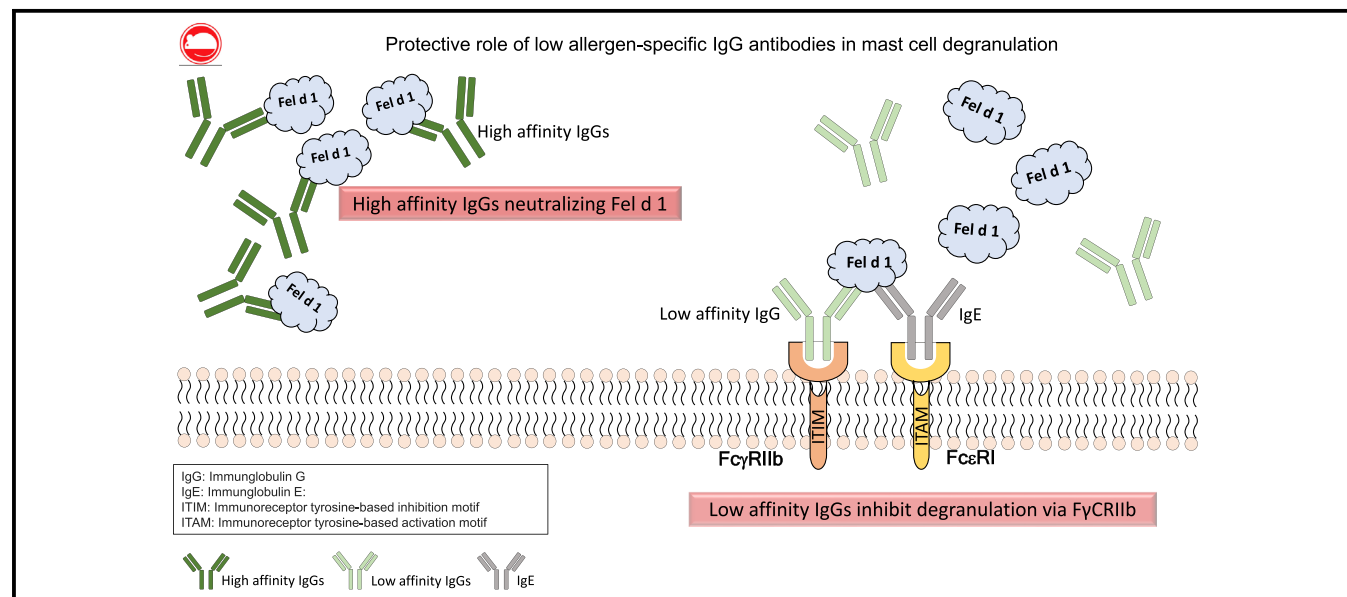


An unexpected protective role of low-affinity allergen-specific IgG through the inhibitory receptor FcγRIIb

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GRAPHICAL ABSTRACT



Background: Induction of allergen-specific IgG antibodies is a critical parameter for successful allergen-specific immunotherapy. IgG antibodies can inhibit IgE-mediated mast cell activation through direct allergen neutralization or through the inhibitory receptor FcγRIIb. The affinity of IgE antibodies to the allergen has been shown to be critical for cellular activation.

Objective: Here we addressed the question of affinity thresholds of allergen-specific IgG antibodies for inhibition of mast cell activation using 2 different mAbs against the major cat allergen Fel d 1 both *in vitro* and *in vivo* in mice.

Methods: Sequences of the 2 high-affinity mAbs were back-mutated to germline, resulting in low-affinity (10^{-7} mol/L) antibodies of the exact same specificity.

Results: Using these newly generated recombinant antibodies, we demonstrate that low-affinity antibodies are still able to

inhibit mast cell activation through FcγRIIb but do not neutralize the allergen.

Conclusion: Antibody affinity dictates the mechanism of mast cell inhibition, and IgG antibodies triggering the inhibitory FcγRIIb pathway can show a broader cross-reactivity pattern than previously thought. This indicates that allergen-specific immunotherapy generates a larger protective umbrella of inhibitory IgG antibodies than previously appreciated. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: Allergy, vaccination, FcγRIIb-mediated inhibition, Fel d 1, low-affinity antibodies

IgE-mediated allergies have reached epidemic proportions, and allergic rhinoconjunctivitis and asthma affect about one third of the population in developed countries.^{1,2} The principal effector

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Abbreviations used

BMMC: Bone marrow–derived mast cell
 DARPIn: Designed ankyrin repeat protein
 FACS: Fluorescence-activated cell sorting
 RU: Relative units
 SIT: Allergen-specific immunotherapy

mechanism of IgE is mediated by the high-affinity IgE receptor (FcεRI), which is expressed by a number of cells, including mast cells and basophils. Free IgE binds to these cells and activates FcεRI on cross-linking by allergen, inducing release mediators (histamines and others) that cause allergic symptoms.³

A number of possibilities exist to treat IgE-mediated allergies. Most currently used therapies consist of symptomatic medications, such as antihistamines or corticosteroids. The only disease-modifying therapy available is allergen-specific immunotherapy (SIT), whereby patients are treated with multiple doses of allergen, changing the immune system in a way to tolerate the allergens better.^{4,5} Even though there is an ongoing discussion about the most critical effector mechanism or mechanisms for this state of enhanced “tolerance,” it is clear that induction of allergen-specific IgG correlates with reduced symptoms and is taken as evidence for successful therapy.^{6,7}

Allergen-specific IgG antibodies can improve allergic symptoms in a number of ways. The most important mechanisms are allergen neutralization and engagement of the inhibitory Fc receptor (FcγRIIb). Allergen neutralization blocks binding of allergen to FcεRI-bound IgE and therefore follows the classical path of neutralizing antibodies blocking, for example, viruses from engaging their receptors. IgG antibodies need to block essentially all epitopes available for IgE binding on the allergen to be effective at neutralizing the allergen. Therefore this mechanism follows the rules of competitive inhibition.^{8–10}

Antibodies engaging the inhibitory receptor block cellular activation by triggering FcγRIIb, leading to recruitment of the SH2 domain-containing inositol 5'-phosphatase, which in turn dephosphorylates signal transduction molecules below FcεRI. Such inhibitory IgG antibodies also must bind the allergen, but it is not necessary to block all available epitopes. Indeed, a single IgG antibody might be sufficient to block polyclonal IgE antibodies of multiple specificities.^{6,11} Thus, in contrast to neutralizing antibodies, which act as competitive inhibitors, inhibiting IgG antibodies are better compared with noncompetitive enzyme inhibitors.^{3,12,13}

The role of antibody affinity in blocking allergen activity is poorly understood. As outlined above, however, one might expect a different effect of affinity for allergen neutralization versus engagement of FcγRIIb because affinity is more important for competitive versus noncompetitive inhibitors. To test this hypothesis, we used 2 well-described high-affinity antibodies for Fel d 1, the major allergen in cats, and generated low-affinity versions by reversing affinity maturation and mutating variable regions back to their germline sequences. Using *in vitro* and *in vivo* models, we demonstrated that high-affinity antibodies block cellular activation through allergen neutralization and engagement of FcγRIIb, whereas low-affinity antibodies do not neutralize the allergen but are still effective through the inhibitory mechanism. Hence, despite inefficient allergen

binding, antibodies with low IgG affinity might still efficiently block allergic responses *in vivo*.

METHODS**Mice**

Female BALB/cOlaHsd mice were purchased from Envigo (London, United Kingdom) at the age of 5 to 7 weeks.

Cloning, expression, and purification of germline monoclonal anti-Fel d 1 IgG_{2a}

Cloning. Two Fel d 1 IgG_{2a}s (A044 and F127) that recognized different epitopes on Fel d 1 were kindly provided by Dr Franziska Zabel and produced, as described previously.⁴ Briefly, BALB/c mice were immunized with 50 μg of rFel d 1 coupled to virus-like particles derived from the bacteriophage Qβ. Fel d 1–specific memory B cells were isolated by using fluorescence-activated cell sorting (FACS). Sorted B cells were used to generate a random combinatorial antibody library that was screened by using the Sindbis-based mammalian cell display,¹⁴ which allowed isolation of the 2 Fel d 1 IgG_{2a} antibodies. Sequences of the variable regions from IgG_{2a} A044 and F127 were aligned with the germline genomic database available at <http://www.imgt.org> to clone germline sequences. Germline sequences of the variable regions from IgG_{2a} A044 and F127 were synthesized by GeneArt (Thermo Fisher, Waltham, Mass), with additional restriction cutting sites (*Xho*I and *Apa*I for the light chain and *Sac*I and *Eco*RI for the heavy chain). Variable regions of light chains from each epitope were digested with *Xho*I and *Apa*I and cloned into the light chain plasmid, which contains the constant region of the kappa chain. Similarly, variable regions of heavy chains were inserted in front of the gamma constant region in the heavy chain plasmid through *Sac*I and *Eco*RI. Afterward, the new constructed heavy and light chain plasmids were digested with *Asc*I, *Pac*I for heavy chain and with *Nhe*I, *Pme*I for the light chain. The fragments were then cloned into the final expression plasmid (pCB15-GL) under 2 independent pCMV promoters (see Fig E1 in this article's Online Repository at www.jacionline.org).

Recombinant monomeric and dimer Fel d 1 proteins were generated, as previously described.⁴ Briefly, a cDNA that encoded a covalent dimer of chains 2 and 1 of Fel d 1, spaced by the 15-amino-acid linker (GGGS)₃, was obtained by using PCR. This amplicon was cloned into a modified version of pET-42a, leading to addition of the sequence LEHHHHHHGGC at the C-terminus of the covalent dimer.

Expression. pCB15-GL, comprising both heavy and light chain germline sequences, was transfected on HEK293T cells. Briefly, 5 × 10⁷ cells were seeded in a T75 flask in complete growth medium (Dulbecco modified Eagle medium [catalog 11965084; Gibco, Carlsbad, Calif] supplemented with 10% FCS and 1% penicillin and streptomycin) 1 day before transfection. The DMEM complete growth medium was changed to serum-free DMEM medium before transfection. Fifteen micrograms of pCB15-GL plasmid DNA was mixed with 45 μL of polyethylenimine and incubated at room temperature for 15 minutes, which was added to the cells dropwise. Serum-free medium was changed to DMEM complete growth medium 6 hours after transfection and collected 24, 48, and 69 hours after transfection.

Purification. Germline IgG_{2a} in supernatants from transfected cells was purified by using the HiTrap Protein G HP column (catalog 7-0402-01; GE Healthcare, Fairfield, Conn) with the ÄKTA Pure protein purification system (GE Healthcare). Expression and purification of germline antibodies were checked on 10% SDS-PAGE.

ELISA of germline A044 IgG_{2a} and F127 IgG_{2a}. ELISA plates were coated with 1 μg/mL mouse Fel d 1 dimer. Purified mature and germline antibodies A044 IgG_{2a} and F127 IgG_{2a} were first added to the wells at increasing concentrations diluted in PBS/casein. Bound antibodies were detected by using horseradish peroxidase–conjugated anti-mouse IgG_{2a} (Southern Bioscience, Birmingham, Ala). The reaction was stopped after 5 minutes with 1 mol/L sulfuric acid, and absorbance was measured at OD 450 nm in a standard ELISA reader (BioTek Microplate Readers; BioTek, Winooski, Vt).

TABLE I. Somatic hypermutation of anti-Fel d 1 antibodies compare with *Mus musculus* germline sequences

	Germline, light chain	Mutations in light chain		Germline, heavy chain	Mutations in heavy chain		Total mutations
		FR	CDR		FR	CDR	
A044	V: IGKV9-120*01		8	V: IGHV1-7*01		14	22
	J: J2*01	6	2	J: JGHJ2*01	9	5	
F127	V: IGKV3-4*01		7	V: IGHV1-82*01F		22	29
	J: IGKJ4*01	6	1	J: JGHJ3*01F	19	3	
				D: IGHD2-2*01F			

Sequences from the variable regions of 2 Fel d 1-specific IgG_{2a} (A044 and F127 antibodies) were aligned with the immunoglobulin germline genomic database by <http://www.imgt.org>. With the alignment, more somatic hypermutations were observed in total in the variable region of the F127 antibody than in the A044 antibody. CDR, Complementarity-determining region; FR, framework region.

Bone marrow-derived mast cells

The method for generating bone marrow-derived mast cells (BMMCs) has been described previously.⁴ Generally, 5×10^8 bone marrow cells were cultured in complete RPMI medium supplemented with recombinant mouse stem cell factor (50 ng/mL; R&D Systems, Minneapolis, Minn) and recombinant mouse IL-3 (30 ng/mL; R&D Systems). The final differentiation status of BMMCs was determined by measuring c-Kit expression (allophycocyanin-anti-mouse c-Kit; BD, Franklin Lakes, NJ) and FcεRI levels (phycoerythrin-anti-mouse FcεRI; eBioscience, San Diego, Calif) by using flow cytometry 4 to 5 weeks after culture.

Flow cytometry

BMMCs were washed with FACS buffer (2% FBS in PBS) and blocked for 1 hour with mouse anti-Fel d 1 IgG_{2a} (5 μg/mL) at 4°C. Afterward, BMMCs were washed twice with FACS buffer and stained with fluorescein isothiocyanate-anti-mouse IgG_{2a} (clone R19-15; BD) antibodies for 30 minutes at 4°C. Cells were then washed twice with FACS buffer and measured by using flow cytometry.

Affinity measurement using surface plasmon resonance

Measurements were performed on a Biacore X100 instrument (GE Healthcare Europe GmbH, Freiburg, Germany). We used HBS-EP+ (10 mmol/L HEPES [pH 7.4], 150 mmol/L NaCl, 3 mmol/L EDTA, and 0.005% surfactant P20) as running buffer. To determine the kinetic parameters of the mature and 1 germline (F127) anti-Fel d 1 antibodies, a CM5 chip was immobilized on both flow cells with 2000 relative units (RU) of Protein A/G and then injected with either mature (A044, F127) or germline (F127) anti-Fel d 1 IgG_{2a} at a concentration of 25 nmol/L, resulting in a coupling level of between 500 and 1000 RU. Fel d 1 monomer and dimer proteins were then applied in a 2-fold serial dilution series (5 concentrations, starting at 100 nmol/L for mature F127 and A044 antibodies and at 2500 nmol/L for germline F127) for 2 minutes at a constant flow rate. For the germline A044, we immobilized 200 RU monomeric and dimeric Fel d 1 protein on flow cell 2 of a CM5 sensor chip. Flow cell 1 remained uncoated and served as a negative control for unspecific binding to the chip surface. Samples were then injected for 3 minutes at different concentrations (500 nmol/L to 31.25 nmol/L) at a flow rate of 30 μL/min. Dissociation rates were measured for another 2 minutes at constant buffer flow. An additional buffer control was measured and subtracted from the sample sensorgram. BIAevaluation software (Biacore) was used based on the 1:1 interaction model (Langmuir) to determine kinetic parameters.

Epitope mapping

To determine whether the germline anti-Fel d 1 antibodies have kept the same epitope specificities as the mature antibodies, we analyzed binding epitopes of the germline antibodies on surface plasmon resonance. Monomeric Fel d 1 was immobilized onto a CM5 sensor chip to a capture

level of 1000 RU. The surface was first saturated with 2 serial injections of each parental mature antibody until most of the epitopes were covered and the sensorgram reached a threshold value. The germline and opposite parental antibodies were then injected once at concentrations of 500 and 50 nmol/L, respectively, for 2 minutes at a flow rate of 30 μL/min.

BMMC degranulation

The degree of BMMC degranulation was determined by measuring β-hexosaminidase release. Briefly, BMMCs were loaded with 40 μg/mL Fel d 1-specific mouse IgE A044 or 40 μg/mL Fel d 1-specific mouse IgE F127 overnight at 37°C. The cells were then incubated with mouse Fel d 1 dimer or mouse Fel d 1 dimer-IgG_{2a} complexes (30, 300, or 3000 nmol/L) for 1 hour at 37°C, followed by washing once with Tyrode buffer (0.1% BSA, 0.1% glucose, 10 mmol/L HEPES, 130 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl₂, and 2 mmol/L MgCl₂, pH 7.4). Cells were spun down to collect supernatants. Meanwhile, cell pellets were lysed with 0.5% Triton X-100 (Sigma, St Louis, Mo) in Tyrode buffer. Both the supernatants and lysed cells were incubated with p-nitrophenyl-N-acetyl-p-D-glucosamine (Sigma) in 0.1 mol/L sodium citrate buffer (pH 4.5) for 90 minutes at 37°C. The reaction was stopped by adding 0.2 mol/L glycine (pH 10.7). ODs at 405 nm were determined as readout. The degree of degranulation was calculated as follows:

$$\% \text{Degranulation} = \text{OD}_{\text{supernatant}} / (\text{OD}_{\text{supernatant}} + \text{OD}_{\text{cell lysis}}).$$

Ear prick test

Mice were given intravenous injections of 10 μg of mouse IgE (A044 or F127 and/or 100 μg IgG_{2a}; clone A044, A044GL, F127, or F127GL). Twenty-four hours later, each mouse was given an intravenous injection of 200 μL of Evans blue dye (0.5% in PBS); designed ankyrin repeat proteins (DARPs) against mouse FcγRIIb receptor (kindly provided by Dr Alex Eggel¹⁵) were used for blocking the receptor by means of local subcutaneous injection on the ears 10 minutes before the ear prick. Twenty-five minutes later, after administration of Evans blue dye, 1 drop of Fel d 1 dimer (200 μg/mL) was placed on the outer ear skin of anesthetized mice after pricking the ears with a needle. Mast cell degranulation was determined 45 minutes after the ear prick and quantified based on the degree of dye extravasation. Briefly, mice were killed, and ears were collected. Dye was extracted from the collected ears by means of digestion with 150 μL of 1 mol/L KOH overnight at 37°C. The next day, 150 μL of 5% H₃PO₄ in acetone was added, and samples were centrifuged. Supernatants were collected and measured at an OD of 595 nm to quantify the extracted dye.

Passive systemic anaphylaxis

Mice were sensitized passively with 10 μg of Fel d 1-specific IgE A044 by means of intravenous tail vein injection. The next day, either 100 μg of IgG_{2a} (A044 or germline A044 or F127 or germline F127) or 100 μg of IgG_{2a}

TABLE II. Affinities of germline and mature antibodies to Fel d 1 monomer and Fel d 1 dimer proteins

Antibodies	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (M)
Fel d 1 monomer			
A044 IgG _{2a} mature*	2.30×10^5	9.81×10^{-4}	4.26×10^{-9}
A044 IgG _{2a} germline*	ND	ND	2.88×10^{-7}
A044 IgG _{2a} germline†	1.58×10^4	2.53×10^{-3}	1.59×10^{-7}
F127 IgG _{2a} mature*	3.03×10^5	3.90×10^{-4}	1.28×10^{-9}
F127 IgG _{2a} germline*	9.14×10^3	7.87×10^{-3}	8.62×10^{-7}
Fel d 1 dimer			
A044 IgG _{2a} mature*	3.54×10^5	3.81×10^{-4}	1.07×10^{-9}
A044 IgG _{2a} germline*	ND	ND	2.77×10^{-7}
A044 IgG _{2a} germline†	2.46×10^4	4.06×10^{-3}	1.64×10^{-7}
F127 IgG _{2a} mature*	3.74×10^5	2.90×10^{-4}	7.76×10^{-10}
F127 IgG _{2a} germline*	1.80×10^4	3.67×10^{-3}	2.03×10^{-7}

K_D , Equilibrium dissociation constant; k_{off} , dissociation rate constant; k_{on} , association rate constant; ND, not determined.

*Kinetic values were obtained by using a CM5 chip displaying captured mature or germline anti-Fel d 1 antibodies by means of immobilization on Protein A/G.

†Kinetic values were obtained by using a CM5 chip immobilized with either Fel d 1 monomer or Fel d 1 dimer.

combined with AT128 mAb against mouse FcγRIIb¹⁶ was injected intravenously. After 24 hours, mice were challenged by means of intravenous injection of 5 μg of Fel d 1. Core body temperature was measured immediately before and every 10 minutes after antigen challenge for 60 minutes.

RESULTS

Binding properties of Fel d 1-specific germline antibodies

Two low-affinity Fel d 1-specific germline antibodies, IgG_{2a} A044 and F127, were generated to investigate the role of antibody affinity in blocking allergen activity. To this end, Fab regions of mature IgG_{2a} A044 and F127 antibodies⁷ were aligned with the germline sequences of both heavy and light variable chains to determine the origins of V, D, and J genes before somatic recombination (Table I). The germline Fabs were then cloned into mouse IgG_{2a} expression vector (see Fig E1) and expressed in 293HEK cells. The purity and size of the germline antibodies after protein G purification were determined by using SDS-PAGE and compared with the parental mature antibodies. As shown in Fig E2 in this article's Online Repository at www.jacionline.org, both germline antibodies bear the same size as the mature heavy and light chains.

To assess binding capacity of the germline antibodies to Fel d 1, we performed ELISA assays (see Fig E3 in this article's Online Repository at www.jacionline.org). Using either monomeric or dimeric Fel d 1 as coating antigen, we found that binding of germline antibodies to Fel d 1 was less efficient than binding of mature antibodies at low concentrations of Fel d 1 but still reached a plateau comparable with that obtained with mature antibodies at high concentrations of Fel d 1.

Kinetic parameters of germline antibodies (germline A044 and germline F127) were assessed by using surface plasmon resonance and compared with those of mature antibodies (A044 and F127, Table II). The mature antibody F127 displayed higher association and lower off-rates than germline F127 on both monomeric and dimeric Fel d 1 (Table II), resulting in more than 100 times lower affinity for the germline antibody compared with the mature one. Similarly, compared to the mature antibody A044, the germline antibody A044 exhibits a 100 times lower affinity for monomeric and dimeric Fel d 1 (Table II).

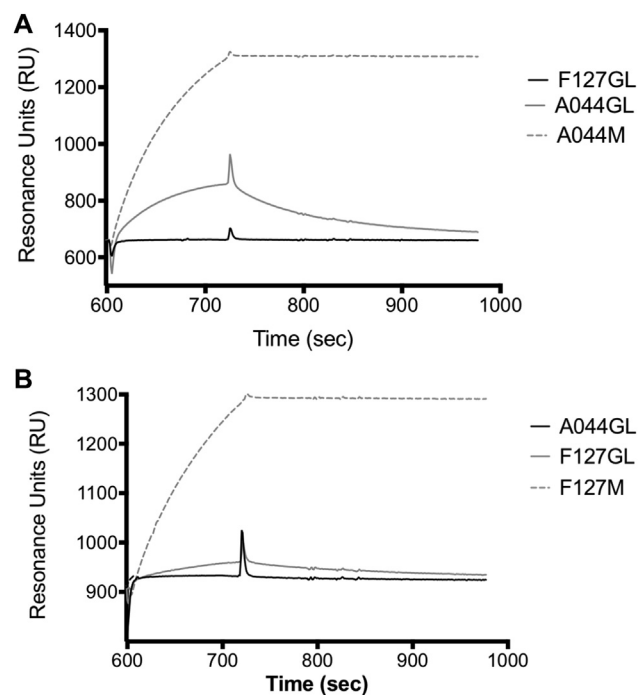


FIG 1. Epitope mapping of germline IgG_{2a} A044 and F127 antibodies. Surface plasmon resonance was used to assess whether germline (GL) antibodies have the same epitope specificity as parental antibodies. The sensor chip immobilized with 1000 RU of Fel d 1 protein was twice saturated with either mature IgG_{2a} A044 (A) or mature IgG_{2a} F127 (B) antibody, each lasting 2 minutes. Subsequently, mature (50 nmol/L) and germline (500 nmol/L) antibodies were injected for 2 minutes at a constant flow rate.

The kinetic parameters for germline A044 could not be measured in the same manner as the others for technical reasons. For this reason, we used a different set-up (ie, direct immobilization of Fel d 1 proteins rather than captured antibodies) (see Fig E4 in this article's Online Repository at www.jacionline.org). Comparable equilibrium dissociation constant (K_D) values were obtained by using both methods, validating the determination of kinetic parameters on immobilized Fel d 1 for the germline A044 antibody.

To confirm that germline antibodies recognized the same epitope as the parental mature antibodies, we performed epitope mapping on a chip exhibiting immobilized monomeric Fel d 1. The chip surface was saturated with either mature A044 or F127 antibodies until the sensorgram reached a maximal response (Fig 1). Subsequently, either germline A044 or F127 antibodies, as well as mature antibodies for control purposes, were injected for an additional 2 minutes. As expected, both germline antibodies were no more able to bind to immobilized Fel d 1 when the chip was saturated with the corresponding mature antibodies, indicating that they recognize the same epitope as their corresponding parental mature antibodies. In contrast, germline antibodies were able to bind, although at a low level, if Fel d 1 was presented by the other parental mature antibody.

Fel d 1-specific germline IgG_{2a} inhibits BMMC degranulation *in vitro*

It has been shown previously that IgG antibodies should bear an affinity high enough to compete with IgE for inhibiting cellular activation. However, the role of antibodies with low affinity in blocking allergen activity has not been studied in detail. Because

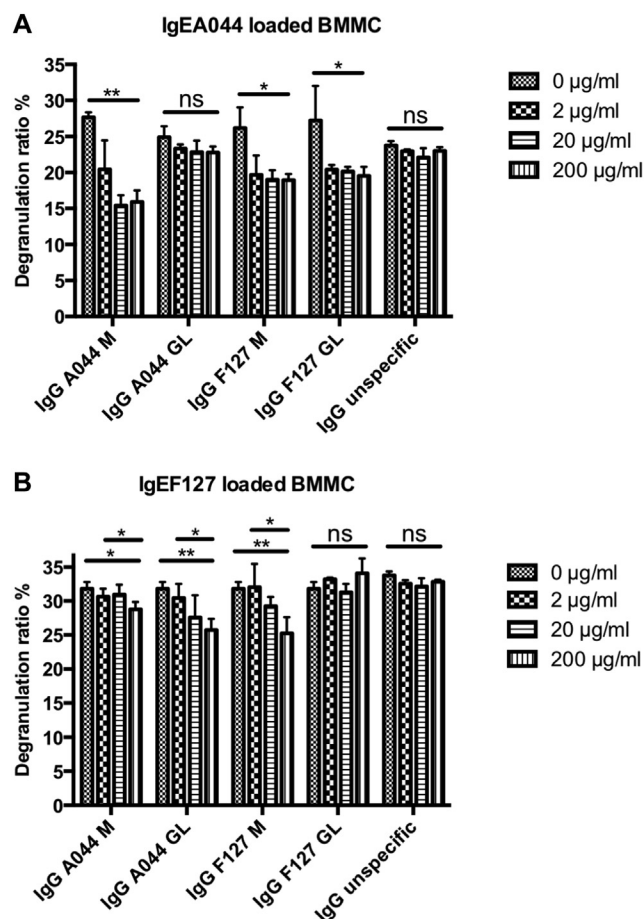


FIG 2. Low-affinity germline mAbs inhibited IgE-induced BMMC activation *in vitro* only if the epitope was different. **A**, BMMCs were loaded with 4 µg/mL Fel d 1-specific mouse IgE A044 antibody overnight and then incubated with Fel d 1-specific mouse IgG_{2a} mature (M)/germline (GL) A044 and IgG_{2a} mature/germline F127 antibodies at different concentrations (3, 30, and 300 µg/mL) with mouse Fel d 1 dimer (3 µg/mL) for 1 hour. **B**, BMMCs were loaded with 4 µg/mL Fel d 1-specific mouse IgE F127 antibody overnight and then incubated with Fel d 1-specific mouse IgG_{2a} mature/germline A044 and IgG_{2a} mature/germline F127 antibodies at different concentrations (3, 30, and 300 µg/mL) with mouse Fel d 1 dimer (3 µg/mL) for 1 hour. Naive BMMCs, which have not been treated with unspecific IgGs, were used as controls. The degree of BMMC degranulation was determined by measuring β-hexosaminidase release. Data shown are representative for 3 independent experiments. **P* ≤ .05 and ***P* ≤ .01. ns, Not significant.

our germline IgG_{2a} antibodies recognize the same epitopes as the mature antibodies, although with low affinity, we next investigated the capacity of germline antibodies to block IgE-induced mast cell degranulation. For this purpose, BMMCs were incubated with IgE A044 or F127 overnight and then challenged with dimeric Fel d 1 alone or with increasing concentrations of IgG_{2a} (A044 mature, A044 germline, F127 mature, and F127 germline antibodies). Interestingly, in case of BMMCs loaded with IgE A044 antibody, IgG_{2a} F127 germline antibody significantly inhibited mast cell degranulation, whereas IgG_{2a} A044 germline antibody, which recognizes the same epitope as IgE A044, did not impede degranulation, even at a concentration of up to 200 µg/mL (Fig 2, A). These data suggest that the inhibitory effect of germline antibodies is not mediated through competition with IgE for binding to Fel d 1 but occurs

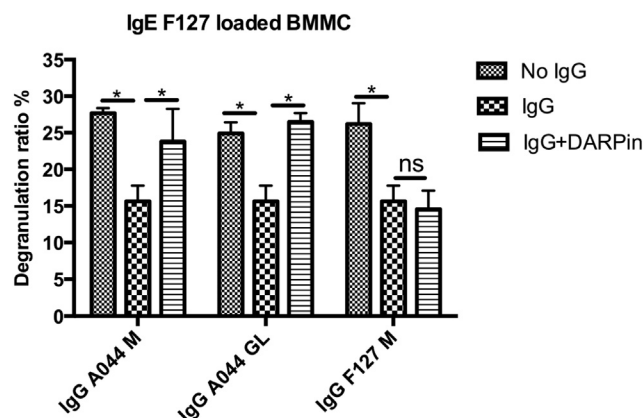


FIG 3. Low-affinity germline mAbs inhibited IgE-induced BMMC activation could be abolished through blocking FcγRIIb. BMMCs were loaded with 4 µg/mL Fel d 1-specific mouse IgE F127 antibody and FcγRIIb DARPin overnight and then incubated with Fel d 1-specific mouse IgG_{2a} mature (M)/germline (GL) A044 and IgG_{2a} mature/germline F127 antibodies at a concentration of 300 µg/mL with mouse Fel d 1 dimer (3 µg/mL) for 1 hour. The group that had not been treated with anti-FcγRIIb DARPin was taken as a control. The degree of BMMC degranulation was determined by measuring β-hexosaminidase release. Data shown are representative for 3 independent experiments. **P* ≤ .05. ns, Not significant.

primarily through engaging the inhibitory receptor FcγRIIb. The same results were obtained when mast cells were loaded with IgE F127 and incubated with either A044 germline or F127 germline antibody (Fig 2, B). Again, the germline IgG_{2a} antibody sharing the same epitope as IgE F127 could not abrogate cell degranulation, whereas the A044 germline antibody could.

Inhibitory effect of germline IgG_{2a} *in vitro* is FcγRIIb dependent

To confirm that the inhibitory effect of germline A044 antibody is mediated through the inhibitory receptor FcγRIIb rather than through competition with IgE for binding to Fel d 1, a biological inhibitor (based on DARPin technology) of mouse FcγRIIb receptor was used for blocking the receptor.¹⁵ As shown in Fig 3, 50 µg/mL DARPin was sufficient to abrogate the inhibitory effect of both mature and germline IgG_{2a} A044 antibodies on IgE F127 antibody-loaded mast cells. In contrast, blocking FcγRIIb receptor had no effect on the neutralizing activity of mature IgG_{2a} F127 antibody. These results indicated that Fel d 1-specific germline IgG_{2a}-induced inhibition of BMMC degranulation is FcγRIIb dependent.

Fel d 1-specific germline IgG_{2a} antibody blocks mast cell activation *in vivo*

Having demonstrated that low-affinity germline antibodies can block mast cell activation *in vitro*, we next addressed whether this concept translates to the *in vivo* situation. BALB/c mice were injected with either A044 or F127 IgEs alone or along with mature A044, germline A044, mature F127, or germline F127 IgG_{2a} antibody (Figs 4 and 5). Skin prick tests were performed with Fel d 1 dimer 1 day after injection of IgE and 25 minutes after injection of Evans blue dye to visualize the allergic reaction (Figs 4, A, and 5, A). In agreement with the *in vitro* results, coadministration of IgG_{2a} F127 germline antibody with IgE

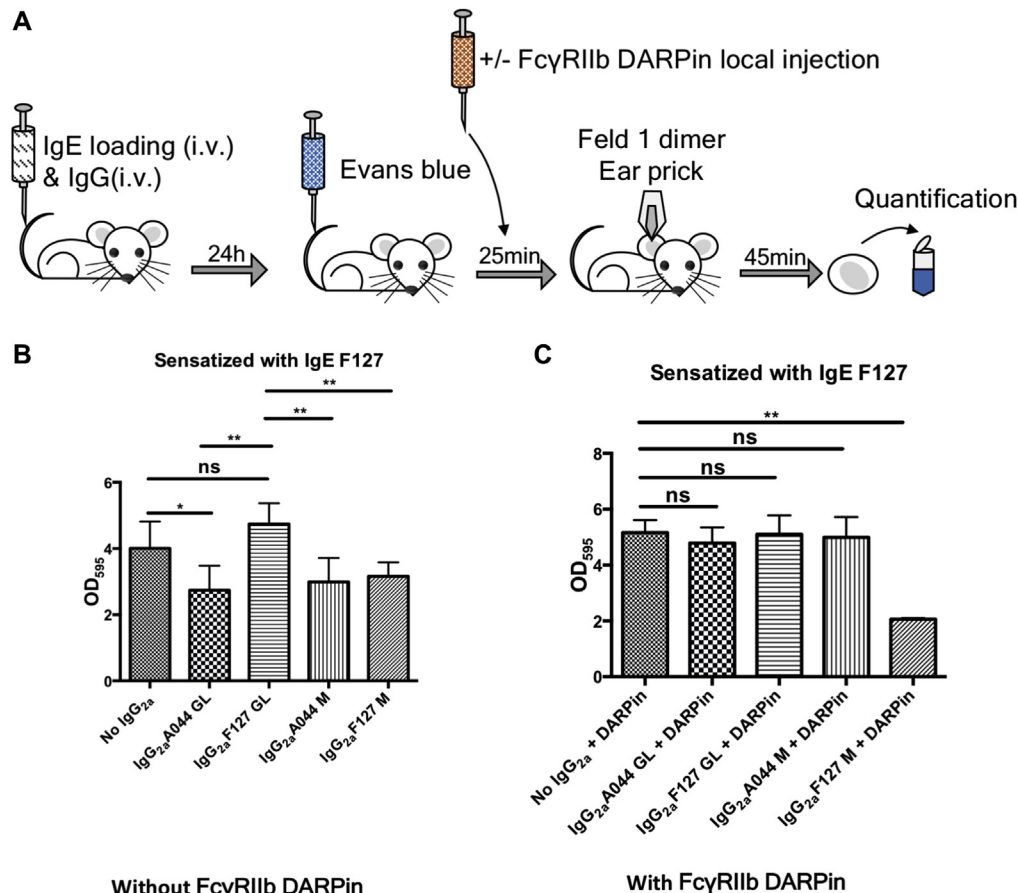


FIG 4. Low-affinity germline mAbs inhibited IgE F127 antibody-induced mast cell activation *in vivo* through blocking FcγRIIb. **A**, Ear prick scheme to determine mast cell degranulation *in vivo*. Mice were given intravenous (*i.v.*) injections of 10 μ g of mouse IgE F127 and/or 100 μ g of IgG_{2a} (A044 mature, A044 germline, F127 mature, or F127 germline) antibodies. Twenty-four hours later, each mouse was injected with 200 μ L of Evans blue dye intravenously with/without receiving local FcγRIIb DARPIn injection. Afterward, mice were challenged with 20 μ g of Fel d 1 dimer on the outer ear skin 25 minutes later. Quantification of mast cell degranulation was determined by the degree of dye extravasation measured at an OD of 595 nm after digestion of the ears. **B**, Severe mast cell degranulation was observed in the mice loaded with only IgE F127 antibody but much weaker when IgE F127 antibody was loaded together with IgG_{2a} A044 mature (M), A044 germline (GL), or F127 mature antibody. However, the combination of IgE F127 and F127 germline antibodies did not show lower degranulation ratio compared with other groups. **C**, Mast cell degranulation was observed in mice loaded with IgE F127 antibody plus DARPIn and was inhibited only when loaded together with IgG_{2a} F127 mature antibody plus DARPIn. IgG_{2a} A044 germline and A044 antibody-induced inhibition were all abolished on local DARPIn treatment. Dye was extracted from collected ears by means of digestion with KOH overnight at 37°C. After full digestion, H₃PO₄ was added, and supernatants were collected and measured at an OD of 595 nm for quantification. Results show means $\pm$ SEMs of OD₅₉₅, and data shown are representative for 5 independent experiments. **P* $\leq$.05 and ***P* $\leq$.01. ns, Not significant.

A044 antibody efficiently prevented mast cell degranulation, whereas no inhibition was observed with germline A044 antibody (Fig 4, B). Using a similar IgG setup but with sensitization with A044 and challenge with F127 confirmed these results as germline IgG_{2a} F127 antibodies with the same epitope did not display any inhibition of mast cell degranulation. In contrast germline IgG_{2a} A044 antibody with a different specificity successfully prevented degranulation (Fig 5, B).

To further confirm the inhibitory capacity of the germline IgG_{2a} A044 antibody in IgE F127-induced mast cell activation, a dose-response experiment was performed. To this end, mice were sensitized with IgE F127 antibody together with different doses of IgG_{2a} A044 germline antibody. As shown in Fig E5 in

this article's Online Repository at www.jacionline.org, strong mast cell degranulation was observed in mice injected with IgE F127 antibody alone and inhibition by IgG_{2a} A044 germline antibody occurred in a dose-dependent manner.

DARPIn against mouse FcγRIIb was used for blocking the receptor by means of local subcutaneous injection into the ears of mice before prick tests to elucidate the mechanisms of germline IgG_{2a} antibody-mediated inhibition of mast cell degranulation *in vivo*. As mentioned above, coadministration of IgG_{2a} of a second specificity (no matter whether germline or mature antibodies were used) efficiently prevented mast cell degranulation in BALB/c mice (Figs 4, B, and 5, B), whereas no IgG-dependent inhibition of mast cell degranulation was

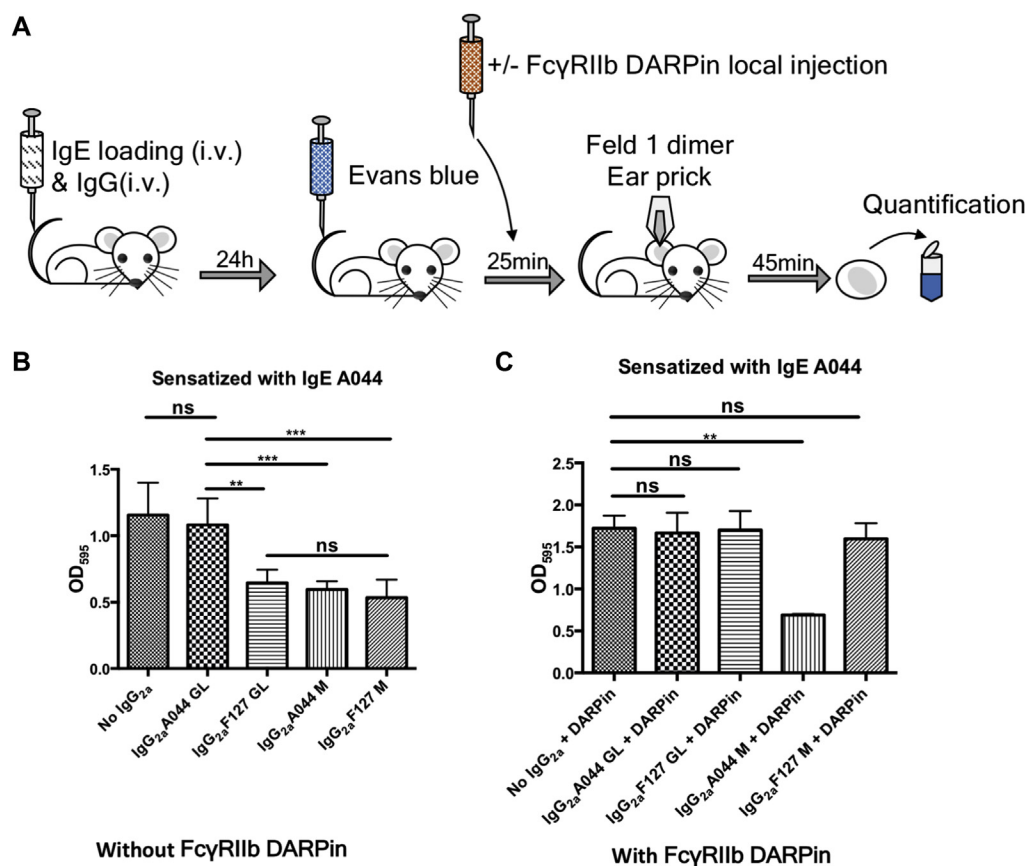


FIG 5. Low-affinity germline mAbs inhibited IgE A044-induced mast cell activation *in vivo* through blocking FcγRIIb. **A**, Ear prick scheme to determine mast cell degranulation *in vivo*. Mice were given intravenous (i.v.) injections of 10 μg of mouse IgE A044 and/or 100 μg of IgG_{2a} (A044 mature, A044 germline, F127 mature, or F127 germline) antibodies. Twenty-four hours later, each mouse was injected with 200 μL of Evans blue dye intravenously with/without receiving local FcγRIIb DARPIn injection. Afterward, mice were challenged with 20 μg of Fel d 1 dimer on the outer ear skin 25 minutes later. Quantification of mast cell degranulation was determined by the degree of dye extravasation measured at an OD of 595 nm after digestion of the ears. **B**, Severe mast cell degranulation was observed in mice loaded with only IgE A044 and IgE A044 plus IgG_{2a} A044 germline (GL) antibodies but was much weaker when IgE A044 antibody was loaded together with IgG_{2a} A044 mature (M), F127 germline, or F127 mature antibodies. **C**, Mast cell degranulation was observed in mice loaded with IgE A044 antibody plus DARPIn and inhibited only when loaded together with IgG_{2a} A044 mature antibody plus DARPIn. IgG_{2a} F127 and F127 germline antibody-induced inhibition was all abolished on local DARPIn treatment. Dye was extracted from collected ears by means of digestion with KOH overnight at 37°C. After full digestion, H₃PO₄ was added, and supernatants were collected and measured at an OD of 595 nm for quantification. Results show means ± SEMs of OD₅₉₅, and data shown are representative for 5 independent experiments. ***P* ≤ .01 and ****P* ≤ .001. *ns*, Not significant.

observed in DARPIn-treated mice (Figs 4, C, and 5, C). Hence Fel d 1–specific germline IgG_{2a} antibody induces inhibition of mast cell activation in a FcγRIIb-dependent manner *in vivo*.

To better mimic the physiologic situation and directly assess whether germline IgG_{2a} antibody mediated inhibition of systematic allergic reaction *in vivo* is FcγRIIb dependent, mice were passively sensitized with Fel d 1–specific IgE A044 antibody with or without IgG_{2a} (A044 or germline A044 or F127 or germline F127 antibodies) combined with or without AT128 mAb against mouse FcγRIIb receptor by means of intravenous injection. Fel d 1 challenge was performed 12 hours after sensitization. In accordance with *in vivo* results from passive cutaneous anaphylaxis, coadministration of IgG_{2a} of a second specificity (both germline and mature antibodies) could dramatically alleviate the systematic anaphylaxis (core temperature decrease) in BALB/c mice (see Fig E6, B, in this

article's Online Repository at www.jacionline.org), whereas no IgG-dependent relief of systematic anaphylaxis was observed when FcγRIIb was blocked by mAb AT128 (see Fig E6, B). These results confirmed again that Fel d 1–specific germline IgG_{2a}–induced inhibition of allergic reaction is FcγRIIb dependent.

In summary, when IgE-induced mast cell degranulation is blocked by engaging the inhibitory receptor, the affinity of IgGs is less important compared with that of IgGs compared with IgE by a classical neutralization mechanism. Thus low-affinity antibodies do not neutralize the allergen but block mast cell activation through FcγRIIb.

DISCUSSION

Allergen-specific IgG antibodies are induced during SIT and correlate with protection against allergic symptoms. IgG

antibodies block mast cell activation either by simply neutralizing the allergen or by engaging the inhibitory FcγRIIb.¹⁷ Here we demonstrate that the 2 mechanisms are governed by different affinity thresholds.

SIT has been shown to induce high-affinity blocking antibodies with somatic mutations in their complementarity-determining region as an indication for affinity maturation.¹⁰⁻¹⁸ These findings are recapitulated here because high-affinity Fel d 1-specific antibodies induced by immunizing mice with Fel d 1 coupled to Qβ-derived virus-like particles showed a high degree of hypermutation, and affinity was dramatically lower on back-mutation of the variable regions to germline sequences. Despite vastly different affinities, the germline antibodies exhibited the same epitope specificity as the parental antibodies; indeed, somatic hypermutation usually does not change the epitopes recognized. This allowed us to compare the importance of antibody affinity without interference by a difference in antibody specificity. Using this system, we found that the allergen-neutralizing pathway was strongly dependent on affinity because germline sequence antibodies did not block mast cell activation either *in vitro* or *in vivo*. In contrast, the inhibitory FcγRIIb pathway was independent of affinity because low-affinity antibodies were similarly effective as high-affinity antibodies.

In contrast to the mouse, in human subjects only the presence of FcγRIIb on the basophil surface is well documented,⁷ but the presence of FcγRIIb on mast cells remains unclear.^{19,20} Thus the described mechanism will likely occur in human basophils, but its importance in human mast cells requires more detailed investigation. Furthermore, because IgA antibodies do not bind to the FcγRIIb our results might only apply to IgG and not IgA antibodies.

The described findings support the mechanistic hypothesis that allergen neutralization is best described by a model analogous to competitive inhibition in enzymology, whereas the inhibitory pathway follows a noncompetitive model.²¹ From a clinical perspective, this might explain why SIT based on allergoids is remarkably effective, even though many of the allergen epitopes are slightly denatured because of their chemical modification.²² Our results might explain these findings because low-affinity antibodies appear to be clinically more important than previously assumed. Indeed, it is known that allergies are often caused by cross-reactive IgE antibodies that recognize a number of different allergens generated by vastly different species.^{23,24} One would expect that allergen-neutralizing IgG antibodies have a similar cross-reactivity pattern.^{7,21,25} However, our data suggest that IgG antibodies triggering the inhibitory FcγRIIb pathway show an even broader cross-reactivity pattern because of the lower affinity-threshold.¹⁹ Therefore SIT can generate a protective umbrella of inhibitory IgG antibodies that is larger than previously thought.

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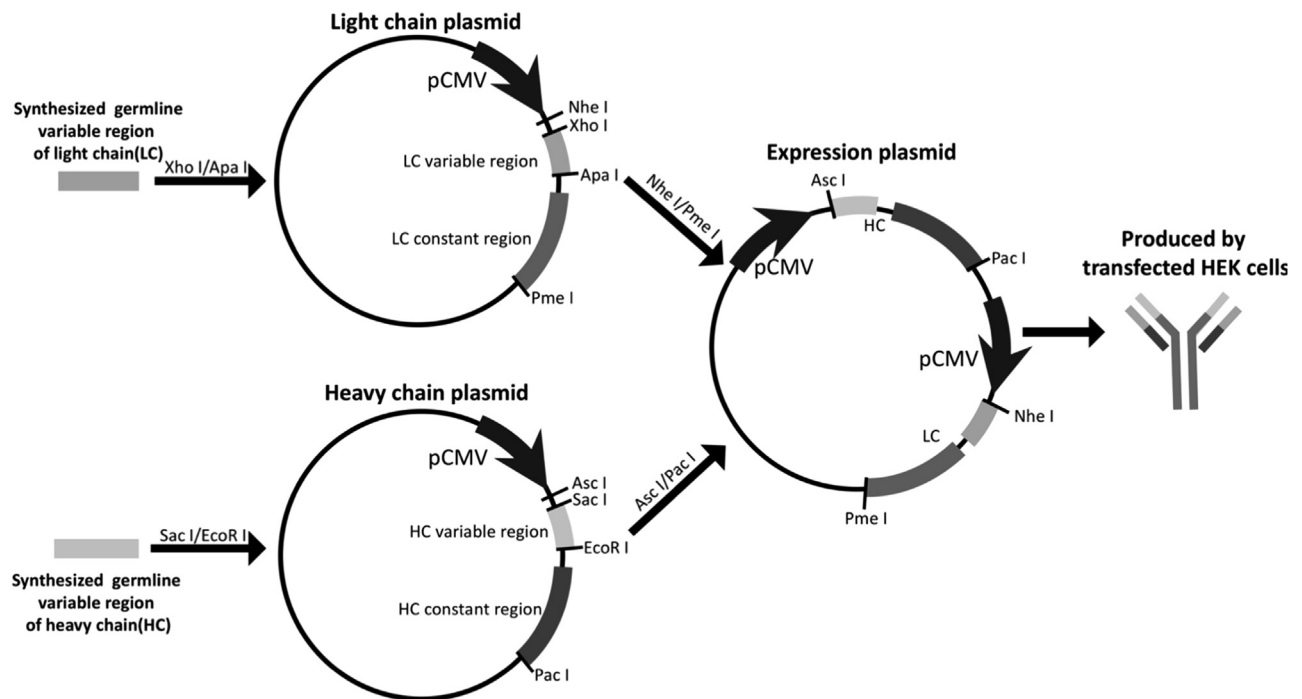


FIG E1. Cloning strategy for germline IgG_{2a} A044 and F127 antibodies. Germline sequences of the variable regions from IgG_{2a} A044 and F127 antibodies were synthesized by GeneArt, with additional restriction cutting sites *Xho*I and *Apa*I for the light chain (LC) and *Sac*I and *Eco*RI for the heavy chain (HC). First, variable regions of the light chain were cloned into the light chain plasmid, which comprises the constant region of the mouse kappa chain. Variable regions of heavy chains were inserted in front of the mouse gamma constant region. Second, the newly constructed heavy and light chain plasmids were digested with *Nhe*I, *Pme*I, *Asc*I, and *Pac*I and cloned into the final expression plasmid (pCB15-GL) under 2 independent pCMV promoters.

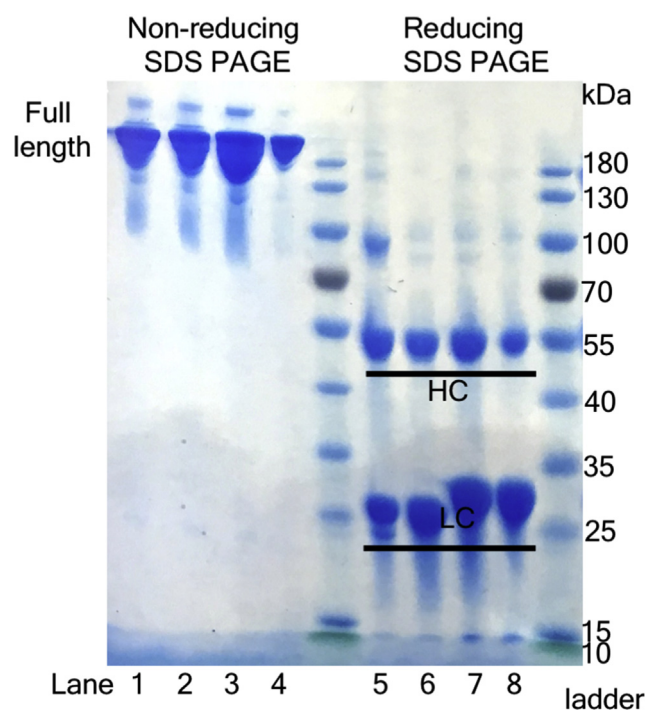


FIG E2. IgG_{2a} germline mAbs bear the same size as mature mAbs. Germline mAbs IgG_{2a} A044 and F127 antibodies after purification showed the same size as the mature IgG_{2a} A044 and F127 antibodies. *Lane 1*, Nonreduced A044 germline mAb; *lane 2*, nonreduced A044 mature mAb; *lane 3*, nonreduced F127 germline mAb; *lane 4*, nonreduced F127 mature mAb; *lane 5*, reduced A044 germline mAb; *lane 6*, reduced A044 mature mAb; *lane 7*, reduced F127 germline mAb; *lane 8*, reduced F127 mature mAb. HC, Heavy chain; LC, light chain.

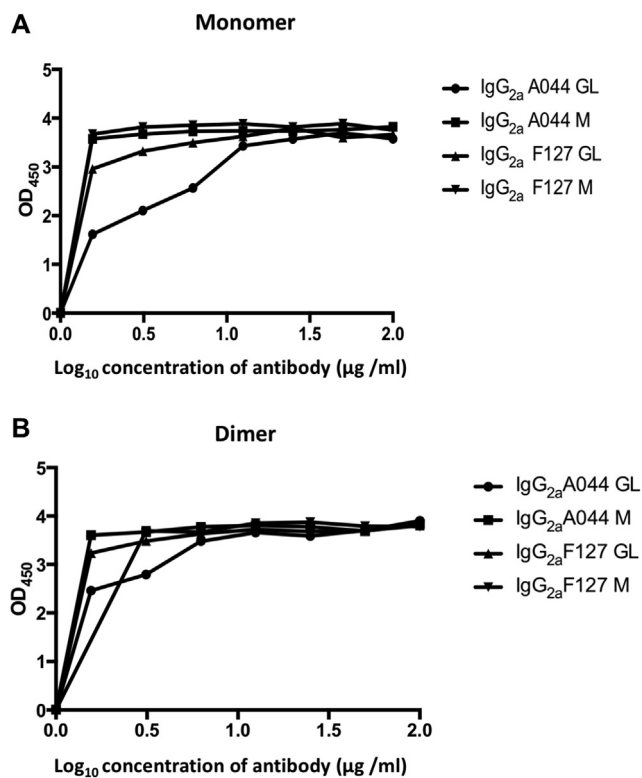


FIG E3. Binding properties of the 2 mAbs (IgG_{2a} A044 and F127) to monomeric Fel d 1 (**A**) and dimeric Fel d 1 (**B**) assessed by means of ELISA. Germline mAbs displayed lower properties of binding to either monomeric Fel d 1 or dimeric Fel d 1, regardless of isotype. *GL*, Germline; *M*, mature.

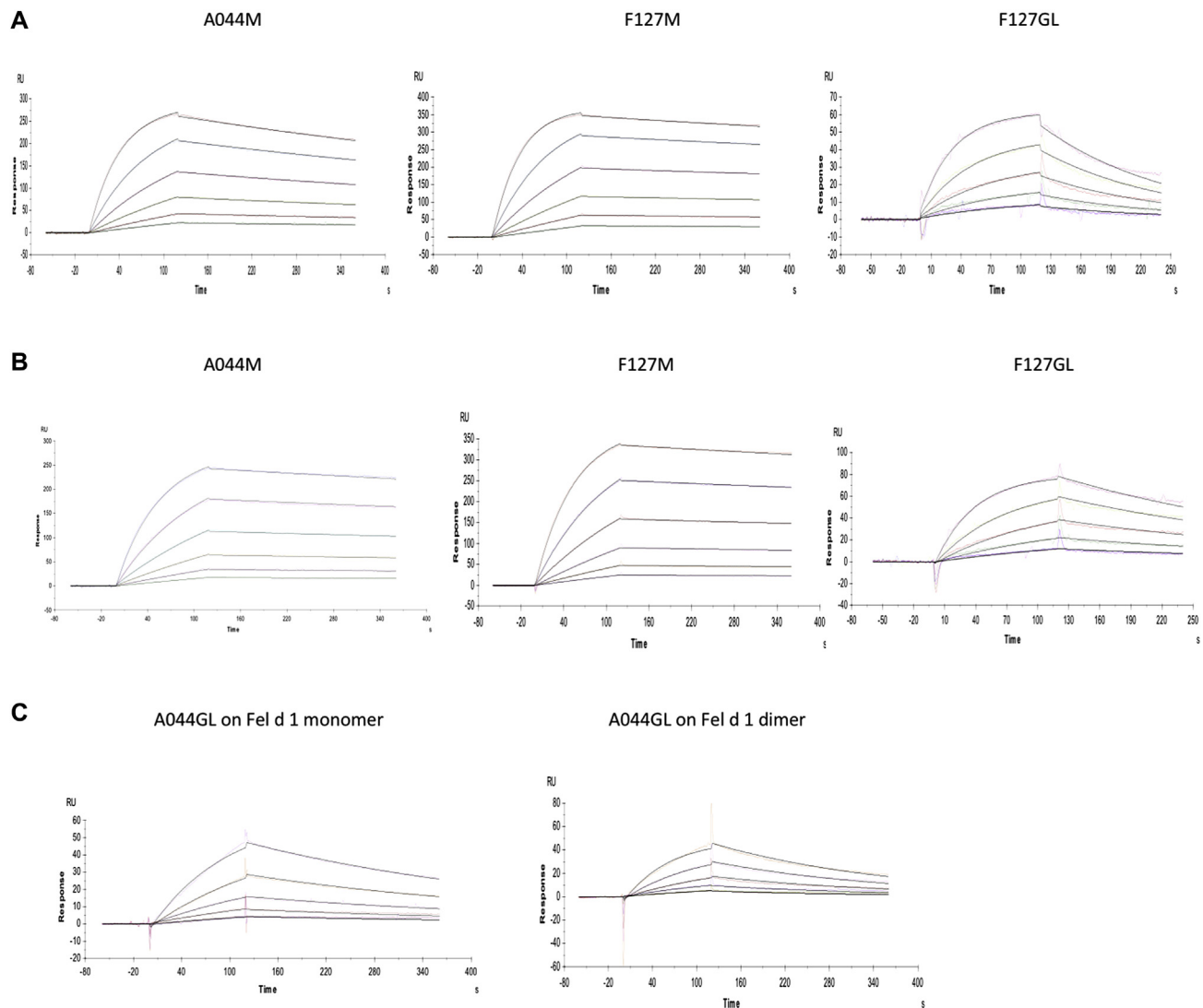


FIG E4. Biacore data of binding to Fel d 1 proteins of captured IgG_{2a} mature (*M*) and germline (*GL*) anti-Fel d 1 antibodies. **A** and **B**, Sensorgrams for binding to the Fel d 1 monomer (Fig E4, *A*) or Fel d 1 dimer (Fig E4, *B*) of a series of 5 antibody concentrations (starting for mature antibodies at 100 nmol/L and for germline antibodies at 2500 nmol/L). Each curve was globally fit (black lines) in a kinetic model at 1:1 binding. **C**, Sensorgrams of binding of A044 germline at different concentrations (500 nmol/L to 31.25 nmol/L) to either Fel d 1 monomer or dimer directly immobilized on the CM5 chip. Each curve were globally fit (black lines) in a 1:1 interaction model.

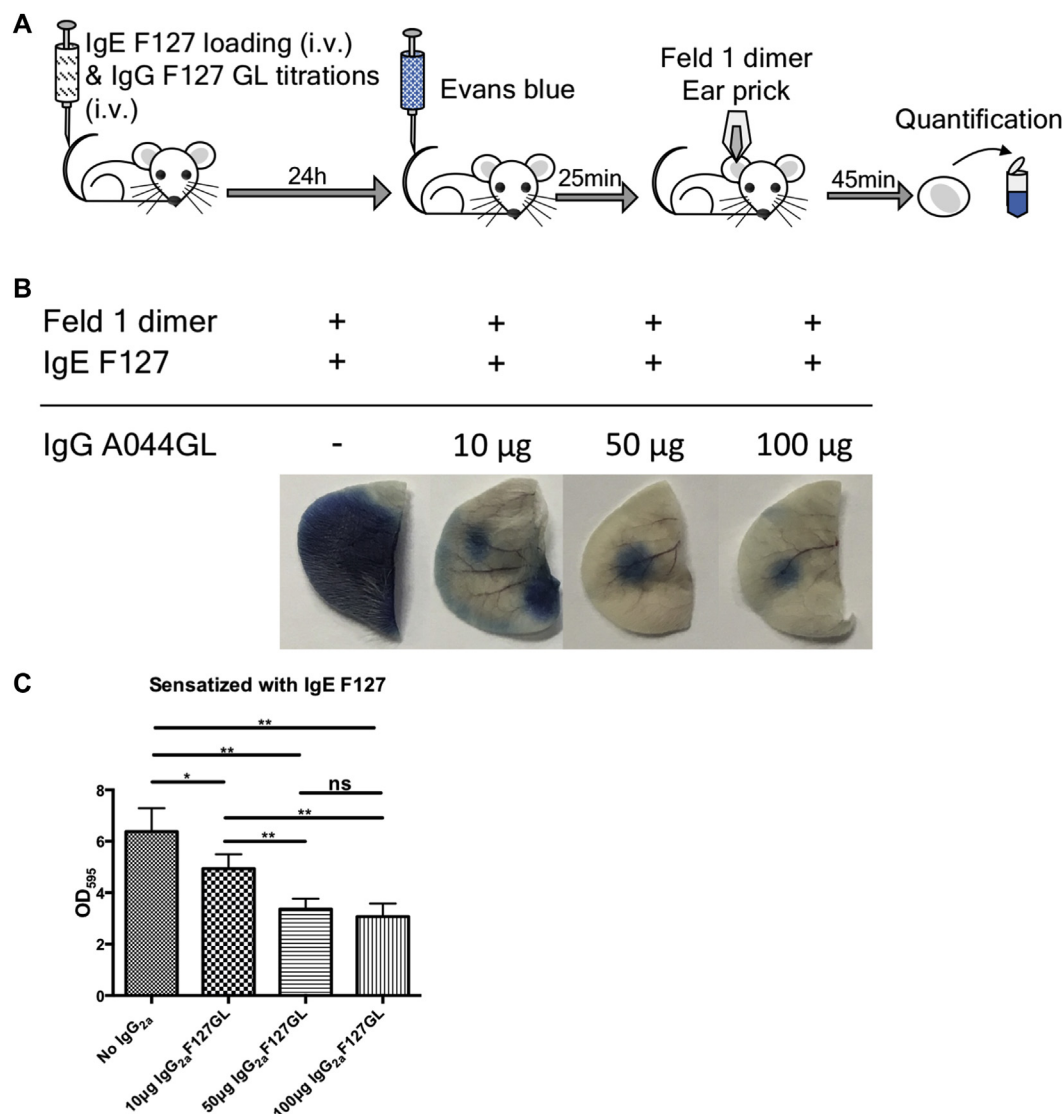


FIG E5. Germline mAb IgG_{2a} A044 but not F127 inhibited IgE F127-induced mast cell degranulation *in vivo* in a dose-dependent manner. **A**, Ear prick scheme to determine mast cell degranulation *in vivo*. Mice were given intravenous (i.v.) injections of 10 μ g of mouse IgE F127 and/or IgG_{2a} germline A044 antibodies (10, 50, and 100 μ g). Twenty-four hours later, each mouse was injected with 200 μ L of Evans blue dye intravenously and challenged with 20 μ g of Fel d 1 dimer on the outer ear skin 25 minutes later. Quantification of mast cell degranulation was determined by the degree of dye extravasation measured at an OD of 595 nm after digestion of ears. **B**, strong mast degranulation was observed in the mice loaded with IgE F127 antibody and was inhibited in combination of IgG_{2a} germline A044 antibody in a dose-dependent manner. **C**, Dye was extracted from collected ears by means of digestion with KOH overnight at 37°C. After full digestion, H₃PO₄ was added, and supernatants were collected and measured at an OD of 595 nm for quantification. Results show means $\pm$ SEMs of OD₅₉₅, and data shown are representative for 5 independent experiments. * $P \leq .05$ and ** $P \leq .01$. ns, Not significant.

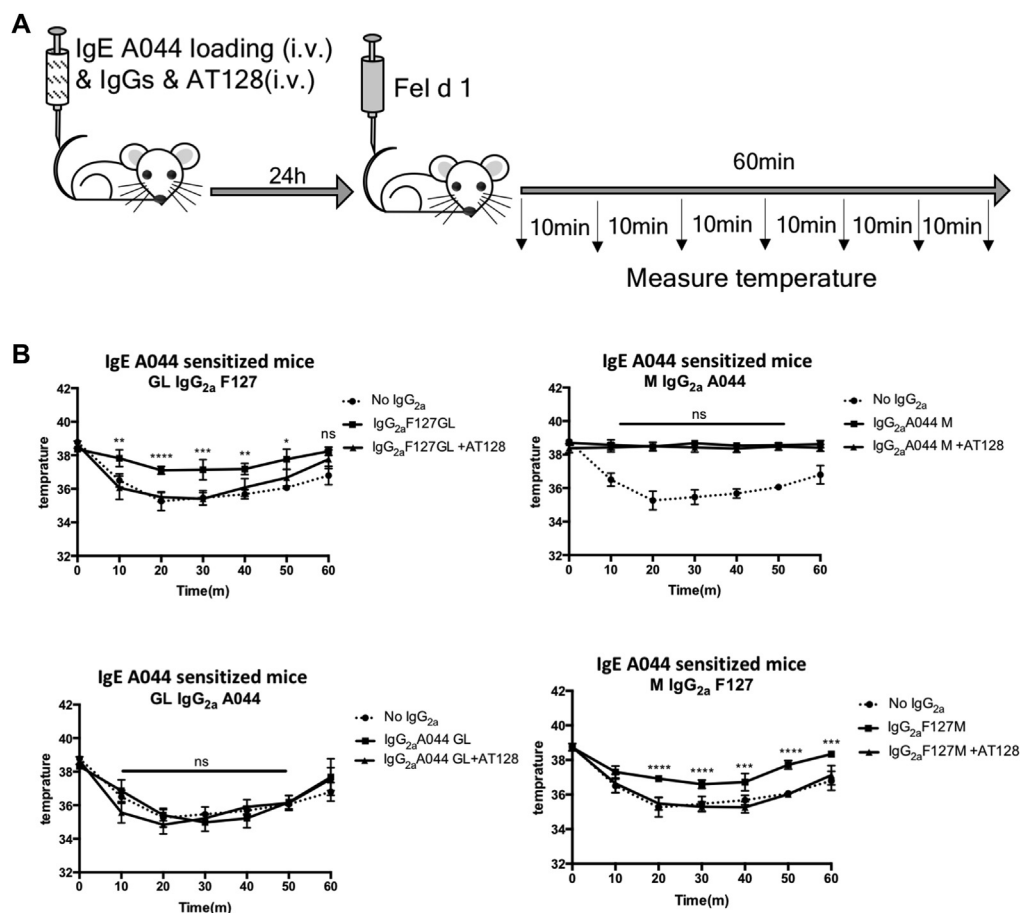


FIG E6. Low-affinity germline mAbs inhibit IgE A044-induced passive systematic anaphylaxis *in vivo* through blocking FcγRIIb. **A**, Scheme of passive systematic anaphylaxis to determine mast cell degranulation *in vivo*. Mice were passively sensitized with 10 μg of Fel d 1-specific IgE A044 by means of intravenous (i.v.) tail vein injection. The next day, either 100 μg of IgG_{2a} (A044 or A044 germline or F127 or F127 germline antibodies) or 100 μg of IgG_{2a} combined with AT128 mAb against mouse FcγRIIb receptor were injected intravenously. After 24 hours, mice were challenged by means of intravenous injection of 5 μg of Fel d 1. Core body temperature was measured immediately before and every 10 minutes after antigen challenge for 60 minutes. **B**, A strong decrease in core body temperature was observed in mice loaded with IgE A044 alone and IgE A044 combined with IgG_{2a} A044 germline (GL) but was inhibited in combination of IgG_{2a} F127 germline/F127 mature (M)/A044 mature. However, inhibition of systematic anaphylaxis induced by F127 germline/F127 mature was abolished on administration of AT128 mAb against mouse FcγRIIb receptor. Results show means ± SEMs of temperatures, and data shown are representative for 5 independent experiments. * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, and **** $P \leq .0001$. ns, Not significant.