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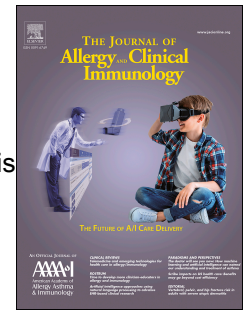

Glycan-specific IgG anti-IgE autoantibodies are protective against allergic anaphylaxis in a murine model

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Title: Glycan-specific IgG anti-IgE autoantibodies are protective against allergic anaphylaxis in a murine model

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Anti-IgE autoantibodies, glycans, IgE regulation, allergy, hypersensitivity

Abbreviations:

IgE-Fel d 1: IgE in complex with Fel d 1
PNG F: Peptide:N-glycosidase F
IgE(PNG)-Fel d 1: Deglycosylated IgE in complex with Fel d 1
BMMC: Bone Marrow-derived Mast cells

APC: Allophycocyanin
FITC: Fluorescein isothiocyanate
PE: Phycoerythrin
HRP: Horseradish peroxidase

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Author contributions: P.E., K.F.B designed, performed and interpreted experiments. K.P and F.S performed experiments. F.T provided monoclonal antibodies and Fel d 1., M.F.B., S.V.G, A.E., and M.V. supervised, designed, and interpreted experiments. P.E. and M.V. wrote the manuscript.

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ABSTRACT

BACKGROUND: IgE causes anaphylaxis in type-1 hypersensitivity diseases by activating degranulation of effector cells such as mast cells and basophils. The mechanisms that control IgE activity and prevent anaphylaxis under normal conditions are still enigmatic.

OBJECTIVE: We aimed to unravel how anti-IgE autoantibodies are induced and understand their role in regulating serum IgE level and allergic anaphylaxis.

METHODS: We immunized mice with different forms of IgE and tested anti-IgE autoantibody responses and their specificities. We then analysed the effect of those antibodies on serum kinetics and their *in vitro* and *in vivo* impact on anaphylaxis. Finally, we investigated anti-IgE autoantibodies in human sera.

RESULTS: Immunization of mice with IgE-immune complexes induced glycan-specific anti-IgE autoantibodies. The anti-IgE autoantibodies prevented effector cell sensitization, reduced total IgE serum levels, protected mice from passive and active IgE sensitization, and resulted in cross-protection against different allergens. Furthermore, glycan-specific anti-IgE autoantibodies were present in sera from allergic and non-allergic subjects.

CONCLUSION: In conclusion, we provide first evidence that in the murine model the serum level and anaphylactic activity of IgE may be down-regulated by glycan-specific IgG anti-IgE autoantibodies.

KEY MESSAGES

- Immunization with IgE-allergen complexes induces anti-IgE autoantibodies
- Those anti-IgE autoantibodies recognize glycan structures on IgE
- The anti-IgE autoantibodies down-regulate endogenous IgE levels and protect against passive and active allergic sensitization

Capsule summary

Glycan-specific anti-IgE autoantibodies are inducible by IgE-immune complex immunization in mice and protect from allergy. Hence, anti-IgE autoantibodies could be a novel therapeutic approach in allergic disease**.**

INTRODUCTION

IgE antibodies are one of the most powerful weapons of the immune system intended to fight threats such as parasites or venoms (1). IgE is capable of activating the degranulation of effector cells such as basophils and mast cells. In this process, antigen-specific IgE first sensitizes the effector cells to an antigen by binding FcεRI receptors with high affinity. Upon antigen re-encounter, FcεRI cross-linking causes degranulation of the cells and leads to inflammatory responses (2,3). When falsely induced against non-hazardous antigens, IgE can cause type I hypersensitivity such as asthma, hay fever, and food allergy (4,5). IgE might also be a contributor to the pathogenesis of several chronic inflammatory disorders such as rheumatoid arthritis bullous pemphigoid, chronic spontaneous urticaria (CSU), and systemic lupus erythematosus (SLE) (6). Direct targeting of IgE with the therapeutic monoclonal anti-IgE antibody omalizumab has shown clinical efficacy in allergic asthma as well as in chronic spontaneous urticaria (7–9). Mechanistically, omalizumab neutralizes free IgE, causing downregulation of FcεRI expression on effector cells and even disrupts IgE:FcεRI complexes (10–14). However, the natural mechanisms of IgE regulation are still poorly understood. An important feature of IgE regulation is its short serum half-life of 2-2.5 days compared to serum IgG, which has a half-life of about 3 weeks in humans (15,16). The two Fc receptors for IgE, FcεRI and CD23 (FcεRII) are thought to regulate IgE serum levels (17,18). B cell-expressed CD23 is thought to further provide a negative feedback signal for the synthesis of new IgE antibodies (19,20). Another interesting attribute of IgE is its adjuvant function. IgE-antigen complexes have been shown to induce strong antigen-specific IgG and T cell immune responses (21–23). Other studies have observed that IgE itself may be a target of anti-IgE autoantibodies. Those antibodies were shown to be functionally heterogeneous, some being able to activate effector cell degranulation while other inhibited degranulation (24–27). These findings have raised the possibility that anti-IgE autoantibodies may exert regulatory functions. Here, we investigated whether immunization with IgE-antigen complexes not only results in antigen-specific responses but also in antibody responses against IgE itself. For our immunization model, we used the major cat allergen Fel d 1 as antigen in complex with a Fel d 1-specific monoclonal IgE (28). We next examined how those antibody responses might functionally impact serum IgE levels, allergic sensitization, and antigen-mediated anaphylaxis.

METHODS

Fel d 1 and Fel d 1-specific monoclonal antibodies

Production of recombinant monomeric and dimeric Fel d 1 is described elsewhere (29,30). Briefly, the sequence encoding a fusion of chains 1 and 2 of Fel d 1 spaced by a 15aa-linker (GGGGS)x3 was linked to a histidine tag and then cloned into plasmid pET42 (Addgene, Watertown, USA). After plasmid transformation into *Escherichia coli* C25661 (New England, Biolabs, Ipswich, UK), recombinant Fel d 1 was produced at 20°C for 20 hours. Cells were then sonicated and cell supernatant was purified by Ni2+ affinity column. To separate monomer and dimers from multimers, a size exclusion chromatography using a Superdex 75 column (GE Healthcare, Chicago, IL, USA) was performed. The monoclonal antibodies were engineered to be expressed as either human or mouse antibodies and were produced in CHO cells (Evitria AG, Zürich, Switzerland) and purified by affinity chromatography over a protein L or protein G Sepharose column (GE Healthcare) respectively. If not otherwise stated, the monoclonal Fel d 1-specific IgE F127 was always chosen for immunization or ELISA coating. In some cases, the non-competitive mouse IgE clone A044 was used. Mouse IgG immunizations and coatings were performed with IgG1 F127 and IgG2a F127. For human IgE, we mainly used the monoclonal human hybridoma-produced IgE SUS11 (31). Antibodies were deglycosylated using PNGase F (New England Biolabs, Ipswitch, MA, USA) under native conditions according to the protocol of the suppliers with slight modifications. Briefly, 25 µg of IgE was mixed with 2.5 µl Glycobuffer 2 (10X) in a final volume of 25 µl adjusted with sterile water. After the addition of 5 µl PNGase F the mix was incubated at 37°C in a shaker with 300 rpm overnight. The deglycosylation with Endo F1 and Endo F2 was performed as described (Sigma-Aldrich, Switzerland). Briefly, 200 µg of IgE was incubated with 2 µl of Endo F1 or Endo F2 in a 50 µl final volume adjusted with reaction buffer (Cat Nr R9025) and incubated for 1 hour at 37°C. Cleavages with PGNase F and Endo F1 or F2 were then monitored on SDS-PAGE and Western blot. The antibodies were additionally purified with Zeba™ Spin Desalting Columns (40K, MWCO) (Thermo Fisher Scientific, Waltham, USA), which are polypropylene devices containing a high-performance size-exclusion chromatography resin allowing removal of the enzymes used for deglycosylation.

Lectin Blots

Two 4-15% Mini-Protean TGX gels (Bio Rad, Hercules, CA, USA) were used for SDS-PAGE analysis of digested IgE. ProSieve Color Protein Marker (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) was used as a ladder. 3 µg IgE was loaded in non-reducing conditions. The gel was first run at 70 V for 15 min. and then at 120 V for 1 h. One SDS-PAGE was incubated overnight in InstantBlue (Abcam, Cambridge, UK). The other gel was used

for the Western Blot, by using the Trans-Blot® Turbo® Transfer System (Bio Rad). The membrane was then incubated with biotinylated Galanthus Nivalis Lectin (Vector Laboratories Inc, Burlingame, CA, USA) to detect high-mannose-type N-glycans for 1h at RT. The gel was washed twice before the secondary antibody Streptavidin HRP (Biolegend, San Diego, CA, USA) was added and incubated for 1h at RT. The gel was again washed twice and developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA).

Mouse immunization and serum collection

BALB/c mice (Envigo, Huntingdon, UK) were used for experiments at the age of 6 weeks and were kept at the central animal facility (Murtenstrasse 31, Bern, Switzerland). All animals were treated for experimentation according to protocols approved by the Swiss Federal Veterinary Office. CD23-/- mice on BALB/c genetic background were kindly provided by Prof. J. Ravetch. Mice on a mixed C57BL/6J–C57BL/6N background provided by Dr. A. Eggel were originally described and received from Prof. JP. Kinet (32). For immunizations, if not otherwise described, mice were injected intravenously (i.v) with 22.5µg IgE, 5µg Fel d 1 in dimeric or monomeric form or 118µg IgG, always dissolved in 100µl PBS. Blood from tail veins was collected using Microtainer® serum tubes (BD Biosciences, Franklin Lakes, NJ, USA). Sera of allergic donors who had a positive ImmunoCAP on timothy grass pollen (class ≥ 4) and of healthy donors (no specific IgE detected in ImmunoCAP) were used for ELISA.

Mast cell binding and activation assay

Isolation of murine bone marrow-derived mast cells (BMMC) for *in vitro* anti-IgE serum blocking assay was previously described by our group (33). Mouse IgE F127 was added at different concentrations in mouse sera of either naïve mice, or IgE-Fel d 1 immunized mice after 14 days. The mixture was then incubated with BMMC for 1h at 37°C. For IgE binding assays, the cells were collected at this step and stained for surface IgE using PE anti-mouse IgE (clone RME-1, Biolegend). To assess degranulation, the cells were washed and incubated with 5nM Fel d 1 diluted in PBS for 30 min at 37°C. The cells were washed and stained with anti-CD63 (clone REA563, Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at RT. The cells were analyzed by flow cytometry. To test the allergenic activity of anti-IgE IgG antibodies, mouse IgE F127 was added at different concentrations to BMMC for

1 h at 37°C followed by subsequent incubation with naïve or immunized sera that have been previously heated for 30 min at 56°C. The cells were washed and stained with anti-CD63 (clone REA563, Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at RT and analyzed by flow cytometry.

Flow Cytometry from murine blood

Blood from tail veins was collected using PBS containing 10mM EDTA (Thermo Fisher Scientific). Red blood cells were lysed using ACK buffer (Thermo Fisher Scientific) according to the manufacturer's protocol. Before antibody staining, the cells were washed three times with PBS. The cells were then stained for 15 minutes at RT. Basophils were marked with APC anti-mouse CD49b (clone HMα2, Biolegend) and PE anti-mouse IgE (clone RME-1, Biolegend) and negative for FITC anti-CD117 (clone 2B8, BD Biosciences) after blocking unspecific binding with mouse Fc gamma block (BD Bioscience). Flow cytometry was performed with BD FACSCanto™ (BD Biosciences) and analyzed using FLOWJO software (TreeStar Inc, Ashland, OR, USA).

Passive and active sensitization

For passive sensitization (IgE re-injection) of immunized mice, 22.5µg IgE F127 was administered per mouse by intravenous injection. The next day, baseline body temperature was measured by MiniTemp rectal probe for mice (Vetronic Services Ltd, Abbotskerswell, UK). IgE sensitized mice were then challenged by intravenous injection with 5µg Fel d 1 and rectal temperature was measured at 10min-intervals for 1 hour. Crude peanut extract was prepared as previously described (34). For active sensitization, mice were injected i.p. with 5 µg crude peanut extract mixed in 200 µl 10 mg/ml Al(OH)3, Alhydrogel, referred to as Alum (InvivoGen, San Diego, CA, USA). For the induction of anaphylaxis, mice were i.v. injected with 10µg peanut extract in PBS.

ELISA

96-well Nunc Maxisorp™ ELISA plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with 2µg/ml antibody in PBS at 4°C overnight. For the determination of mouse IgE-specific IgG, plates were coated with mouse IgE F127. For the detection of human IgG anti-IgE, previously described IgE clone SUS11(31) was coated. Alternatively, native human IgE protein (Abcam, Cambridge, UK) was coated. For mouse IgE capture ELISA, rat anti-mouse anti-IgE (clone R35-7, BD Biosciences) was used for coating. For human IgE capture ELISA, preciously

described anti-IgE clone Le27 (35) was used. After blocking with PBS/0.15% Casein solution for 2 hours, plates were washed five times with PBS/0.05% Tween. Serial dilutions of sera were added to the plates and incubated for 2 hours at RT. Plates were then washed eight times with PBS. Thereafter, HRP-labeled goat anti-mouse IgG (The Jackson Laboratory, Bar Harbor, ME, USA) antibodies were incubated at RT for 2 hours. To detect IgG subclasses, rat anti-mouse IgG1 (clone X56, BD Biosciences), rat anti-mouse IgG2a (clone R19-15, BD Biosciences), rat anti-mouse IgG2b (clone R12-13, BD Biosciences) rat anti-mouse IgG3 (clone R40-82, BD Biosciences) were used and developed with polyclonal HRP-labeled goat anti-rat IgG (Biolegend). For IgE detection, polyclonal HRP-labeled goat anti-mouse IgE (Bio-Rad Laboratories) was used. ELISAs were developed with TMB (3,30,5,50-tetramethylbenzidine) and H_2O_2 and stopped with 1 mol/L sulfuric acid. Optical densities were measured at 450 nm. Half-maximal antibody titers (OD50) are defined as the reciprocal of the dilution leading to half of the OD measured at saturation.

Glycan microarray and analysis

Glycan microarray screening, using slides from Semiotik LLC, Moscow, Russia was performed as previously reported (36). Briefly, pooled sera (10 mice per group) of Fel d 1, IgE-Fel d 1, IgE or IgE(PNGase treated)-Fel d 1 immunized BALB/c mice were diluted 1 in 30 (v/v) in PBS containing 1% Tween 20 and 3% BSA and applied onto array microchips for 1.5 h at 37°C. Biotinylated goat anti-mouse IgG (SouthernBiotech), followed by 5µg/mL of streptavidin conjugated with Alexa Fluor 633 (Thermo Fisher Scientific, Waltham, USA) were used to develop the fluorescent signal. Scanning of Semiotik slides was performed on a GenePix® 4100A Microarray Scanner (Molecular Devices) with 5µm resolution. Values that were below two-fold changes respect to the control group and in the lower 25 percentile of fluorescence intensity were excluded from the analysis. Heatmap representation of the data was performed using the function heatmap.2 from the package gplots2 of the "R" environment (The R Foundation for Statistical Computing, Vienna, Austria, Version 3.0.2). Other representations were done using Microsoft Excel (Microsoft Corporation, 2011, Version 14.0.0).

Statistical analysis

All statistical tests were performed using GraphPad PRISM 6.0 (GraphPad Software, Inc.). For all experiments throughout the manuscript, $\alpha=0.05$ and statistical significance are displayed as $p\leq0.05$ (*), $p\leq0.01$ (**), $p\leq0.001$

(***), p≤0.0001 (****). Two groups were analyzed by Two-tailed Student‘s t-test. All data in graphs are displayed as mean ± SEM. Dose- and time-dependent comparisons were performed by 2-way ANOVA followed by Tukey testing.

RESULTS

IgE-Fel d 1 complexes induce IgG autoantibodies against IgE

We first investigated the IgE-specific IgG response induced by immunization of mice with IgE-Fel d 1 complexes (Figure 1A-F). All respective Fel d 1 specific IgG responses are shown in Figure E1A-D. First, either IgE-Fel d 1 complexes or monomeric IgE as a control was injected intravenously. On day 14 after immunization mice were bled for the detection of anti-IgE autoantibodies by ELISA (Figure 1A). Mice treated with IgE-Fel d 1 complexes generated anti-IgE autoantibodies as well as anti-Fel d 1 antibodies of the IgG isotype (Figure E1), whereas injection of monomeric IgE did not result in detectable anti-IgE autoantibodies. We also observed IgG anti-IgE responses when mice were immunized intraperitoneally and subcutaneously (Figure E2A). Next, we assessed whether the induction of anti-IgE autoantibodies is Fcε and not variable region dependent and used the same anti-Fel d 1 antibody clone (F127) expressed as IgG. IgG1-Fel d 1, IgG2a-Fel d 1, or IgE-Fel d 1 complexes were injected and mice bled after 14 days. Anti-IgE antibodies were exclusively generated upon IgE-Fel d 1, but not IgG1-Fel d 1 or IgG2a-Fel d 1 complex injection (Figure 1B). Immunization with IgG-Fel d 1 complexes did also not lead to IgG anti-IgG responses (Figure E2B). The Fel d 1 used for IgE-Fel d 1 complex immunizations is a dimer providing two IgE binding sites and thus allowing the formation of complexes (28). Injection of IgE in complex with monomeric Fel d 1 did not induce anti-IgE responses (Figure 1C). We next tested a different Fel d 1 specific IgE clone (A044) to evaluate whether the anti-IgE response depends on the IgE clone used (Figure 1D). IgE-Fel d 1 complexes with A044 and F127 clones induced equal anti-IgE responses while IgE A044 alone, like IgE F127, did not induce anti-IgE antibodies. Interestingly, the complexation of IgE with a rat IgG anti-mouse IgE also induced anti-IgE responses in absence of Fel d 1 (Figure E2C). The most frequently induced IgE-specific IgG subclass upon IgE-Fel d 1 immunization was IgG1 and IgG2b (Figure E2D). Finally, we evaluated whether the induction of anti-IgE responses is dependent on IgE Fc receptors. To this end, we immunized mice lacking CD23 (FcεRII) or FcεRIα

(Figure 1E and 1F) with IgE-Fel d 1. Surprisingly, in both FcεR deficient mice, the induction of anti-IgE antibodies was significantly enhanced, indicating that FcεRs suppress rather than facilitate anti-IgE responses.

The IgE-Fel d 1 induced IgG anti-IgE autoantibodies are glycosylation-specific

We next investigated whether the anti-IgE sera are cross-reactive and react with another mouse IgE (isotype-specific), human IgE (species-specific), or only the immunized IgE (idiotype-specific). As shown in Figure 2A, sera from IgE F127-Fel d 1 complex immunized mice recognize IgE A044 and IgE F127 indicating that anti-IgE sera are isotype-specific. Since IgE is heavily glycosylated (37), we speculated that the anti-IgE response could be directed against glycans on IgE. To test this, we digested mouse IgE F127 with deglycosylation enzyme PNGase F, an amidase that cleaves between the innermost GlcNAc and asparagine residues of N-linked oligosaccharides and was previously used to deglycosylate IgE (38). Deglycosylated IgE is here referred to as IgE(PNG) and Figure E3 shows that the PNG digestion was efficient. Figure 2B demonstrates that the coating of ELISA plates is not significantly altered for IgE(PNG), as the signal of polyclonal anti-IgE developing antibody is equal for both untreated IgE and deglycosylated IgE. However, we observed a clear loss of binding to IgE(PNG) by sera of IgE-Fel d 1 complex immunized mice (Figure 2C). Additionally, when mice were immunized with IgE(PNG)-Fel d 1, the anti-IgE response was much weaker than that obtained with non-deglycosylated IgE-Fel d 1 (Figure 2D). To assess whether glycans may be recognized independent of IgE, we performed a glycan-array analysis with sera from IgE, IgE(PNG)-Fel d 1 or IgE-Fel d 1 immunized mice (Figure 2E). We found a number of glycans that were recognized by IgG upon IgE-Fel d 1 immunization, including Man5 (Mannose), one of the previously described oligomannose N-glycans found at the N394 of IgE (38). Some of the recognized glycan structures contain monosaccharides which are generally of low abundancy (Kdn (3-deoxy-D-glycero-D-galacto-nonulosonicacid)) or not present (Kdo (3-deoxy-D-manno-octulosonic acid) and Rha (rhamnose)) in mammalian glycans. This finding suggests some cross-reactivity of the IgG anti-IgE antibodies. All glycan structure hits are additionally summarized in Table 1. Finally, we also digested IgE F127 with Endoglycosidase F1 (Endo F1) or Endoglycosidase F2 (Endo F2). Endo F1 preferentially cleaves oligomannose-type and hybrid structures whereas Endo F2 cleaves complex biantennary structures (38). As seen in Figure 2F, Endo F1 digestion had a similar impact on anti-IgE recognition as PNGase digestion whereas Endo F2 digestion had less impact on IgE recognition suggesting that oligomannose or hybrid glycan structures are preferentially recognized. Together, these data clearly show that immunization with IgE-Fel d 1 complexes induces glycan-specific anti-IgE responses.

IgE-Fel d 1 immunization down-regulates serum and basophil IgE levels

We next aimed to evaluate the impact of induced anti-IgE autoantibody responses on total endogenous IgE levels in mice. To this end, we immunized mice with monomeric IgE, IgE-Fel d 1 or IgE(PNG)-Fel d 1 complexes. Total IgE levels in serum as well as surface IgE levels on blood basophils (gated as $CD117^{-}CD49b^{+}IgE^{+}$) were evaluated 14 days post-immunization. Figure 3A shows that total IgE levels were reduced in IgE-Fel d 1 immunized mice whereas no reduction of IgE levels was observed in mice immunized with IgE(PNG)-Fel d 1 or with IgE only. The same was true for basophil surface IgE levels, which are shown as raw dot plots in Figure 3B and as anti-IgE MFI in Figure 3C. We next investigated the impact of anti-IgE antibodies on IgE serum clearance of secondary injected IgE. Therefore, mice were immunized with either monomeric IgE, IgE-Fel d 1 or IgE(PNG)-Fel d 1 complexes and re-injected with IgE 14 days later. Total serum IgE levels were measured after three hours (Figure 3D). IgE-Fel d 1 complex immunization increased clearance of passively injected IgE in comparison to free IgE or IgE(PNG)-Fel d 1 complex immunized mice. The next day, we assessed basophil surface IgE levels in these mice. In line with the serum clearance, basophils carried significantly less IgE on their cell surface in IgE-Fel d 1 complex immunized groups as compared to the other groups (Figure 3E, raw data are shown in Figure E4A). From these experiments, we conclude that actively induced anti-IgE antibodies are physiologically active, as they down-regulate endogenous and passively administered IgE.

IgE-Fel d 1 immunization protects from passive IgE sensitization and Fel d 1 challenge

Given that anti-IgE autoantibodies can regulate IgE levels in the serum, we assessed whether they also protect mice from allergen challenge. As anti-IgE antibodies could potentially crosslink cell surface bound IgE and cause systemic anaphylaxis, we measured body core temperature upon IgE re-injection after IgE:Fel d 1 complex immunization. Figure 4A shows that the mice showed a small drop in core temperature upon i.v. injection with a high IgE dose, but no sign of severe systemic anaphylaxis. We next investigated, whether induced anti-IgE autoantibodies may be protective during allergen challenge. Therefore, mice immunized with IgE-Fel d 1 complex were challenged with Fel d 1 one day after IgE-reinjection and systemic anaphylaxis was measured. Strikingly, IgE-Fel d 1 immunization protected mice from Fel d 1 re-challenge whereas mice immunized with IgE alone showed a severe anaphylactic reaction to Fel d 1 challenge (Figure 4B). Figure 4C shows that immunization with IgG-Fel d 1

failed to induce protection from passive systemic Fel d 1 challenge in contrast to IgE-Fel d 1, which protected from anaphylaxis. In a last step, we compared IgE(PNG)-Fel d 1 immunized mice with IgE-Fel d 1 immunized mice. Figure 4D shows, that IgE-Fel d 1 immunization facilitates significant protection compared to IgE(PNG)-Fel d 1 immunization. In summary, these data provide strong evidence that IgE-Fel d 1 immunization confers increased protection against allergen challenge in a passive sensitization model by inducing anti-IgE autoantibodies that reduce serum and basophil surface IgE levels. To evaluate whether allergen challenge induces anti-IgE autoantibodies, we passively sensitized mice with IgE, followed by Fel d 1 challenge the next day. Allergen challenge of sensitized mice did not induce anti-IgE autoantibodies and did also not lead to resistance against secondary IgE sensitization and challenge (Figure E4B and E4C). Using bone marrow-derived mouse mast cells (BMMCs), we also tested whether sera from IgE-Fel d1 complex immunized mice (anti-IgE serum) display *in vitro* blocking activity of IgE binding to FcεRI. As shown in Figure 4E, IgE binding to BMMCs was inhibited by anti-IgE serum from immunized mice but not by naïve serum. Upon Fel d 1 challenge, BMMCs incubated with anti-IgE serum displayed reduced degranulation as measured by CD63 up-regulation (Figure 4F). To further investigate the allergenicity of anti-IgE IgG antibodies, we heated naïve and immunized sera to inactivate heat-labile IgE and tested them on BMMCs. Figure E4D shows no difference in activation between heated naïve or immunized sera demonstrating that anti-IgE IgG antibodies per se do not induce mast cell activation. Together these data show that IgE-Fel d 1 immunized mice are protected from secondary passive Fel d 1 sensitization and systemic anaphylaxis and serum from IgE-Fel d 1 immunized mice inhibits IgE mediated activation of FcεRI on allergic effector cells in vitro.

IgE-Fel d 1 immunization cross-protects from active peanut extract sensitization and challenge

Given that IgE-Fel d 1 immunization protects from passive anaphylaxis, we next investigated whether anti-IgE immunization might protect against active, polyclonal sensitization to a different allergen than Fel d 1 (cross-protection). Therefore, we used a common model of active peanut sensitization where alum is mixed with the allergen extract to induce IgE upon i.p. injection (39). Figure 5A-C shows a clear increase in serum as well as basophil surface IgE upon peanut sensitization. We then investigated whether IgE-Fel d 1 immunization 14 days before sensitization has a protective effect on sensitization. To this end, we immunized mice with monomeric IgE, IgE-Fel d 1 or IgE(PNG)-Fel d 1 complexes 14 days before inducing active sensitization with peanut. We collected

blood 28 days after immunization and evaluated total IgE and basophil bound IgE. We monitored anti-IgE autoantibody titers during the sensitization period. Figure 5D shows that IgG anti-IgE titers were stable over the sensitization phase and only detectable in IgE-Fel d 1 immunized mice. Figure 5E shows that total IgE levels were reduced in IgE-Fel d 1 complex immunized mice but not in untreated, monomeric IgE or IgE(PNG)-Fel d1 complex immunized mice. The same effect was also observed for basophil IgE levels, as seen in Figure 5F and 5G. The mice were then intravenously challenged with peanut extract and systemic anaphylaxis was assessed by measuring body core temperature. As shown in Figures 5H and I, IgE-Fel d 1 immunized mice were protected from peanut extract challenge whereas this was not the case when mice were immunized with IgE or IgE(PNG)-Fel d 1. Altogether our results show that induction of anti-IgE autoantibodies by IgE-Fel d 1 complex immunization leads to a universal reduction of IgE levels and thereby cross-protects against active sensitization to other allergens.

Glycosylation-specific anti-IgE autoantibodies are present in human serum

Having demonstrated the induction, specificity, and functional impact of anti-IgE responses in mice, we next aimed to investigate the presence and specificity of anti-IgE autoantibodies in human serum. We first used human IgE SUS11(31) in untreated or deglycosylated form to determine whether natural anti-IgE autoantibodies are glycosylation-specific in sera of a mix population of allergic and non-allergic donors. In line with our findings in mice, human IgG anti-IgE autoantibodies were also glycosylation-specific (Figure 6A). We next deglycosylated a purified native polyclonal IgE and Figure 6B shows that recognition of native polyclonal IgE was equally glycan-dependent as for SUS11. Figure 6C shows that recognition of untreated and deglycosylated human IgE by polyclonal anti-IgE antibody in ELISA is comparable supporting the fact that reduced binding of human serum to deglycosylated IgE is due to the absence of glycan. To evaluate whether IgG antibodies against other Ig isotypes are present, we coated IgE, IgA, or IgM and investigated the presence of anti-Ig IgGs. As shown in Figure 6D, only IgE-specific IgG is present in human serum while no IgA- or IgM-specific IgG antibodies are present. To evaluate the physiological relevance of IgG anti-IgE in human serum we coated the mouse monoclonal anti-IgE antibody Le27 to capture IgE. We then detected IgG with either a polyclonal anti-human IgE or a polyclonal anti-human IgG antibody. Figure 6E shows that not only IgE but also IgE-IgG complexes were detected in high amounts in human serum. In contrast, when high doses of SUS11 were added instead of human serum, only anti-IgE but no anti-IgG signal was detected, eliminating a possible cross-reactivity of the developing antibody. Next, we compared 10 non-

atopic individuals with no history of atopy and 10 allergic donors with specific IgE to grass pollen in terms of IgG anti-IgE autoantibodies. We found no significant difference in IgG anti-IgE antibody titers in healthy and allergic donors. However, in both healthy and allergic donors we detected less reactivity against deglycosylated than against untreated IgE suggesting a dominant anti-glycan response independent to allergic status. Nevertheless, further studies with an increased sample size are required to draw definitive conclusions (Figure E5).

Altogether our data show that glycan-specific IgG ant-IgE antibodies are present in human serum and a large amount of them are found in complex with IgE. Hence, anti-IgE autoantibodies potentially have a similar physiological relevance in humans as we have described in mice.

DISCUSSION

Even though the existence of natural anti-IgE autoantibodies has often been described, their role within the immune system has long been enigmatic. IgE-specific IgG autoantibodies have been detected in atopic patients as well as healthy human donors and most of them have been shown to recognize protein epitopes within the Cε2, Ce3, and Cε4 domains (24–26). However, the mechanisms by which these antibodies are induced are still not understood. While other studies have shown that viral antigens can induce anti-antibody responses (40) we here show that a single immunization with IgE-allergen complexes in absence of adjuvant induces IgE-specific IgG autoantibodies.

It has never been clear whether anti-IgE autoantibodies are idiotype- or isotype-specific. Here, we report that anti-IgE autoantibodies in mice and human donors are generally glycan-specific, yielding the open question as to whether IgE “glycotypes” may exist. That said, it has been shown that IgE is the most heavily glycosylated Ig (with around 12% carbohydrate) in the serum with seven N-linked glycosylation sites in the epsilon chain (37,41). Independent of IgE glycans, the presence of glycan-specific IgG antibodies is also well described (36). The immunogenicity of the IgE glycosylation sites requires further in-depth investigations, especially as we show that induction of anti-IgE autoantibodies is not dependent on FcεRs (FcεRI and CD23). It seems plausible, that glycan-recognizing receptors could be involved in positively regulating this process, also because it is generally established that glycsosylations regulate serum kinetics of proteins (42,43). For IgE specifically, glycans also seem to regulate its inflammatory activity as the N-linked glycan N394 in the IgE Cε3 domain is critical for FcεRI binding and for triggering anaphylaxis (38). A recent study has shown an increased level of sialic acid and galactose on allergic IgE sustaining the role of glycans in the IgE biology (44). The targeting of IgE by administration of anti-IgE antibodies

such as Omalizumab for therapy is an established approach to reduce IgE-dependent hypersensitivity (14). Our study shows that natural IgG anti-IgE autoantibodies may represent an actual physiological mechanism that could provide a balancing factor between IgE-dependent inflammation and homeostasis. With anti-IgE antibodies, there is typically the potential of side effects, as anti-IgE antibodies could cross-link FcεRI-bound IgE. However, the fact that our immunized mice tolerated intravenously injected IgE at exceptionally high doses without severe signs of side effects and anaphylaxis suggests that the antibodies do not efficiently cross-link FcεRI-bound IgE. This is in line with our latest finding showing that IgE in complexed form is generally non-inflammatory (45). Thus, serum anti-IgE could capture IgE leading to complexation resulting in reduced FcεRI sensitization and increased serum clearance. The reason for non-reactivity with FcεRI-bound IgE is still not clear. Since glycans are critical for FcεRI binding, we speculate that they may be hidden in FcεRI-bound IgE and can thereby not be recognized by anti-IgE autoantibodies. Thus, a vaccine inducing non-anaphaylactogenic anti-IgE autoantibodies that prevent the binding of IgE to FcεRI would provide clinical benefits in the treatment of allergic diseases.

In summary, we have uncovered a novel mechanism in the regulation of IgE serum levels in a murine model. Further work in human to examine whether human anti-glycan IgE autoantibodies play a similar regulatory role as mice anti-glycan in clearing IgE serum and preventing IgE binding to FceRI will give us perspective on uncovering new light on IgE biology and on paving the way for the design of novel therapeutic strategies in type I hypersensitivity diseases.

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FIGURE LEGENDS

Figure 1. IgE-Fel d 1 complexes induce IgG autoantibodies against IgE

Shown are scatter plot of IgG anti-IgE titers of individual mice as measured from serum dilutions at OD_{450} by ELISA of individual mice from at least two independent experiments, 14 days after i.v immunization. **(A)** IgG responses of mice immunized with IgE F127 or IgE F127-Fel d 1 (n=10/group) **B)** IgG anti-IgE responses of mice immunized with IgG1 F127-Fel d 1, IgG2a F127-Fel d 1 or IgE 127-Fel d 1 (n=4/group). **C)** IgG anti-IgE responses of mice immunized with Fel d 1 dimer, IgE F127-Fel d 1 monomer, IgE F127-Fel d 1 dimer (n=8/group) **D)** IgG anti-IgE responses of mice immunized with IgE A044, IgE A044-Fel d 1, IgE F127- Fel d 1 (n=5/group). **E)** IgG anti-IgE responses of mice immunized with IgE 127-Fel d 1, BALB/c mice or CD23-/- (BALB/c background) mice

immunized with IgE 127-Fel d 1 (n=5/group) **F)** IgG anti-IgE responses of mice immunized with IgE 127-Fel d 1 C57BL/6 mice or FcεRI -/- (C57BL/6 background) mice immunized with IgE 127-Fel d 1 (n=3/group).

Figure 2. The IgE-Fel d 1 induced IgG anti-IgE autoantibodies are glycosylation-specific

In A), C), D) and F) results are shown as scatter plots of IgG anti-IgE titers as measured from serum dilutions at OD_{450} by ELISA of individual mice from at least two independent experiments, 14 days after i.v immunization. **A)** Cross-recognition of IgE clones by IgE F127-Fel d 1 induced anti-IgE responses was analyzed on coated IgE clones from mouse IgE F127 and IgE A044 (n=4/group). **B)** Shown is the effect of deglycosylation on mouse IgE coating to ELISA plates and recognition by polyclonal anti-mouse IgE antibody. **C)** IgE F127 or IgE F127(PNG) recognition of IgE F127-Fel d 1 induced anti-IgE sera (n=10/group). **D)** IgG anti-IgE responses of individual mice immunized with IgE F127-Fel d 1 or IgE F127(PNG)-Fel d 1 (n≥5/group). **E)** Heatmap of glycan structures recognized by pooled sera (10 mice per group) of Fel d 1, IgE F127-Fel d 1, IgE F127 or IgE(PNG) F127-Fel d 1 immunized BALB/c mice. Values represent the relative fluorescence units (RFU). GID refers to the glycan identification number. **F)** Recognition of IgG anti-IgE responses of IgE F127 deglycosylated with either PNGase F (removes all N-linked glycans), Endo F1 (removes oligomannose and hybrid structures), Endo F2 (removes complex biantennary structures) (n=5/group).

Figure 3. IgE-Fel d 1 immunization down-regulates serum and basophil IgE levels

In A) C) D) and E) total IgE levels and basophil surface IgE levels from individual mice from least two independent experiment are shown as dots. **A)** Total IgE levels of naïve mice and mice immunized with IgE F127, IgE F127-Fel d 1 or IgE F127(PNG)-Fel d 1 after 14 days (n≥4/group). **B)** Representative flow cytometry dot plots of basophils in mice immunized with IgE F127, IgE F127(PNG)-Fel d 1, or IgE F127-Fel d 1 after 14 days showing anti-IgE staining versus anti-CD49b staining intensity. **C)** Mean ± SEM IgE MFI on basophils from naïve mice and mice immunized with IgE F127, IgE F127-Fel d 1 or IgE F127(PNG)-Fel d 1 (n≥4/group). **D)** IgE, IgE F127(PNG)-Fel d 1, or IgE F127-Fel d 1 immunized mice were re-injected with IgE after 14 days. Shown are total serum IgE levels from IgE F127, IgE F127-Fel d 1 and IgE F127(PNG)-Fel d 1 immunized mice 3 hours after IgE F127 re-injection (n≥5/group). **E)** IgE binding to basophils was measured by flow cytometry 24h after IgE F127 re-injection. Shown are mean ± SEM IgE MFI values of CD49b+IgE+ basophils (n≥5/group).

Figure 4. IgE-Fel d 1 immunization protects mice from passive systemic anaphylaxis

A-D) Mice were i.v immunized with IgE F127 or IgE F127-Fel d 1 for 14 days and re-injected with IgE. Shown are mean ± SEM temperature changes from at least 4 mice per group from two individual experiments **A)** The impact of IgE F127-Fel d 1 immunization on systemic anaphylaxis in response to IgE re-injection. 1 day after IgE F127 re-injection, the mice were challenged i.v. with 5µg Fel d 1 and systemic anaphylaxis was assessed by measuring rectal body temperature in 10 minute intervals (n=5/group). **(B)** IgE F127 or IgE F127-Fel d 1 **C)** IgG1 F127-Fel d 1, IgG2a F127-Fel d 1 or IgE F127-Fel d 1 **D)** IgE F127(PNG)-Fel d 1 or IgE F127-Fel d 1. **E)** IgE F127 binding to mouse bone marrow-derived mast cells (BMMC) after 1 hour at 37°C in pure naïve serum or anti-IgE serum raised by IgE F127-Fel d 1 immunization (n=5/group). IgE binding was assessed by surface anti-IgE staining. Shown are mean ± SEM percent IgE positive cells. **F)** Activation of BMMCs upon Fel d 1 challenge as measured by CD63 up-regulation in presence of naïve serum or anti-IgE serum raised by IgE-Fel d 1 immunization (n=5/group). Shown are mean ± SEM percent CD63 positive cells.

Figure 5. IgE-Fel d 1 immunization cross-protects from active peanut extract sensitization and challenge

A-C) To induce peanut allergy, mice were sensitized for 14 days during two independent experiments (n=5/group). **(A)** Shown are representative flow cytometry dot plots of basophils, **(B)** mean ± SEM IgE MFI on basophils from individual mice and **(C)** mean ± SEM total IgE levels of untreated and sensitized individual mice (n≥5/group) . **D-G)** Mice were immunized with IgE F127, IgE F127-Fel d 1 or IgE F127(PNG)-Fel d 1 for 14 days, peanut-sensitized and at day 28, total IgE, basophil IgE and IgG anti-IgE was assessed (two independent experiments) (n≥5/group). Shown are: (**D)** mean ± SEM OD_{50} IgG anti-IgE titers from individual mice, **(E)** mean ± SEM total IgE, **(F)** mean ± SEM IgE MFI on basophils from individual mice, **G)** representative flow cytometry dot plots of basophils. **H-I)** The mice were i.v. challenged with peanut extract at day 35. Systemic anaphylaxis was assessed by measuring rectal body temperature in 10 minute intervals from 5 mice per group. **H)** Shown are mean ± SEM changes in body temperature in mice immunized with IgE or IgE-Fel d 1. **I)** Shown are mean ± SEM changes in body temperature in mice immunized with IgE(PNG)-Fel d 1 or IgE-Fel d 1 immunized mice.

Figure 6: Glycosylation-specific anti-IgE antibodies are present in human serum

A) Human hybridoma IgE SUS11 was coated in untreated or deglycosylated form, displayed as SUS11(PNG). Shown are scatter plot of IgE-reactive IgG titers in the serum of a mix of 10 allergic and 10 non-allergic human donors **B)** Purified native human IgE was coated in untreated or deglycosylated form. Shown are IgE-reactive IgG in serum of 7 normal healthy human donors **C)** Shown is the effect of deglycosylation on human IgE coating to ELISA plates and recognition by polyclonal anti-human IgE antibodies. **(D)** Human hybridoma IgE, IgA or IgM were coated. Shown are mean ± SEM IgG anti-IgE, anti-IgA or anti-IgM the serum of 5 normal healthy human donors. **E)** IgE capture antibody LE27 was coated to ELISA plates. IgE:IgG complexes in the serum of normal human donors were detected by polyclonal anti-human IgG antibodies. As a control, IgE was detected using polyclonal anti-human IgE antibodies.

Table 1. Glycans recognized by anti-IgE response upon immunization with IgE-Fel d 1

Glycans recognized by sera of Fel d 1, IgE-Fel d 1, IgE or IgE(PNG)-Fel d 1 immunized mice. Glycan identification number (GID) with the determined relative fluorescence unit (RFU) values and the corresponding glycan structures according to the IUPAC Nomenclature of Carbohydrates. Sp represents the specific linkers used to conjugate the glycans to the array, while Su represents sulfation at the indicated oxygen atom. Monosaccharide abbreviations: Fuc (fucose), FucNAc (N-acetyl-L-fucosamine), Gal (D-Galactose), GalNAc (N-fcetyl-D-galactosamine), Glc (glucose), GlcA (D-glucuronic acid), GlcNAc (N-ccetyl-D-glucosamine), Kdn (3-deoxy-D-glycero-D-galacto-nonulosonic acid), Kdo (3-deoxy-D-manno-octulosonic acid), Man (Mannose), ManNAc (N-acetyl-D-mannosamine), Neu5Ac (N-acetylneuraminic acid), Rha (rhamnose) and Rib (ribose).

Table 1. Glycans recognized by anti-IgE response upon immunization with IgE-Fel d 1

GID	Fel d 1	IgE	IgE(PNG)-Fel d 1	IgE-Fel d 1	Glycan Structure
362	130.5	1150.5	303.3	5258.3	Galα1-3(Fucα1-2)Galβ1-3GalNAcα-sp
48	133.5	174.5	123.0	4667.5	Neu5Acα-sp
1016	132.0	389.5	142.8	4165.3	Ribfβ1-4Galβ1-4GlcNAcα1-4Galβ1-3GlcNAcα1-sp
822	127.0	858.5	133.8	3967.8	GalNAcα1-4Galβ-sp
509	131.0	140.5	120.5	3951.5	Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ-sp
1004	150.5	289.3	139.8	3898.8	Fucα1-2Galβ1-3GalNAcα1-3GlcNAcα1-sp
1810	134.5	370.8	131.5	3822.0	Glcβ1-3GalNAcβ1-4GalNAcβ1-4Galβ1-sp
211	137.5	307.0	259.3	3344.5	Kdoα2-8Kdoα-sp
215	129.0	206.5	123.3	2878.3	Fucα1-2Galβ1-3GlcNAcβ-sp
1416	211.5	540.0	307.3	2529.8	Rhaα1-3FucNAcα1-3FucNAcα1-3DFucNAcα1-sp
62	332.0	983.3	408.0	2392.8	GlcAβ-sp
288	125.5	146.3	120.5	2257.5	3-O-Su-Galβ1-4(Fucα1-3)GlcNAcβ-sp
307	131.0	145.3	132.0	2107.8	Kdnα2-3Galβ1-3GlcNAcβ-sp
383	136.5	194.3	146.5	1315.5	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp
117	130.0	277.5	130.3	1283.5	GlcNAcβ1-4GlcNAcβ-sp
1254	129.0	467.0	716.3	1167.3	ManNAcα1-3Rhaα1-3Rhaα1-3Rhaα1-3GalNAcα1-sp
204	691.0	513.8	485.0	1135.8	4-O-Su-GalNAcβ1-4GlcNAcβ-sp
217	194.5	336.0	187.3	847.8	Fucα1-2Galβ1-3GalNAcα-sp
202	131.5	195.5	133.8	706.0	6-O-Su-GalNAcβ1-4(6-O-Su)GlcNAcβ-sp
814	281.0	150.3	135.0	589.0	Manα1-6Manα-sp
495	129.0	160.5	119.5	543.5	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ-sp
164	135.0	146.3	124.0	541.3	GlcAβ1-3GlcNAcβ-sp

Fig 1

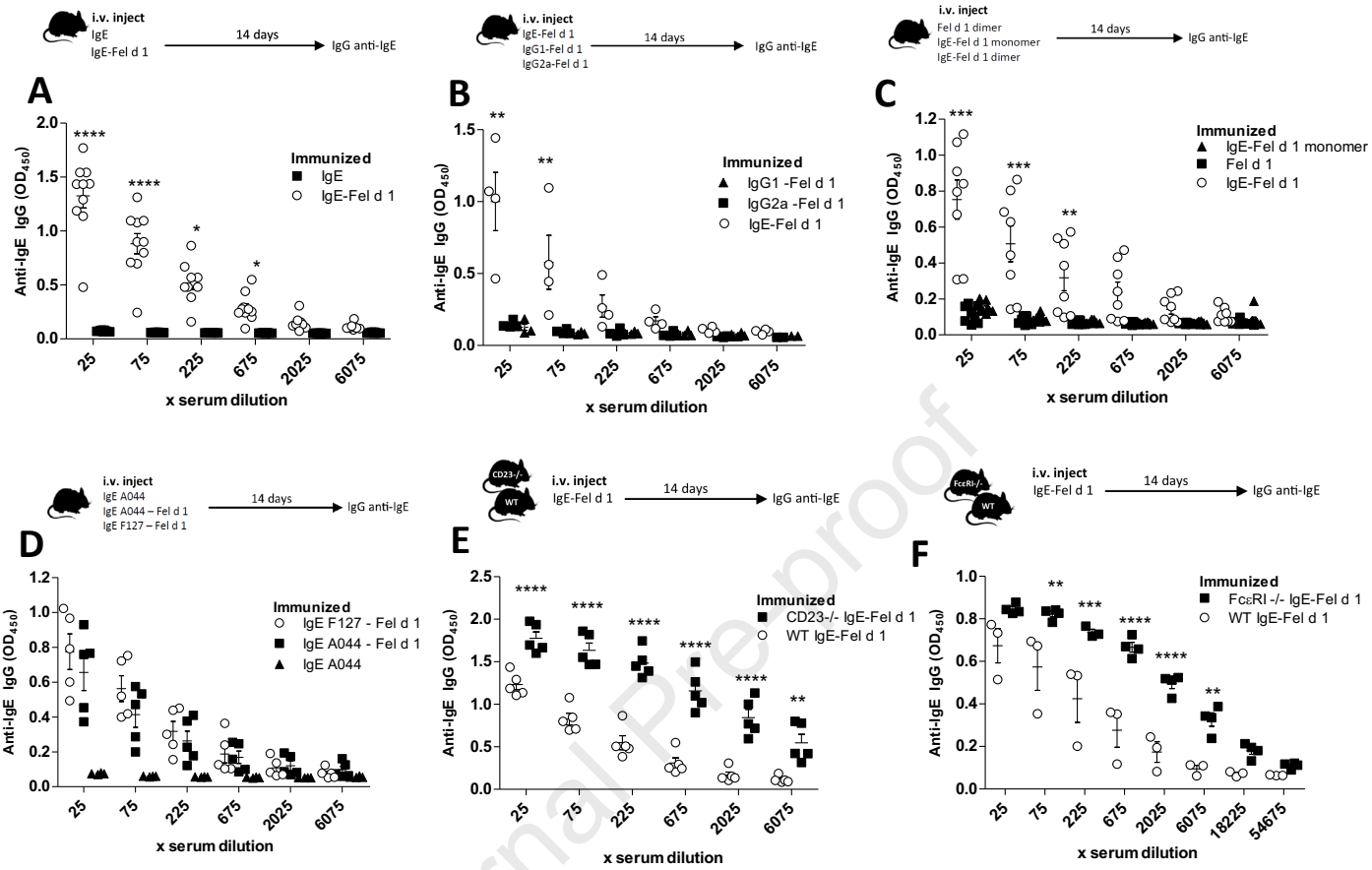
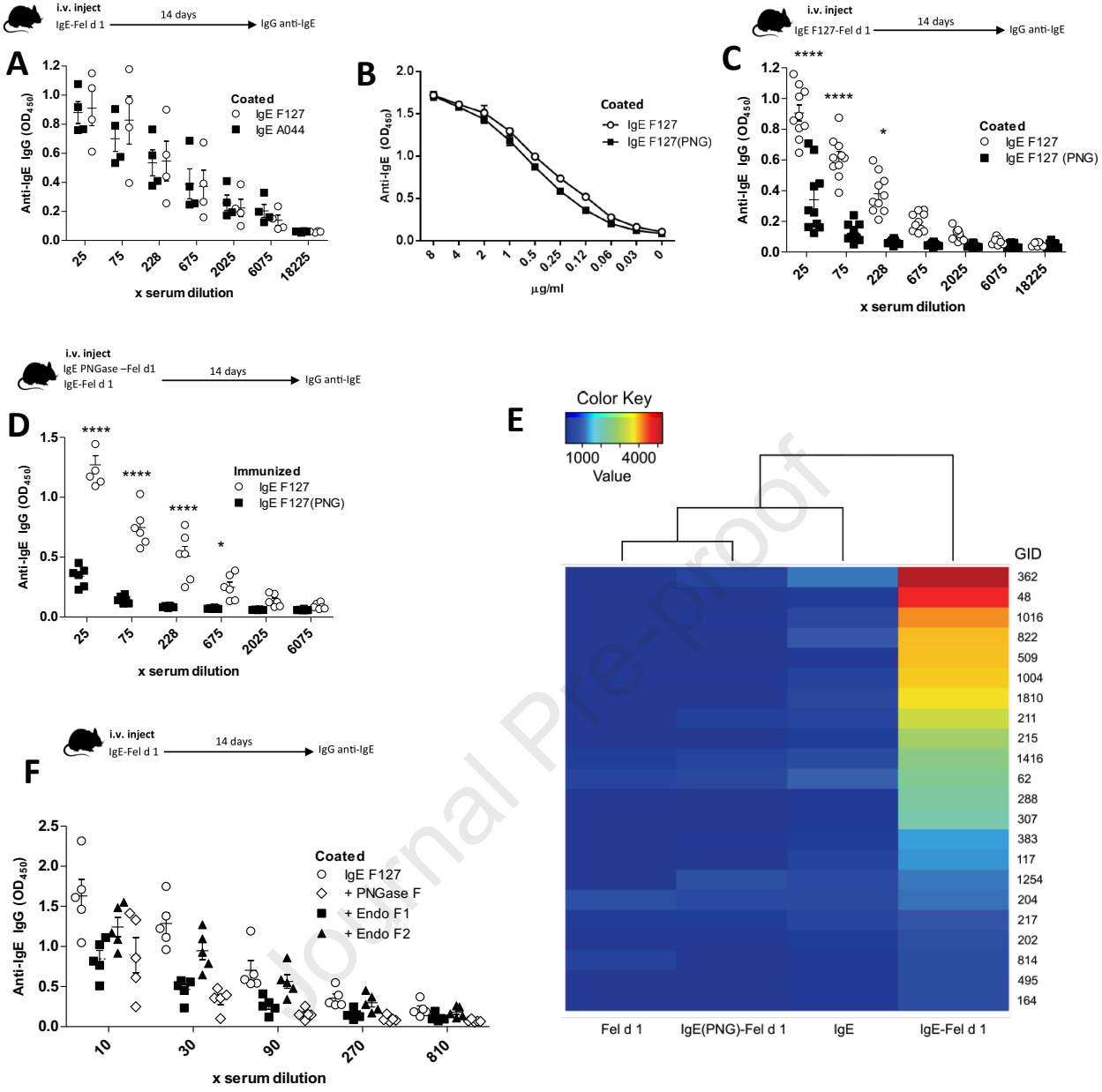


Fig 2



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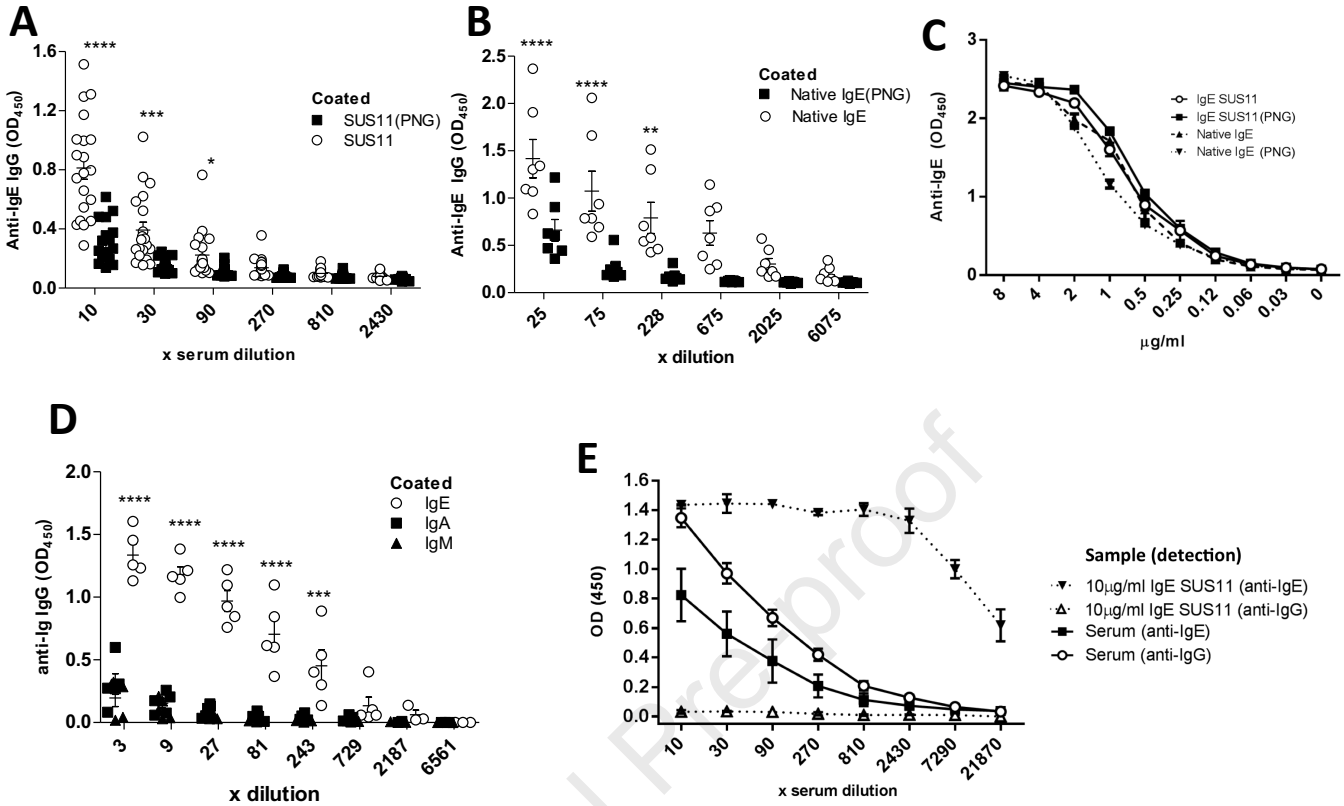
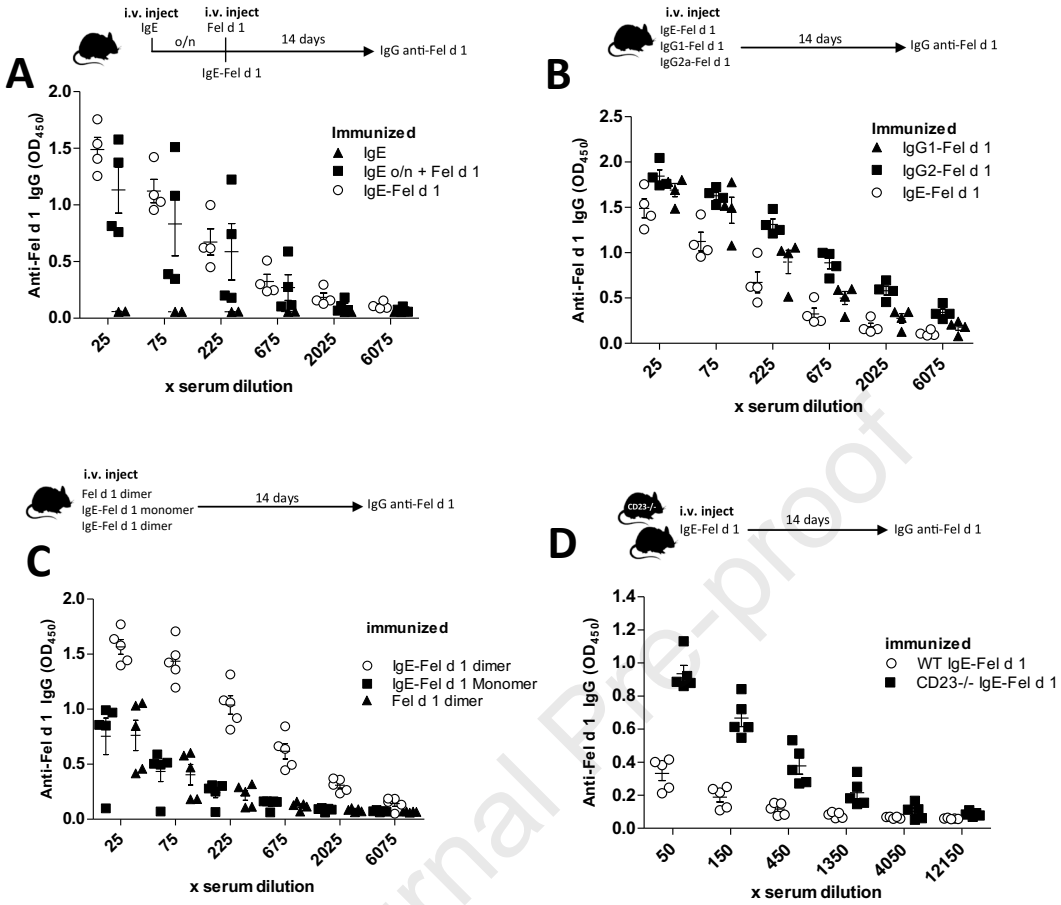


Fig E1



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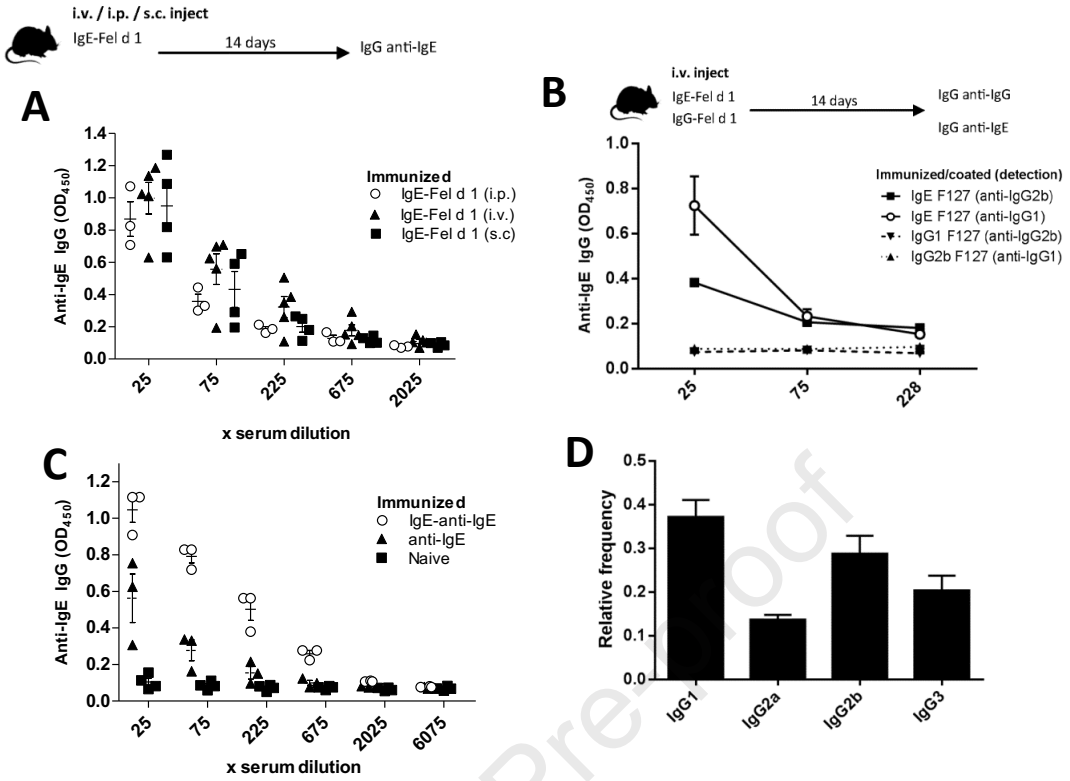

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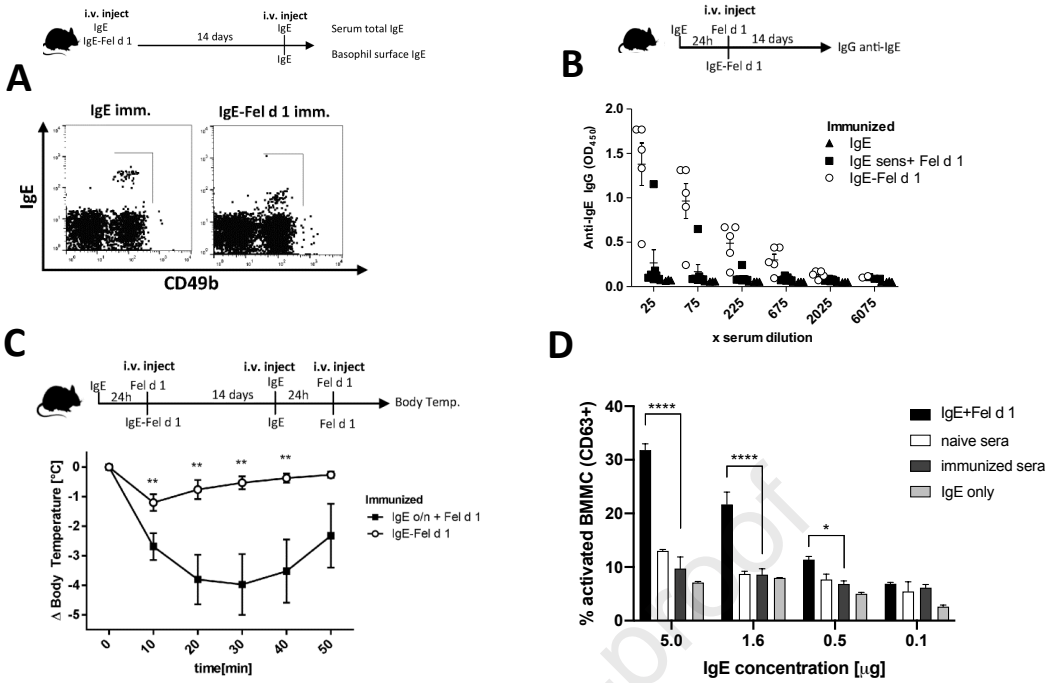

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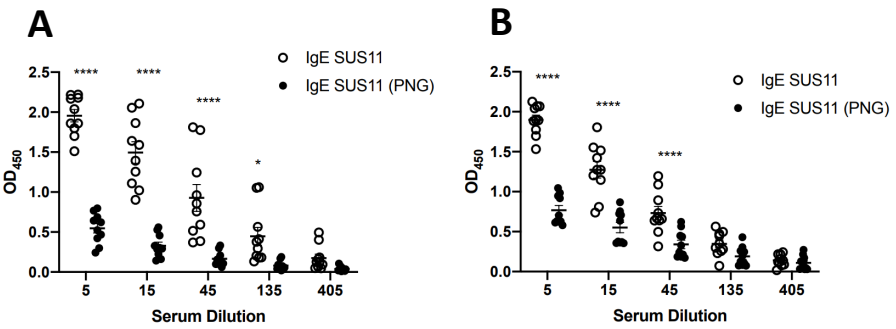
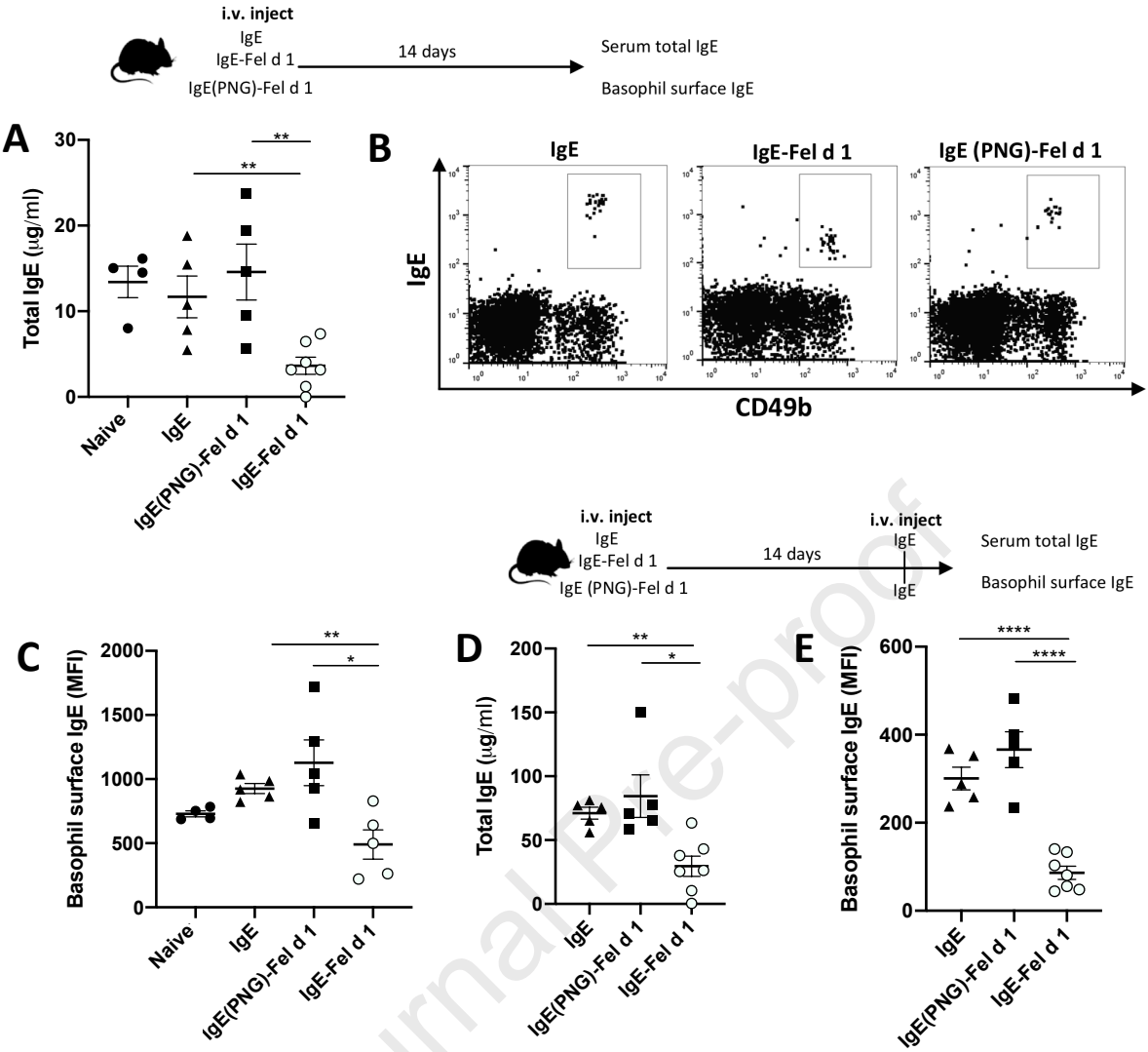


Fig 3



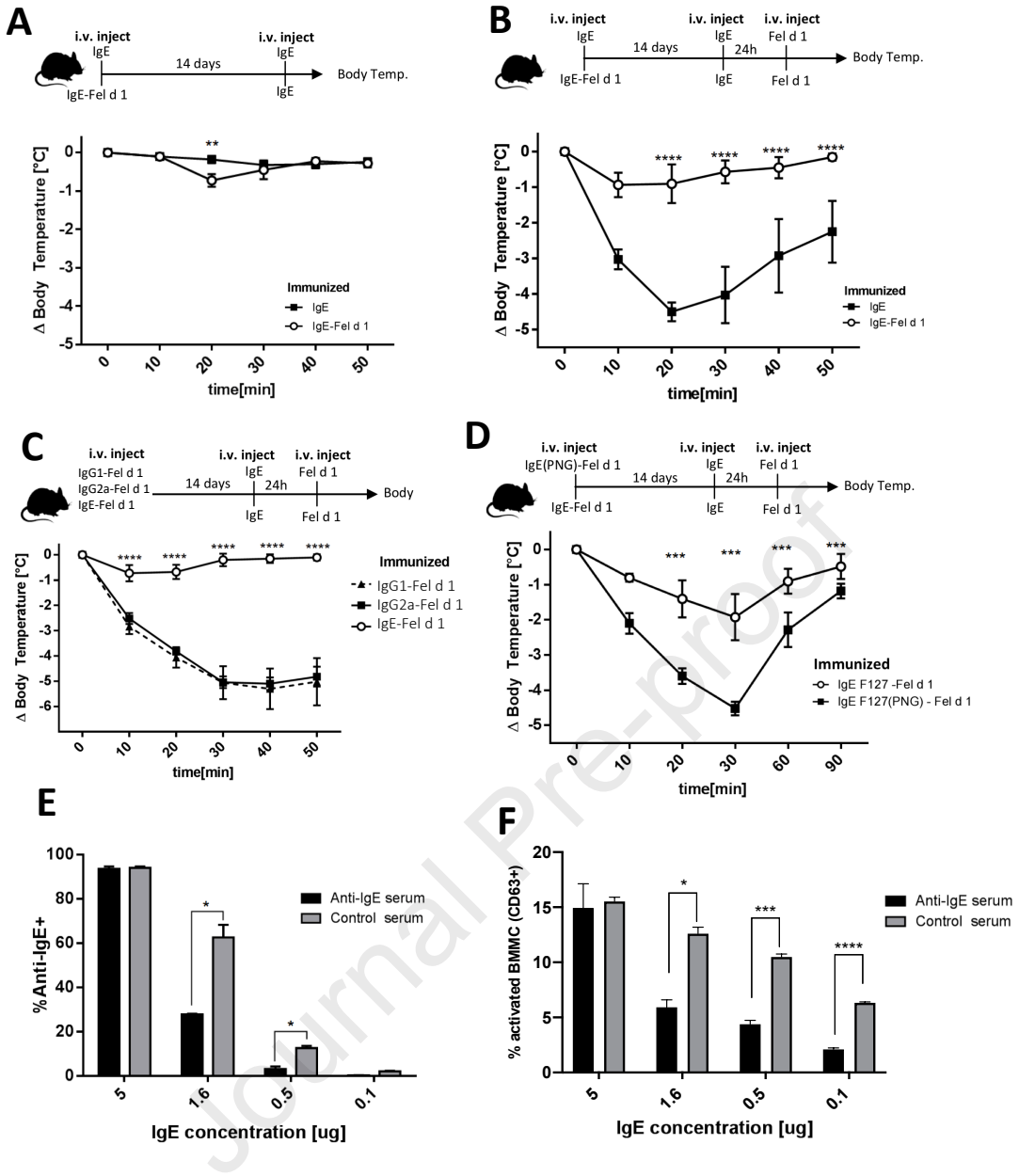


Fig 5

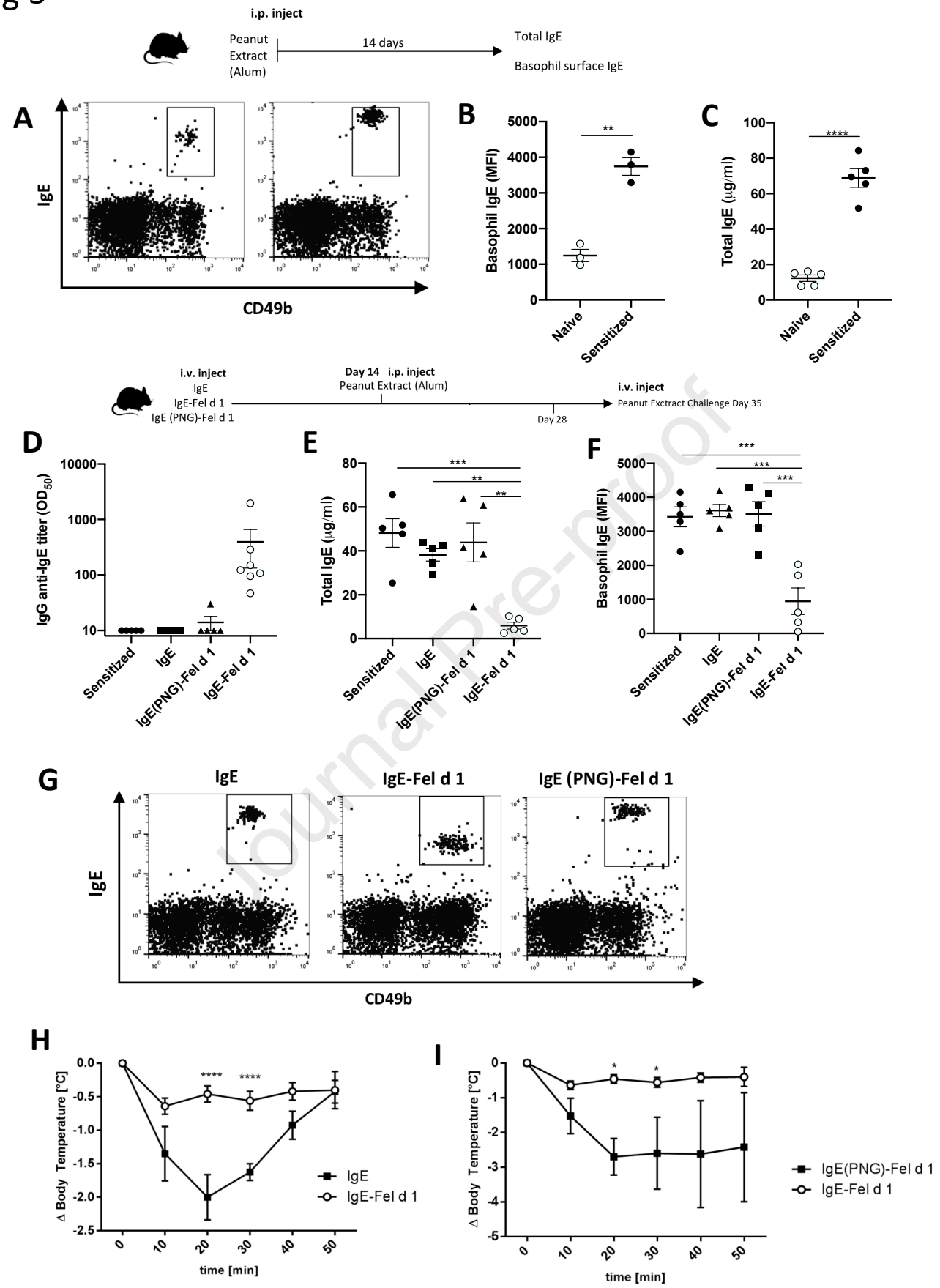

i.p. inject
Peanut Extract (Alum)
14 days
Total IgE
Basophil surface IgE
A
IgE
CD49b
B
Basophil IgE (MFI)
Naive
Sensitized
C
Total IgE (µg/ml)
Naive
Sensitized
i.v. inject
IgE
IgE-Fel d 1
IgE (PNG)-Fel d 1
Day 14 i.p. inject
Peanut Extract (Alum)
Day 28
i.v. inject
Peanut Exctract Challenge Day 35
D
IgG anti-IgE titer (OD50)
Sensitized
IgE
IgE(PNG)-Fel d 1
IgE-Fel d 1
E
Total IgE (µg/ml)
F
Basophil IgE (MFI)
G
IgE
IgE-Fel d 1
IgE (PNG)-Fel d 1
CD49b
H
Δ Body Temperature [°C]
time [min]
IgE
IgE-Fel d 1
I
Δ Body Temperature [°C]
time [min]
IgE(PNG)-Fel d 1
IgE-Fel d 1

Fig E3