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# Vaccine-Induced Anti-IgE Antibodies Neutralize Free IgE but Fail to Bind and Activate Mast Cell-Displayed IgE

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## ABSTRACT

**Background:** In Type I hypersensitivity, IgE is the antibody that contributes to the onset of anaphylaxis through the activation of effector cells, causing the release of allergic mediators. Blocking IgE activity with anti-IgE autoantibodies induced by active immunization could potentially offer an efficient and cost-effective method to treat allergic disorders. We recently developed an effective and safe anti-IgE vaccine in mice, but the mechanisms by which the vaccine protects and the explanation for its safety remained largely unknown. Here, our objective was to better understand those mechanisms.

**Methods:** $CuMV_{TT}$-Cε3-Cε4 is a virus-like particles (VLP)-based vaccine designed to target domains 3 and 4 of IgE. Here, we studied the role of FcγRIIb and CD23 in vaccine-mediated protection from allergy using FcγRIIb KO and CD23 KO mice. Moreover, vaccine-induced IgGs were purified for passive immunization studies and for in vitro experiments with murine bone marrow-derived mast cells (BMMCs).

**Results:** Immunized mice generated high titers of IgG antibodies specific for IgE that conferred protection against allergic responses, independently of FcγRIIb and CD23. Sera of immunized mice blocked IgE binding to BMMC FcεRI and suppressed degranulation, independent of FcγRIIb. Purified anti-IgE antibodies did not provoke an anaphylactic response when transferred into allergic mice but protected against subsequent allergen challenge. Interestingly, the purified IgG antibodies were unable to recognize receptor-bound IgE in BMMCs, explained by the finding that recognition by these anti-IgEs depends on the same mannose moieties on IgE that are essential for FcεRI binding and thus hidden when receptor-bound.

**Conclusions:** In conclusion, the $CuMV_{TT}$-Cε3-Cε4 vaccine induces high titers of anti-IgE IgGs that confer protection by neutralizing IgE through its glycan epitopes, without causing FcεRI cross-linking. Our results imply that an anti-IgE vaccine may represent a promising therapeutic strategy, offering effective protection while minimizing the risk of adverse reactions similar to the monoclonal antibody omalizumab.

**Abbreviations:** Fc, fragment crystallizable; FcεRI, Fc epsilon receptor I; Ig, immunoglobulin E; VLP, virus-like particle.

## 1 | Introduction

Type I hypersensitivity reactions are primarily mediated by IgE antibodies [1, 2]. Both mast cells and basophils become sensitized by IgE when the high-affinity receptor, FcεRI, binds IgE with nanomolar affinity. Allergic reactions occur as a result of cross-linking of the receptor-bound IgE by allergens [3, 4]. This results in cellular degranulation and the release of both preformed and newly synthesized inflammatory mediators [5, 6]. It has been shown that IgE levels can be controlled, among other factors, by the IgE Fc receptors FcεRI and FcεRII (CD23). CD23, known as the low-affinity IgE receptor, is mainly expressed on B cells. CD23 plays an important role in the regulation of IgE levels, while its precise function in allergy is still not completely understood [7, 8]. It has been shown previously that CD23 provides a non-inflammatory pathway of IgE elimination upon binding of IgE-allergen immune complexes to CD23 [9].

An important therapeutic approach for IgE-mediated diseases relies on the use of anti-IgE antibodies. Omalizumab, a monoclonal anti-IgE antibody, which has been approved for treating allergic conditions like moderate to severe persistent allergic asthma, chronic idiopathic urticaria, and nasal polyps, has demonstrated clinical effectiveness in managing allergic diseases [10–12]. In terms of mechanism, omalizumab mainly functions by neutralizing free serum IgE, but it also disrupts IgE-FcεRI complexes. Consequently, over time, fewer IgE molecules are bound on effector cells, and IgE-dependent cellular activation will therefore be inhibited [13, 14].

Interestingly, anti-IgE IgG antibodies can also be produced naturally by the body's immune system. Both atopic and healthy individuals have been found to express these endogenous anti-IgE IgG autoantibodies, suggesting another potential mechanism for the regulation of IgE [15, 16]. Some of these anti-IgE IgG autoantibodies can activate effector cells by cross-linking FcεRI-bound IgE, plausibly leading to an anaphylactic reaction [17]. Other such antibodies may prevent Type I allergic reactions by either reducing IgE antibody levels [18] or preventing basophil sensitization [19].

These anti-IgE IgG autoantibodies can impede the activation of basophils and/or mast cells by at least three distinct pathways: (i) direct IgE neutralization as the most straightforward mechanism, by blocking IgE binding to FcεRI, thus preventing sensitization of the cells, (ii) disrupting receptor-bound IgE as was shown for omalizumab, and (iii) engagement of IgG antibodies with the inhibitory FcγRIIb, suppressing FcεRI activation and promoting IgE internalization by mast cells.

We have recently developed a VLP-based anti-IgE vaccine that suppresses allergy by reducing serum IgE levels [20]. Surprisingly, high titers of anti-IgE antibodies were well tolerated by the allergic mice, without any apparent side effects. The reason why these antibodies fail to activate FcεRI is poorly understood. Potential mechanisms could be the targeting of IgE and IgG-IgE complexes to non-inflammatory receptors such as CD23 and/or FcγRIIb, or by competition between anti-IgE and FcεRI for IgE binding [21]. In the current study, we performed in vivo and in vitro studies in transgenic mice lacking CD23 or FcγRIIb to elucidate the mechanism of action of the anti-IgE antibodies induced by our VLP-based anti-IgE candidate vaccine and delineate IgE neutralization as the key pathway for protection and the failure of recognizing FcεRI as a key mechanism for safety.

## 2 | Material and Methods

### 2.1 | Generation of $CuMV_{TT}$-Cε3-Cε4 Vaccine

Mouse IgE-Fc fragment consisting of Cε3-Cε4 was conjugated to $CuMV_{TT}$ using the cross-linker SMPH (Succinimidyl 6-((beta-maleimidopropionamido) hexanoate)), (Thermo Fisher Scientific, Waltham, MA, USA) at a 10 M excess to $CuMV_{TT}$ for 30 min at 25°C. $CuMV_{TT}$ and Cε proteins were coupled at a molar ratio of 1:1 by shaking at 25°C for 3 h at 400 rpm on a DSG Titertek (Flow Laboratories, Irvine, UK). Any residual SMPH and Cε proteins were eliminated using Amicon Ultra 0.5, 100 K (Merck Millipore, Burlington, MA, USA).

### 2.2 | Mouse Immunization and Sampling

Mice aged between 8 and 12 weeks were housed at the central animal facility in Murtenstrasse 31, Bern, Switzerland, for experimentation. BALB/c mice, obtained from Envigo (Envigo, Huntingdon, UK), and CD23−/− mice, graciously provided by Prof. J. Ravetch, along with FcRIIb −/− mice (Jackson Laboratory, Bar Harbor, Maine, MA, USA) purchased at 6 weeks and subsequently bred in our facility, were used in the study. The treatment protocols for these experimental subjects were approved by the Swiss Federal Veterinary Office. All animals were allowed a minimum acclimatization period of 1 week in the facility. Mice were injected subcutaneously (s.c.) with either 5 μg $CuMV_{TT}$-Cε3-Cε4 vaccine or 5 μg $CuMV_{TT}$ as the control. For boosting purposes identical doses were administered on Days 14 and 28 and the mice were bled every other week via tail veins until Day 42. To measure IgG anti-IgE levels, serum was isolated using Microtainer Tube (BD Biosciences, USA).

### 2.3 | ELISA Assay for Investigating Serum Anti-IgE IgG

ELISA plates (CORNING half area 96-well, Costar, USA) were coated with 2 μg/mL mouse IgE antibody in PBS and left overnight at 4°C. After blocking with PBS/0.15% Casein solution for 2 h at room temperature, the plates were washed 5 times with PBS. Incubation of sera was performed for 1 h at RT. Afterward, the plates were washed 5 times with PBS/0.05% Tween, and HRP-labeled goat anti-mouse IgG (Jackson Laboratory) was added for 1 h at RT. Finally, the plates were developed with tetramethyl benzidine (TMB) in citrate buffer, stopped with 1 M H2SO4 solution, and OD values were recorded at a wavelength 450 nm.

Mannose-deficient F1 IgE was generated using the native protein deglycosylation kit (Sigma) according to the

manufacturer's protocol. To test the ability of purified IgG from the sera of mice immunized with CuMVTT-Cε3-Cε4 to recognize IgE glycans, three ELISA assays were performed. In the first ELISA setup, ELISA plates were coated with 2 μg/mL of either mouse wild-type (non-deglycosylated, WT) IgE or F1 (removal of mannose) treated IgE. The plates were then incubated with purified antibody preparation for 1 h at RT after blocking and washing. In the next assays, ELISA plates were coated with 2 μg/mL of mouse WT IgE. In the second assay, the plates were incubated with either mannose-binding *Galanthus Nivalis* lectin (GNL, vector laboratories) or as a control ovalbumin (Sigma) at different concentrations before adding purified IgG. In the third assay, the plates were incubated with different concentrations of WT IgE or F1-treated IgE before adding purified IgG. All assays were developed with HRP labeled goat anti-mouse IgG (Jackson Laboratory) as described above.

### 2.4 | Passive Sensitization and Challenge

Before allergen challenge, passive sensitization was carried out on either immunized or control mice groups with an intravenous (i.v.) injection of 6 μg IgE F127 (house-made recombinant monoclonal anti-Fel d 1 antibody). The following day, their baseline body temperature was recorded using a miniTemp rectal probe designed for mice (Vetronic Services Ltd., Devon, United Kingdom). These IgE-sensitized mice were subsequently challenged through an i.v. injection of 3 μg Fel d 1 (major cat allergen) diluted in PBS. Their rectal temperature was then monitored every 10 min for a duration of 1 h.

### 2.5 | Active Sensitization and Passive Immunization

To achieve active sensitization, naïve Balb/c mice were administered an intraperitoneal (i.p.) injection of 0.3 μg of Fel d 1, combined with 200 μL of 10 mg/mL Al (OH)3, also known as Alhydrogel or Alum (InvivoGen, San Diego, Calif). To assess the anaphylactogenic potential of the serum purified IgGs, these allergic mice were injected i.v. either with 200 μg or a higher dose (400 μg) of purified total serum IgG, obtained from $CuMV_{TT}$-Cε3-Cε4 vaccinated or $CuMV_{TT}$ control mice sera. The method for measuring core body temperature remained the same as described above.

### 2.6 | Flow Cytometry for BMMCs Experiments

To assess whether IgG anti-IgE antibodies generated by vaccination are able to block mast cell activation upon allergen challenge, murine bone marrow cells were isolated from femur and tibia bones. Bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate (Gibco, ThermoFisher Scientific, MA, USA), 2.5 μg/mL amphotericin B (Sigma-Aldrich) and 1 mM penicillin/streptomycin. This medium was additionally supplemented with 30 ng/mL IL-3 and 50 ng/mL SCF (stem cell factor) as murine-derived mast cell growth factors. The cell culture flasks were maintained at 37°C in 5% $CO_2$ for 4 wks, and differentiation of BMMCs was confirmed by flow cytometry after staining with anti-mouse stem cell factor receptor (c-KIT) CD117 and FcεRI antibodies. To check in vitro anti-IgE inhibition upon allergen challenge, 500 ng/mL mouse IgE F127 was added to the sera of either $CuMV_{TT}$-Cε3-Cε4 immunized or $CuMV_{TT}$ control mice after 42 d. The mixture was then incubated with BMMCs for 1 h at 37°C in 5% $CO_2$. IgE binding to the cell surface was assessed by collecting the cells at this stage and staining for PE anti-mouse IgE (BioLegend). To assess degranulation, the cells were washed and incubated with 50 ng/mL Fel d 1 diluted in PBS for 30 min at 37°C in 5% $CO_2$. The cells were washed and stained with anti-mouse-CD63 conjugated with Vio Bright FITC (Miltenyi Biotec) for 15 min at 37°C in 5% $CO_2$. Eventually, the cells were washed, and degranulation was analyzed by flow cytometry with CytoFLEX S 4 L 13C (B2-R3-V4-Y4) plus 96 DW plate loader (Beckman Coulter Life Sciences, CA, USA) and using FLOWJO software (TreeStar Inc., Ashland, OR, USA).

To test the anaphylactogenicity of the purified IgG from the sera of mice immunized with $CuMV_{TT}$-Cε3-Cε4 or $CuMV_{TT}$ after 42 d, BMMCs were incubated with the antibody preparations for 30 min at 37°C in 5% $CO_2$. The cells were then washed and stained with anti-mouse CD63 conjugated as described above.

To assess the capacity of the purified IgGs to recognize cell surface-bound IgE, BMMCs were preloaded overnight with IgE at a concentration of 2 μg/mL. The cells were then washed and incubated with purified IgG at a concentration of 20ug/ml for 30 min either at 4°C or 37°C in 5% $CO_2$. As a control, the cells were incubated with 500 ng/mL polyclonal anti-IgE (STAR10). Following incubation, the cells were washed and stained with labeled anti-mouse IgE (BD Biosciences), anti-mouse IgG (Thermo Fisher), and anti-goat IgG (Jackson) for 15 min at 37°C in 5% $CO_2$. Surface IgE and IgG were analyzed using CytoFLEX S 4 L 13C (B2-R3-V4-Y4) plus 96 DW plate loader (Beckman Coulter Life Sciences, CA, USA) and using FLOWJO software (TreeStar Inc., Ashland, OR, USA).

### 2.7 | IgG Isolation and Purification

Following the instructions provided by the manufacturer, total serum IgG was isolated from 5 mL of pooled serum obtained from vaccinated and/or control mice via terminal bleeding. This was done using protein G column chromatography (HiTrap Protein G HP, 1 mL, Cytiva, Marlborough, MA, USA). Subsequently, both the flowthrough and the eluate were collected. Following that, buffer exchange to PBS was carried out using Vivaspin 6 50 kDa MWCO (Cytiva).

### 2.8 | Statistical Analysis

Data were analyzed and presented as mean ± SEM using unpaired *t*-test, two-way, or one-way ANOVA as mentioned in the figure's legend, with GraphPad PRISM 9 (GraphPad Software Inc., San Diego, CA, USA). The values of $p < 0.05$ were considered statistically significant; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

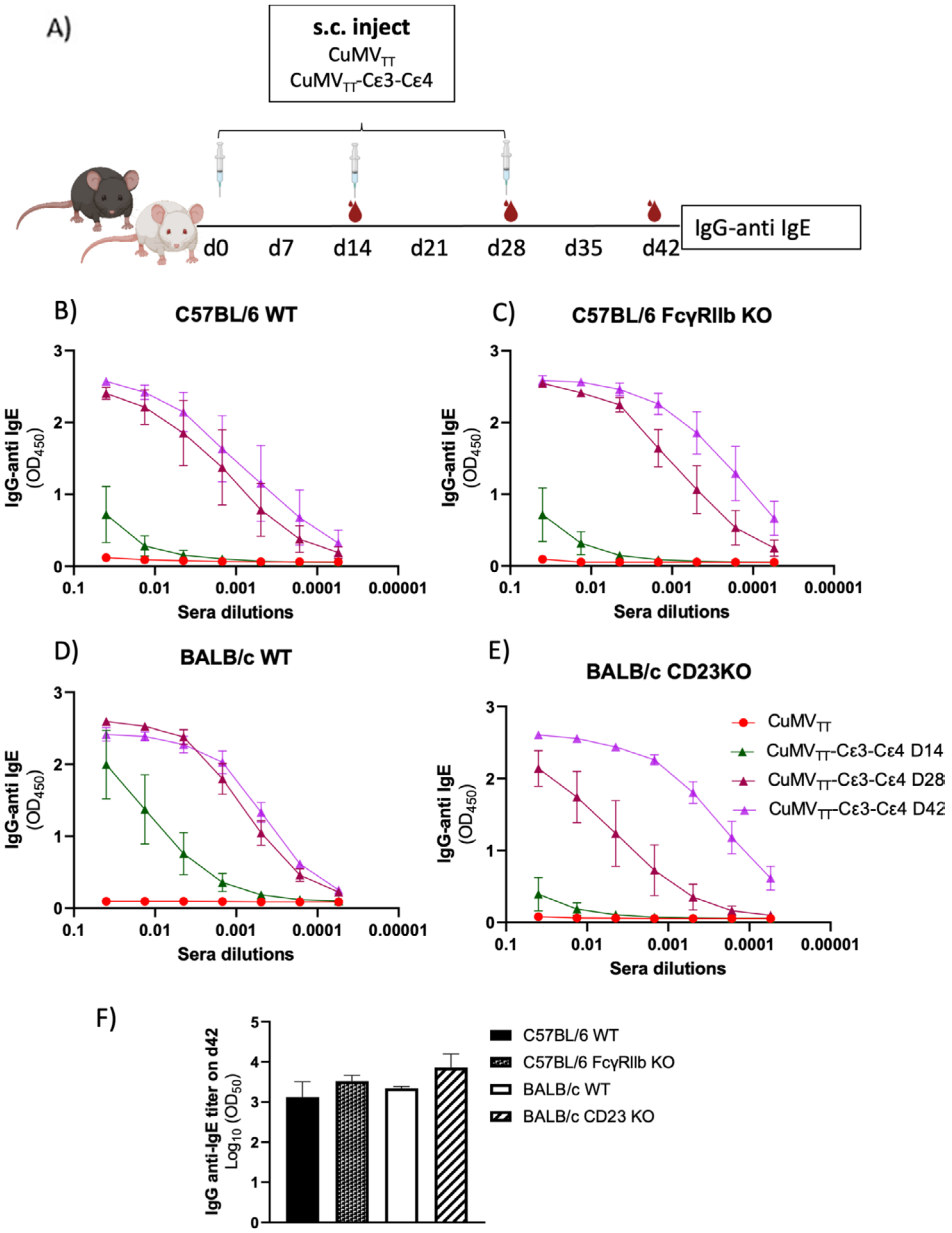


**FIGURE 1** | Immunization with CuMV$_{TT}$-Cε3-Cε4 vaccines induce IgG anti-IgE response. (A) Schematic overview of the immunization regimen and sample collections for ELISA assay. (B–E) IgG anti-IgE response of immunized mice groups measured by ELISA at different time points. (F) Comparison of IgG anti-IgE OD50 titers among all the immunized mouse groups on Day 42.

## 3 | Results

### 3.1 | CuMV$_{TT}$-Cε3-Cε4 Induces Anti-IgE IgG Antibodies Independently of FcγRIIb and CD23

The anti-IgE vaccine candidate CuMV$_{TT}$-Cε3-Cε4 was produced as previously described [20]. We demonstrated earlier that this vaccine downregulates IgE levels and suppresses allergic responses in mice. Additionally, we have shown that CD23 can control the serum level of free IgE and absorb IgE-IgG immune complexes [22]. Similarly, FcγRIIb has the potential to internalize IgE–IgG complexes [23]. Both CD23 and FcγRIIb are also immunomodulatory receptors and could impact the antibody titers generated by vaccination.

Thus, we investigated immune responses induced by our vaccine in wild-type, FcγRIIb KO, and CD23KO mice. The mice were immunized with CuMV$_{TT}$-Cε3-Cε4 vaccine and CuMV$_{TT}$ as a control on Day 0 and then boosted on Days 14 and 28 (Figure 1A). As depicted in Figure 1B–E, all CuMV$_{TT}$-Cε3-Cε4 immunized mice groups generated anti-IgE IgG antibodies. In all mice, IgG anti-IgE titers increased following the first and second boosters. However, after the first booster, fewer anti-IgE antibodies were induced in the CD23KO mice, while FcγRIIb-deficient mice showed increased responses compared to WT. After the second booster, no significant difference between the groups was observed (Figure 1F). As expected, immunization with the CuMV$_{TT}$ alone induced no anti-IgE antibody response. Hence, the induction of anti-IgE

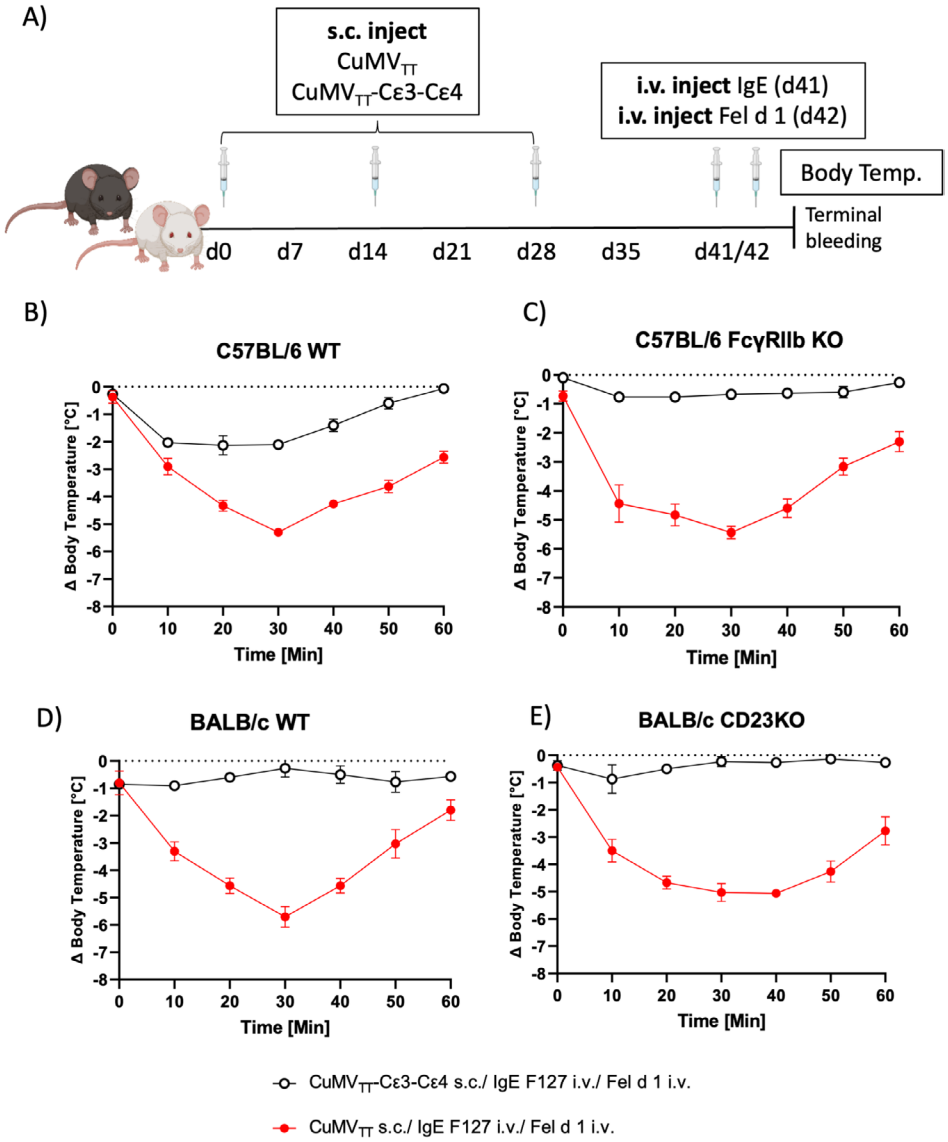


**FIGURE 2** | CuMV$_{TT}$-Cε3-Cε4 immunization protects IgE sensitized mice from systemic anaphylaxis. (A) Schematic overview of immunization regimen and allergen challenge. (B–E) Assessment of core body temperature at 10-min intervals upon challenge with Fel d 1 after passive sensitization with IgE F127.

responses by the CuMV$_{TT}$-Cε3-Cε4 vaccine candidate was only minimally altered in the absence of FcγRIIb and CD23 compared to wild-type mice.

### 3.2 | CuMV$_{TT}$-Cε3-Cε4 Immunization Blocks Anaphylaxis Upon Allergen Challenge

Having shown that CuMV$_{TT}$-Cε3-Cε4 generates equal anti-IgE titers in the different gene-deficient mouse models, we then assessed whether CuMV$_{TT}$-Cε3-Cε4 immunization confers protection against anaphylaxis. To this end, wild-type as well as FcγRIIb KO and CD23KO mice were immunized with CuMV$_{TT}$-Cε3-Cε4 vaccine or CuMV$_{TT}$ and then challenged with Fel d 1 one day after passive sensitization with anti-Fel d 1 IgE.

As shown in Figure 2A, CuMV$_{TT}$-Cε3-Cε4 immunization protected all mouse groups from Fel d 1 challenge, whereas mice injected with CuMV$_{TT}$ showed anaphylactic reaction upon allergen challenge (Figure 2B–E). These data imply that the principal mechanism of protection can occur independently of FcγRIIb and/or CD23. We thus concluded that the most likely mechanism of protection is through the neutralization of free IgE antibodies.

### 3.3 | CuMV$_{TT}$-Cε3-Cε4 Immunization Reduces IgE Binding to Murine BMMCs and Inhibits Degranulation Independently of FcγRIIb

As CuMV$_{TT}$-Cε3-Cε4 suppresses anaphylaxis in vivo, we next aimed to study the mechanism of suppression further by performing functional in vitro tests with serum antibodies derived from CuMV$_{TT}$-Cε3-Cε4 immunized mice. The effect of serum antibodies was tested in bone-marrow-derived mast cells (BMMCs), which express high levels of FcεRI. The BMMCs can be sensitized with recombinant mouse IgE anti-Fel d 1 antibodies and challenged with recombinant Fel d 1 to trigger degranulation [9, 24]. To study the role of FcγRIIb

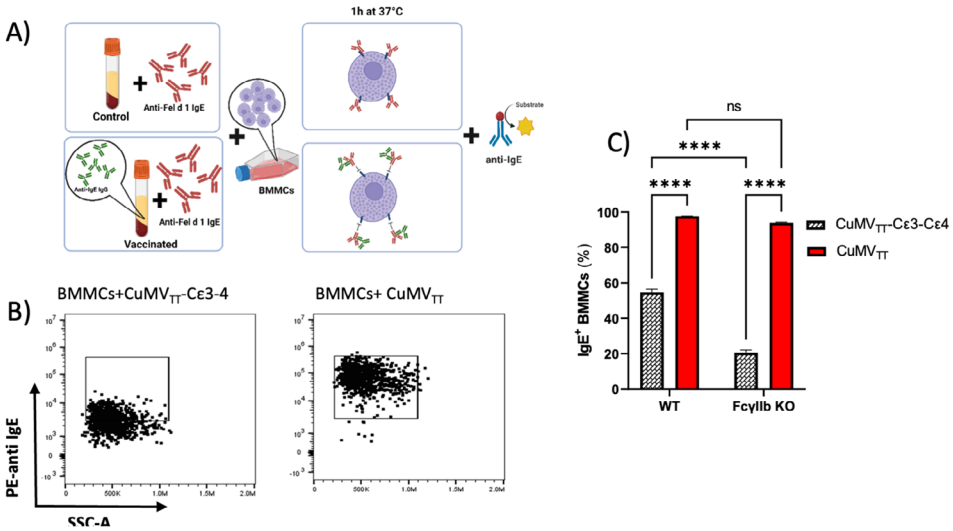


**FIGURE 3** | CuMV$_{TT}$-Cε3-Cε4 immunized sera reduces binding of IgE to mice BMMCs in vitro. (A) Schematic overview of the experiment. (B) Illustration of flow cytometry dot plots showing anti-IgE staining intensity. (C) (mean ± SEM) IgE mean fluorescence intensity (MFI) on BMMCs isolated from C57BL/6 WT and FcγRIIb KO mice. Statistical analysis was performed using two-way ANOVA: 0.0001 (****), $n = 5$, from at least two similar experiments.

in this process, we likewise cultured BMMCs derived from FcγRIIb KO mice.

BMMCs were incubated with a mixture of IgE and sera of mice immunized with CuMV$_{TT}$-Cε3-Cε4 and/or CuMV$_{TT}$ and subsequently stained with anti-IgE antibodies (Figure 3A). As seen in Figure 3B,C, regardless of FcgRIIb-expression, IgE binding was notably lowered on cells treated with sera from CuMV$_{TT}$-Cε3-Cε4 immunized mice, indicating that the anti-IgE IgG antibodies in these sera can block the binding of IgE to BMMCs.

Interestingly, cells lacking FcγRIIb also showed significantly less IgE binding in the presence of anti-IgE serum. Lower FcεRI-displayed IgE densities on BMMCs typically translate to a reduction in degranulation in response to allergen challenge. To confirm this, we incubated murine BMMCs with Fel d 1-specific IgE and the mouse sera as described above, and then challenged the cells with Fel d 1 for triggering FcεRI cross-link and degranulation. We then stained BMMCs for CD63, a marker of cell degranulation (Figure 4A). As depicted in Figure 4B,C, BMMCs derived from both wild-type and FcγRIIb KO mice, when incubated with sera of CuMV$_{TT}$-Cε3-Cε4 immunized mice, showed significantly reduced degranulation. This confirms that the presence of IgE neutralizing anti-IgE antibodies in these sera not only suppresses FcεRI sensitization but likewise reduces BMMC sensitivity to allergen challenge independently of FcγRIIb.

These findings suggest that the anti-IgE antibodies induced by the CuMVTT-Cε3-Cε4 vaccine effectively neutralize IgE binding to FcεRI, thus inhibiting allergic sensitization.

### 3.4 | Purified IgG From CuMV$_{TT}$-Cε3-Cε4 Vaccinated Mice Does Not Induce Anaphylaxis

A main concern related to immunization against IgE is the potential anaphylactic reaction that could be triggered by anti-IgE IgG antibodies due to their ability to bind and cross-link cell-bound IgE. As our vaccine candidate did not induce any symptoms of anaphylaxis during active immunization, we wondered whether purified anti-IgE IgGs had the capacity to induce such an anaphylactic reaction in an acute setting.

To investigate this, we isolated total IgG from pooled sera of immunized and control mice by Protein G chromatography and injected 200 μg of purified IgG antibodies into Fel d 1 allergic mice (Figure 5A). The mice receiving IgGs from both control and immunized mice did not show any signs of anaphylaxis, confirming that the anti-IgE IgG present in the sera of immunized mice does not cross-link or activate FcεRI-bound IgE (Figure 5B). We then assessed whether passive immunization with purified IgG preparations can protect against Fel d 1 challenge, which was indeed the case (Figure 5C,D).

To better understand the non-anaphylactogenicity of the IgG anti-IgE seen above in vivo, we conducted a corresponding experiment in vitro using BMMC with purified IgG rather than serum. We sensitized murine BMMCs with IgE and subsequently challenged them with titrated doses of purified anti-IgE IgGs or with Fel d 1 (Figure 5E). As shown in Figure 5F, purified IgG from mice immunized with CuMVTT-Cε3-Cε4 induced only a minimal degranulation at very high antibody concentrations, whereas Fel d 1 efficiently degranulated the cells at very low concentrations.

As BMMCs derived from FcγRIIb KO mice displayed intrinsic differences to WT BMMCs in terms of overall IgE binding and degranulation, we blocked FcγRIIb in WT BMMCs using a receptor-blocking antibody rather than FcγRIIb-deficient mast cells (Figure 5F). The low level of degranulation was not increased when FcγRIIb was blocked, suggesting that FcγRIIb does not contribute to the prevention of cellular activation.

Hence, purified IgG anti-IgE antibodies from *CuMV$_{TT}$-Cε3-Cε4* vaccinated mice only trigger minimal levels of degranulation

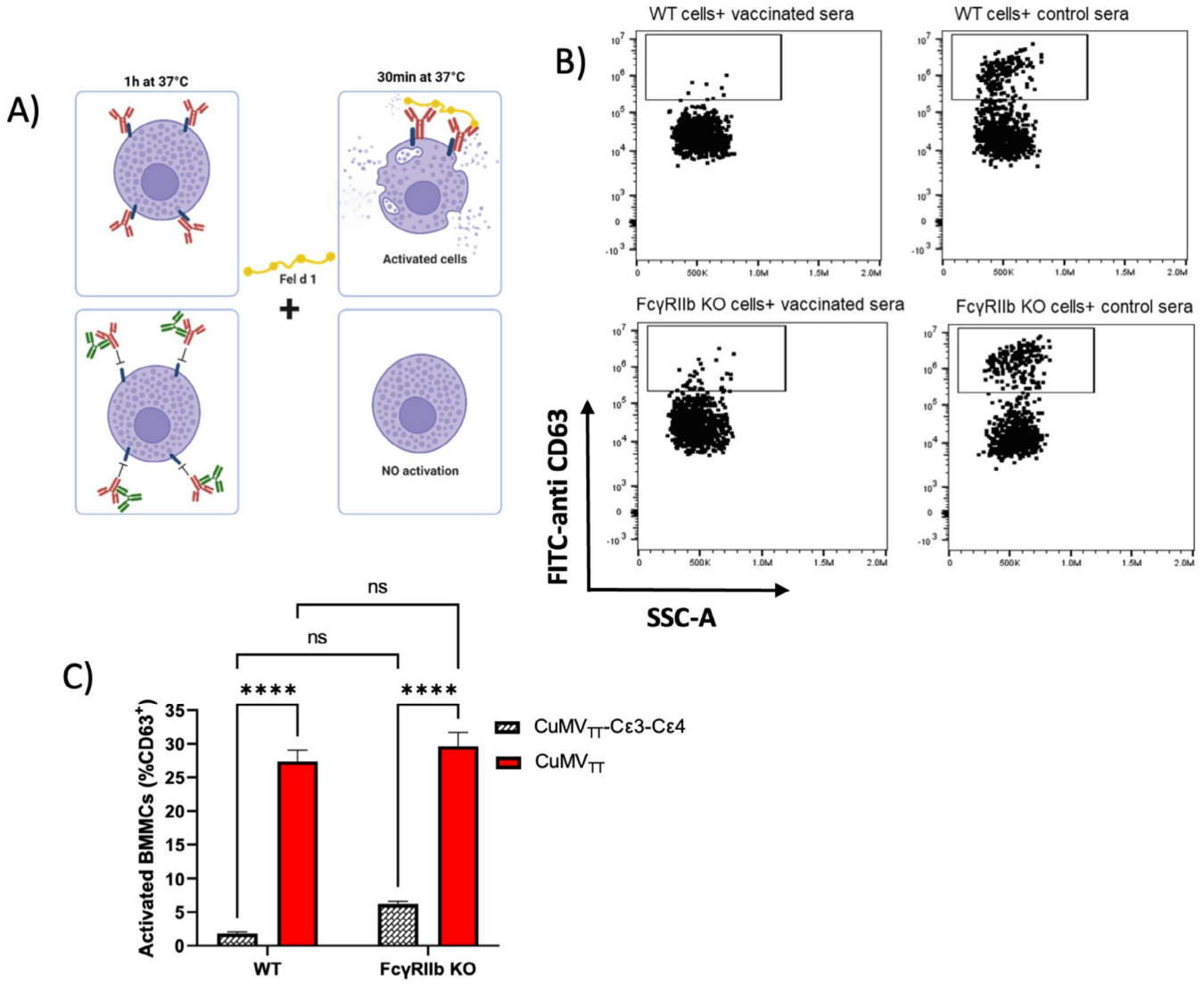


**FIGURE 4** | $CuMV_{TT}$-Cε3-Cε4 immunized sera diminishes activation and degranulation of mice BMMCs upon allergen challenge in vitro. (A) Schematic overview of the experiment. (B) Illustration of flow cytometry dot plots showing anti-CD63 staining intensity. (C) (mean ± SEM) $CD63^+$ BMMCs isolated from C57BL/6 WT and FcγRIIb KO mice. Statistical analysis was performed using two-way ANOVA: 0.0001 (****), ns: Not significant, $n = 5$, from at least two similar experiments.

compared to allergens. This lack of anaphylactogenicity is not due to FcγRIIb engagement.

### 3.5 | Purified IgGs From $CuMV_{TT}$-Cε3-Cε4 Immunized Mice Sera Recognize Glycans on IgE

Having shown that protection by antibodies derived from CuMVTT-Cε3-Cε4 vaccines occurs independently of FcγRIIb, we hypothesized that a direct competition for the same IgE epitope between anti-IgE antibodies and FcεRI could explain the lack of FcεRI cross-linking via anti-IgE antibodies. We previously observed that glycan structures, specifically a single conserved mannose region on IgE, are an immunodominant epitope for natural anti-IgE autoantibodies [25]. The same conserved mannose region is indispensable for FcεRI binding and is involved in the binding of omalizumab [26].

We therefore investigated whether CuMVTT-Cε3-Cε4 induced antibodies, like natural anti-IgE autoantibodies, have a similar tendency to target those glycan structures on IgE. We conducted three ELISA assays to assess the direct binding of purified IgGs to mannose, using untreated wild-type IgE or mannose-deficient (demannosylated by F1- treatment) IgE (Figure 6A–C). As shown in Figure 6A, mannose-deficient (F1) IgE was not recognized by vaccine-induced IgG. Moreover, the mannose binding lectin *Galanthus Nivalis* lectin (GNL) blocked IgG binding to wild-type IgE (Figure 6B). Finally, mannose-deficient (F1) IgE failed to block IgG binding to wild-type IgE displayed on ELISA plates (Figure 6C). This suggests that purified IgG primarily recognizes the single conserved mannose region on IgE that is essential for FcεRI binding. This competition for the same IgE mannose region could ensure both FcεRI-blockade for free IgE and absence of FcεRI-bound IgE cross-linking reflecting the specificity of Omalizumab.

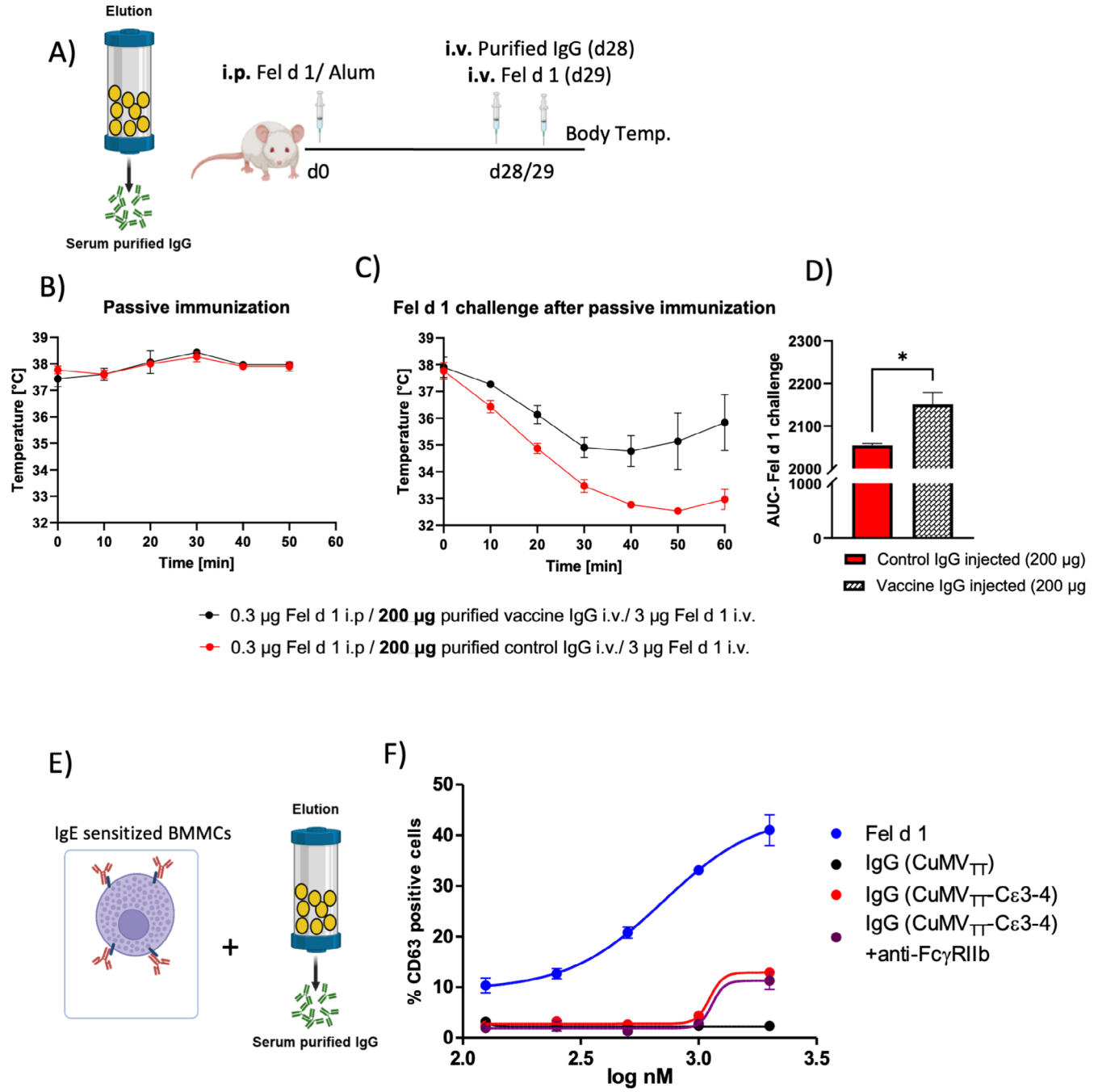


**FIGURE 5** | Purified IgGs from CuMV$_{TT}$-Cε3-Cε4 immunized mice do not induce anaphylaxis. (A) Schematic overviews of active sensitization with Fel d 1 and passive immunization with purified IgGs (vaccinated and/or control) and allergen challenge. (B) shows no drop in core body temperature at 10-min intervals upon passive immunization with 200 µg/mL serum purified IgGs. (C, D) Measurement of temperature changes upon Fel d 1 challenge in the allergic mice which are passively immunized. (E) IgE sensitized BMMCs were incubated with purified IgG from CuMV$_{TT}$-Cε3-Cε4 vaccinated or CuMV$_{TT}$ control mice sera or Fel d 1 as positive control. (F) Shown is the mean ± SEM of CD63+ BMMCs. Statistical analysis was performed (mean ± SEM) using unpaired *t* test: $p < 0.05$ (*), ns: Not significant, $n = 3$/group.

### 3.6 | Purified IgGs From CuMV$_{TT}$-Cε3-Cε4 Do Not Recognize FceRI-Displayed IgE

The mannose glycan has been shown to be crucial for binding to FcεRI [26]. After demonstrating that purified IgG recognizes mannose, we questioned whether these IgGs could still recognize IgE when it is already bound to FcεRI. To investigate this, we conducted flow cytometry experiments to examine the binding of purified IgGs at 4°C and 37°C to BMMCs preloaded with IgE. As shown in Figure 7C, at both temperatures, the purified IgGs were unable to bind to surface-bound IgE. In contrast, a commercially available polyclonal anti-IgE showed clear binding to the cells in both conditions.

In a next step, we tested if surface IgE staining is altered by the presence of purified IgGs, which may occur through IgE removal, IgE internalization, or simply competition with the staining antibodies. The purified IgGs were unable to alter IgE surface levels,

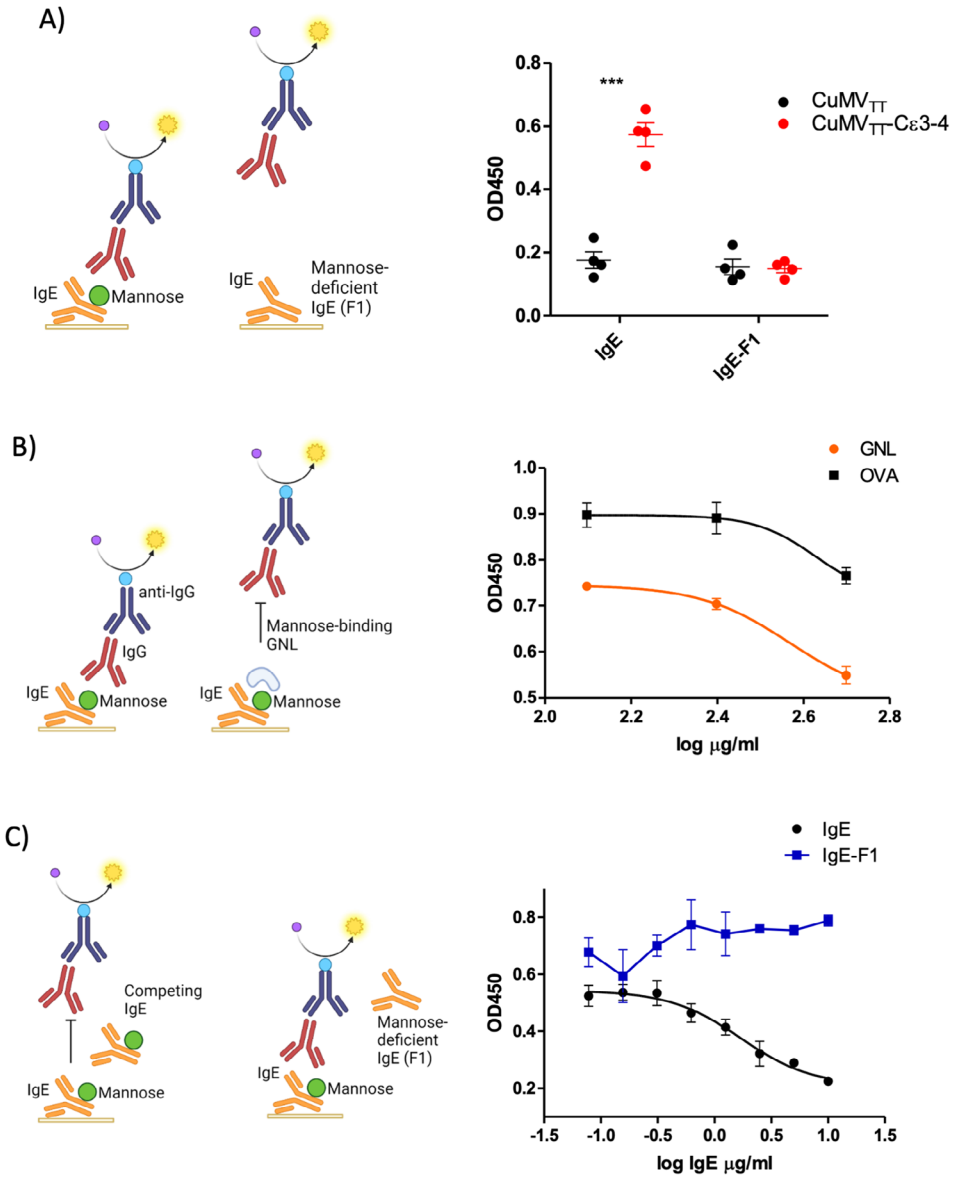


**FIGURE 6** | Purified IgGs from CuMV$_{TT}$-Cε3-Cε4 recognize IgE-mannose. Purified IgG was characterized by ELISA. (A) Shown is the ELISA setup to detect mannose-specific IgG (left panel) and mean ± SEM OD450 values (right panel). (B) Shown is the ELISA setup to block IgG binding using mannose-binding *Galanthus Nivalis* Lectin (GNL) and mean ± SEM OD450 values. (C) Shown is the ELISA setup to block IgG binding using WT IgE or mannose-deficient IgE (F1) to block IgG binding and mean ± SEM OD450 values.

unlike the commercially available control anti-IgE antibodies (Figure 7D,E), which induced a reduction of surface IgE levels.

These results confirm that the purified IgGs do not recognize IgE once it is bound to the cell, which is in line with the absence of allergic reactions when sensitized effector cells are challenged with anti-IgE IgGs both in vitro and in vivo. Moreover, these results highlight the importance of IgE-mannose for the recognition of IgE by vaccine-induced IgGs. Hence, mannose competition is an explanation for why vaccine-induced IgGs are well tolerated and fail to cause anaphylaxis.

## 4 | Discussion

An IgE-targeted immunotherapy aims to prevent or block the ongoing allergic response. Unlike traditional desensitization therapy, which is allergen-specific and requires identifying specific allergens like pollens, dust mites, or molds, this approach does not depend on allergen identification. We previously developed a VLP-based anti-IgE vaccine that induces a polyclonal IgG anti-IgE response [20]. Here, we elucidated the mechanisms that underlie its protective effects. Allergen-specific desensitization has been reported to induce, in addition to regulatory B and T cells [27, 28], allergen-specific IgGs, which function via allergen-neutralization and engaging FcγRIIb [27, 29–31].

For vaccine-induced anti-IgE antibodies, since FcγRIIb KO mice display a similar level of protection as the wild-type (WT) mice, we conclude here that the primary protective mechanism operates through the neutralization of free serum IgE. This conclusion was sustained by in vitro experiments in which sera from the CuMV$_{TT}$-Cε3-Cε4 immunized mice were able to neutralize

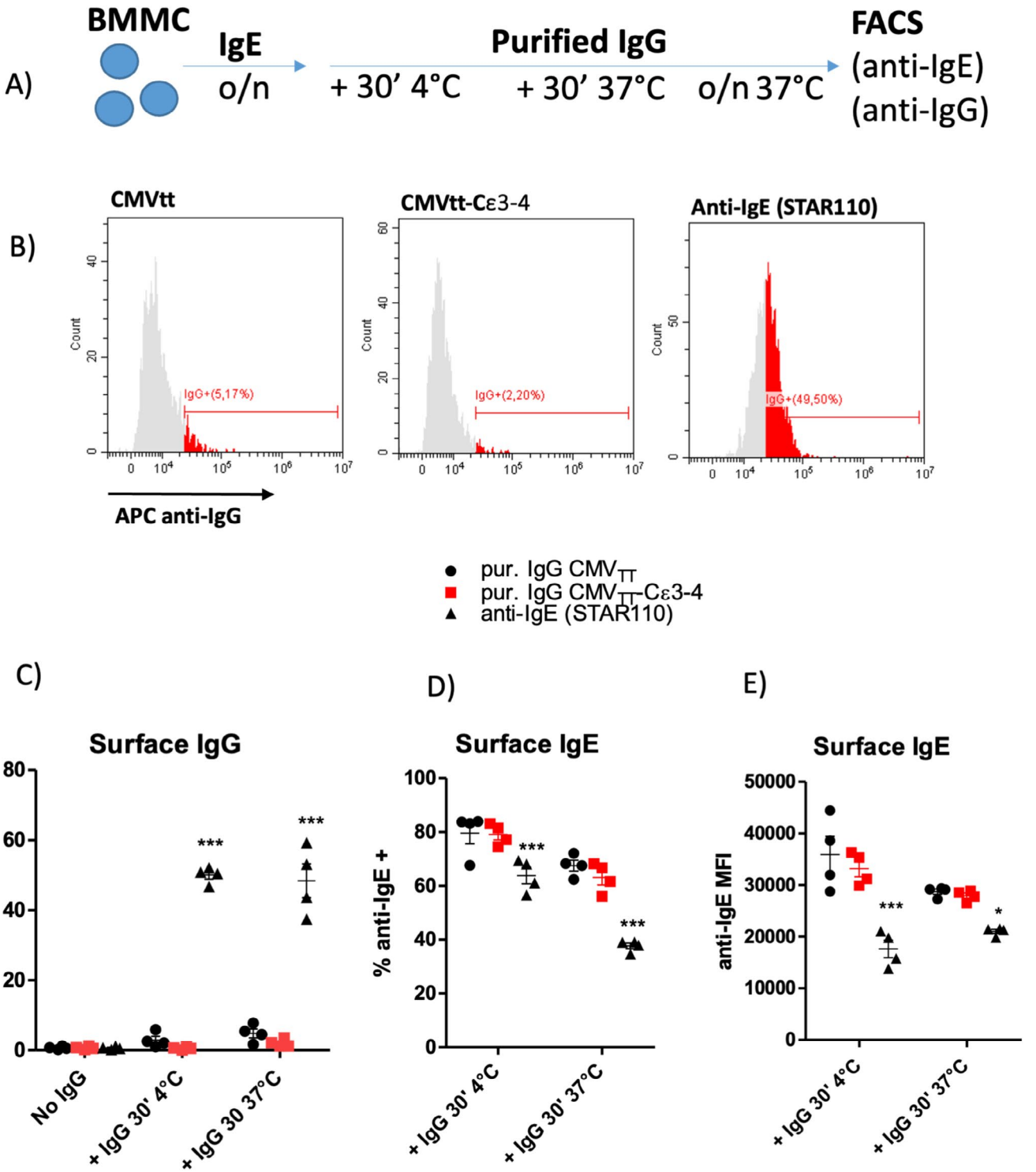


**FIGURE 7** | Purified IgGs from CuMVTT-Cε3-Cε4 do not recognize FcεRI-displayed IgE. BMMCs were sensitized o/n with IgE. (A) Purified IgG was added at 4°C and 37°C to BMMC as shown. (B) Representative flow cytometry histograms show IgG binding to BMMCs measured by anti-IgG staining. (C) mean ± SEM percent IgG-positive BMMCs. (D) mean ± SEM percent IgE-positive BMMCs. (E) mean ± SEM percent anti-IgE MFI.

free IgE. Specifically, BMMC isolated from both wild-type and FcγRIIb KO mice demonstrated equivalent levels of protection upon allergen activation.

In our previous study, we demonstrated that protection against anaphylaxis induced by passive immunization with IgG anti-IgE autoantibodies is mediated by CD23-dependent serum elimination [32]. Nevertheless, we here show that anti-IgE IgGs induced through active immunization conferred protection in both wild-type and CD23KO mice. This observation suggests that protection can occur independently of CD23 and that clearance of IgE can also be mediated in the absence of CD23. It is worth noting that CD23 and FcγRs could potentially both clear IgE-IgG complexes, thereby functionally compensating for each

other. In addition, clearance could be mediated by other types of IgG receptors.

Finally, we sought to assess the potential of vaccine-induced antibodies to cross-link cell-bound IgEs and cause anaphylactic reactions in allergic mice. Even by injecting high doses of vaccines, we could not observe any signs of anaphylaxis in these mice. We further demonstrate that IgG anti-IgE antibodies induced by the vaccine candidate also fail to trigger IgE bound to mast cells. Indeed, in an in vitro experiment, we observed minimal to no degranulation upon incubation of IgE-loaded BMMCs with purified anti-IgE IgG. An explanation for this non-anaphylactogenicity is offered by the observation that a significant amount of anti-IgE IgG is dependent on mannose glycans, which is in line with our previous findings that IgE glycans are key epitopes for non-inflammatory anti-IgEs [25]. Indeed, our ELISA assays revealed that the majority of purified IgG specific for IgE recognizes mannose on IgE. The competition between FcεRI and vaccine-induced anti-IgE IgG for the same conserved mannose residues indicates a role for this epitope, as the conserved IgE mannose region is essential for FcεRI binding and probably not accessible on IgE bound to mast cells. This hypothesis was confirmed in a flow cytometry assay where the purified anti-IgE IgGs induced by vaccination were not able to bind to surface-bound IgE on mast cells. This indicates that purified anti-IgE IgGs, which mainly target mannose glycan, cannot effectively engage IgE when it is already bound to the receptor. This highlights the importance of mannose accessibility and its role in mediating interactions between IgE and its receptor. Passive immunization with purified IgG anti-IgE antibodies from vaccinated mice resulted in protection. However, the protection was not complete, suggesting that there was still cell-bound IgE that could be cross-linked upon allergen challenge. Thus, the vaccine-induced antibodies likely need to act on free IgE and cannot desensitize mice rapidly. This is in line with our previous findings that actively sensitized mice were desensitized slowly rather than rapidly [20]. These findings suggest that a "turnover" of FcεRI-bound IgE needs to occur before the effects of the anti-IgE vaccine on newly produced IgE are visible.

Recent advancements have focused on the creation of specific anti-IgE monoclonal antibodies such as omalizumab that do not cross-link FcεRI-bound IgE, thus preventing degranulation and anaphylaxis caused by antibody therapy [32–38]. However, an active immunization approach against IgE could offer even greater potential due to its vastly better cost-effectiveness and broader applications compared to passive immunization methods.

An important element to the success of omalizumab is its excellent safety profile, as it does not appear to cause immunodeficiency and susceptibility to cancer or parasite infection [39, 40], which should translate to the here presented active vaccination approach as well. A potential reason for the safety of anti-IgE therapy is that the regulation by IgE via anti-IgE antibodies appears to be a natural physiological process. We have shown that natural anti-IgE antibodies are continuously present to surprisingly high levels in normal mice and in healthy humans [25]. Our findings could explain why these natural anti-IgE autoantibodies do not cause anaphylaxis in healthy individuals. Of note, human natural anti-IgE autoantibodies and omalizumab depend on the same conserved mannose regions as the mouse anti-IgE antibodies [21].

Taken together, our findings demonstrate that active immunization against IgE is a viable therapeutic approach. Most likely, this VLP-based anti-IgE approach could also be applied in companion animals, as we have shown for other targets [41, 42]. The VLP platform employed here offers a distinct advantage due to its robust immunogenicity and lack of need for adjuvants [43]. Potentially, the principle of anti-IgE vaccination could translate to other types of vaccination approaches and platforms. Those may need to focus on the conserved mannose region on IgE in order to prevent IgE from binding to FcεRI without cross-linking FcεRI-bound IgE. This specificity ensures the safety and success of our approach, which could pave the way for broader applications in preventing and treating allergies without the need for allergen-specific therapies.

### Author Contributions

M.F.B., M.V., Z.G., and P.E. designed the experiments, Z.G. and P.E. performed experiments, acquired data, and visualized data. All authors interpreted experiments. Z.G. and M.V. wrote the initial draft of the manuscript; P.E. and M.F.B. revised and edited the manuscript. M.F.B., M.V., and P.E., acquired funding. M.V., P.E., and M.F.B. supervised the study. All authors read and approved the final manuscript.


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### Conflicts of Interest

M.F.B. is under consultancy agreements with Allergy Therapeutics Plc (ATLp). M.F.B. is a co-founder of Saiba AG, which has out-licensed vaccine development of $CuMV_{TT}$ to ATLp within allergy and other disease indications. All other authors declare no conflicts of interest.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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