizumab (Xolair) neutralizes circulating IgE in humans, providing relief from allergic symptoms, apparently without reducing the underlying IgE stimulus. In a human trial of Xolair, the serum concentration of free IgE dropped rapidly (to 13.9 ng/ml), whereas the total IgE concentration increased over time (to >1000 ng/ml for 120 days) (29). By contrast, in the present study pR1E4 treatment suppressed both free IgE and total IgE and RNA analysis indicated that it also suppressed new IgE synthesis. These results may suggest that Xolair fails to suppress mIgE⁺ B cells. If this is the case, our data suggest the possibility that more effective anti-IgE mAbs may be found with the potential for longer-term benefit. Such Abs would ideally share Xolair's ability to block IgE-Fc ϵ RI interactions, but also be able to suppress IgE⁺ B cells. Our preliminary studies indicate that Xolair is able to trigger Ca²⁺ mobilization in a B cell line carrying human mIgE. However, it may be that Xolair promotes rather than inhibits IgE production because of the quality of this signal or through its ability to generate high-order immune complexes.

We presume that in vivo expression of rR1E4 mediates negative regulation of developing memory IgE B cells, probably by induction of apoptosis. However, the numbers of mIgE⁺ B cells were too low to directly demonstrate this. Indeed, there is some controversy over whether mIgE⁺ B cells stably exist in vivo (5). Several studies demonstrate that IgE class switching often occurs through an IgG1 intermediate (30–33). On the other hand, there are clear indications that B cells giving rise to IgE responses must carry mIgE and functionally signal through mIgE, at least for a short time. The membrane exons and protein sequences of mammalian IgEs are well conserved and similar to other membrane Igs (34). Moreover, mutations or truncations of the membrane exons of IgE severely inhibit IgE responses, particularly secondary responses (7). The membrane form of ϵ H chain mRNA may be poorly expressed owing to inefficient polyadenylation signals (35), suggesting that IgE memory B cells may express relatively low mIgE⁺ levels. Nevertheless, Abs directed to the membrane form of IgE can have toleragenic effects (8). In the present studies, we detected mIgE⁺ cells and showed that their numbers increased in appropriately immunized mice. Moreover, mIgE⁺ cells were specifically reduced in pR1E4-treated mice (Fig. 3C), indicating that negative regulation of developing IgE B cells by IgE-reactive ligands is possible.

The potency of IgE in allergic reactions is a result of the extraordinarily high affinity of Fc ϵ RI for monomeric IgE ($K_a = 10^{10}$ M⁻¹) combined with the powerful biological consequences of Fc ϵ RI ligation on mast cells and basophils (2). But the levels of IgE in blood were very low compared with other types of Igs,

facilitating the effectiveness of treatment with passively administered IgE-neutralizing Abs and, as we show here, gene therapy using recombinant anti-IgE plasmid. We expressed the anti-IgE-neutralizing Ab as scFv fusion protein with a naked DNA injection method. The merit of this method is that gene expression is potentially long-lasting and it also permits the expression of membrane-bound proteins in vivo. Importantly, soluble rR1E4 expression lasted >3 wk after plasmid injection and could completely neutralize serum IgE, as measured by the level of free IgE and the levels of IgE bound to mast cells and basophils. Although convenient for proof of principle, plasmid injection provides only transient expression. Other modes of gene transfer, including retroviral transduction, should ultimately be more effective and practical in a clinical setting and may provide permanent IgE suppression. Long-term anti-IgE gene expression is predicted to not only suppress new plasma cell formation but also to neutralize IgE secreted by long-lived plasma cells that are no longer subject to regulation by surface Ig.

An unexpected finding was that even when IgE was neutralized mice still underwent anaphylaxis upon challenge with an activating anti-IgE stimulus, presumably through a Fc γ RIII-mediated pathway triggered by cross-linking IgE-rR1E4 complexes. We also showed that rR1E4 administration induced anaphylaxis in mice with IgE-Ag complexes. A comparable reaction may occur in a small fraction of anti-IgE-treated patients. According to the U.S. Food and Drug Administration (<http://www.fda.gov/Cder/drug/InfoSheets/HCP/omalizumabHCP.htm>), the frequency of anaphylaxis attributed to Xolair use in patients was $\geq 0.2\%$. Of reported cases, 39% occurred after the first dose of Xolair, while only 19% occurred with the second dose, indicating the cause of anaphylaxis was not anti-Id (anti-Xolair) Ab. Xolair is a humanized IgG1 mAb that makes complexes with IgE at various ratios, the dominant complex being a trimer (36). If patients have both IgE and IgG Abs against the same Ag or IgE-bound foreign Ag itself, Xolair infusion may form higher order immune complexes and induce anaphylaxis through clustering of Fc γ RIII (Fig. 6). Conceivably, such reactions may be influenced by polymorphisms in Fc γ RIII and Fc γ RII (37, 38). To prevent anaphylaxis, mutagenesis of therapeutic anti-IgE to prevent Fc γ RIII binding may be desirable. Alternatively, expression of soluble monovalent or membrane-tethered anti-IgE may be used. Overall, our data suggest several ways that anti-IgE therapy can be improved to facilitate safety and longer-term effectiveness. We also show the feasibility of providing the Ab by gene therapy, which may provide a strategy to permanently suppress IgE reactions.

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Disclosures

The authors have no financial conflict of interest.

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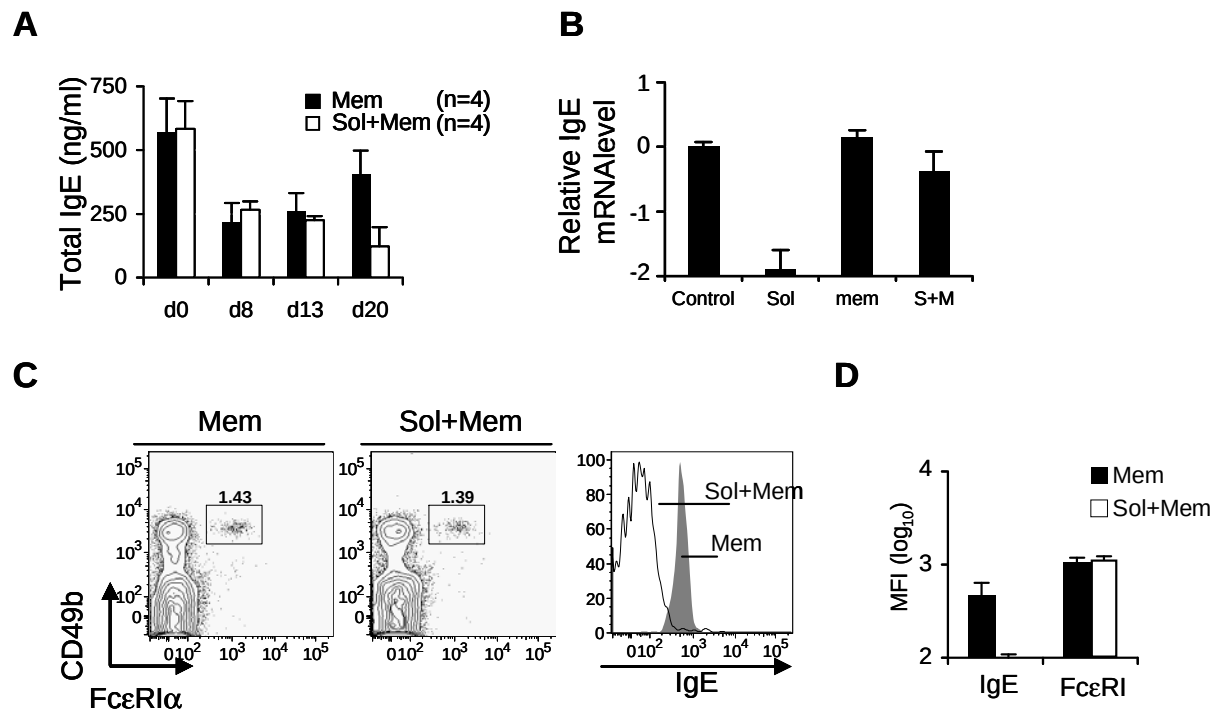
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Light chain
DIVLTQSPAL AVSLGQRATI SCRASQSVSI SRYTLMHWYQ KKPGQRPKVL
               Q       P       L
IYRASNLASG IPARFSGSGS GTDFTLTINP VQADDIATYY CHQSRESPPT
      Linker                      Heavy chain
FGGGTKLELK RGGGGSGGGG SGGGGSQVTL KESGPGILQP SQTLSLTCSF
SGFLLSASSV RVAWIRQPSG KGLEWLATIG WEDVKHYNPS LKSRLTISKD
TSNNQVFLKI SSVDTADTGT YYCAHSSFDQ GTYFDYWGGQ VMVTVSS

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Supplementary Figure 1. Development of recombinant R1E4.

Recombinant R1E4 plasmid scFv amino acid sequence. From R1E4 hybridoma cells, Ig heavy and light chain variable region sequences was determined. Light and heavy chain sequences were fused with (Gly4Ser)₃ linker. To decrease the immunogenicity, 3 amino acids were replaced (to the amino acids shown below).



Supplementary Figure 2. Analysis of the effects of plasmids encoding membrane-bound or secreted forms of R1E4 anti-IgE on markers of IgE expression.

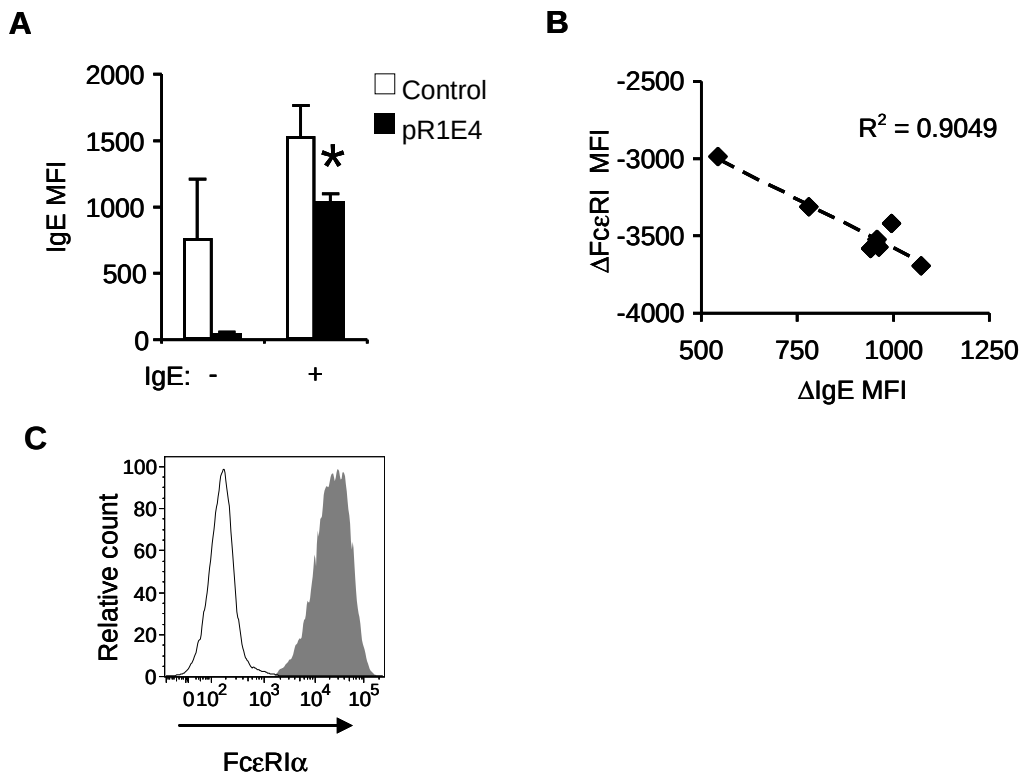
Each mouse was injected with 30 μ g of the indicated plasmids encoding the membrane form of recombinant R1E4 (Mem), the soluble form (Sol), both (Sol+Mem), or a control plasmid lacking scFv insert (Control). Each treatment group consisted of 4 mice.

(A) Total serum IgE levels measured on the indicated days post plasmid injection.

(B) qPCR analysis of the levels of IgE H-chain mRNA in spleens of mice injected with the indicated plasmids 13 days previously.

(C) Representative comparison of IgE and FcεRI levels on splenic basophils of the indicated mice 13 days post plasmid injection.

(D) Mean fluorescence intensity (MFI) of staining for (in vivo bound) IgE or FcεRI on splenic basophils from the indicated mice at day 13 post treatment.



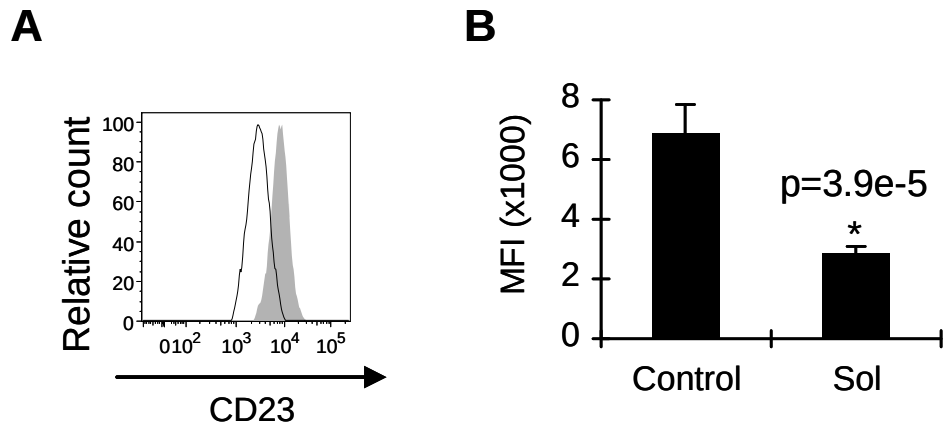
Supplementary Figure 3. Lower Fc ϵ RI expression level on basophils in pR1E4-treated mice and properties of anti-Fc ϵ RI α antibody MAR-1 .

Two month old BALB/c mice were treated with pR1E4 or control plasmid and analyzed 13 days later. In some experiments, harvested bone marrow cells were incubated with free IgE to saturate free Fc ϵ RI as shown in materials and methods. Control (n=3) or pR1E4 (n=4) plasmid treated mouse bone marrow cells were incubated with purified IgE at 10 μ g/ml for 30 minutes on ice and bound IgE assessed. The difference of surface IgE and Fc ϵ RI MFI on basophils between non-saturated and IgE-saturated cells was calculated.

(A) Comparison of surface IgE level on bone marrow basophils with (+) or without (-) exogenous IgE addition.

(B) Surface Fc ϵ RI and IgE level change on bone marrow basophils caused by exogenous IgE addition was quantitated. In the plot shown, each data point represents the value measured in an individual mouse.

(C) Characteristics of anti-Fc ϵ RI α antibody MAR-1. Bone marrow derived mast cells expanded in WEHI3 cell culture supernatant (with IL3 bioactivity) were stained with anti-Fc ϵ RI α antibody after incubation with IgE (10 μ g/ml) (black line). Histogram marked by gray shadow shows binding by MAR-1 in the absence of IgE.



Supplementary Figure 4. CD23 down-regulation on B cells of pR1E4-treated mice.

(A) CD23 expression level was compared between Ova/alum immunized mice receiving either pR1E4 (black line) or control plasmid (gray fill).

(B) Analysis of CD23 staining in each group. Note: staining was carried out in the presence of 1 mM EDTA to strip bound IgE.