**An unexpected protective role of low affinity allergen-specific IgG via the inhibitory receptor FcRIIb**

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**Abstract**

Background: Induction of allergen-specific IgG antibodies is a critical parameter for successful specific immunotherapy (SIT). IgG antibodies may inhibit IgE-mediated mast cell activation by direct allergen-neutralization or via the inhibitory receptor FcRIIb. The affinity of IgE antibodies to the allergen has been shown to be critical for cellular activation. Objective: Here we addressed the question of the affinity thresholds of allergen-specific IgG antibodies for inhibition of mast cell activation by using 2 different monoclonal antibodies (mAbs) against the major cat allergen, Fel d 1, both *in vitro* and *in vivo* in mice. Methods: The sequences of the two high-affinity mAbs were back-mutated to germ-line, resulting in low affinity (10-7M) 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 via FcRIIb but fail to neutralize the allergen. Conclusion: Antibody affinity dictates the mechanism of mast cell inhibition and IgG antibodies triggering the inhibitory FcRIIb-pathway may show a broader cross-reactivity pattern than previously thought. This indicates that SIT generates a larger protective umbrella of inhibitory IgG antibodies than previously appreciated.

**Abbreviations**

SIT: Allergen-specific immunotherapy

FcγRIIb: Fc γ receptor—IIb

FcεRI: Fc ε receptor one—I

Fel d 1: the major cat allergen

BMMCs: bone marrow–derived mast cells

GL antibodies: germ—line antibodies

**Introduction**

IgE-mediated allergies have reached epidemic proportions and allergic rhino-conjunctivitis and asthma affect now about one third of the population in developed countries 1,2. The principal effector 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 upon cross-linking by allergen, inducing the release of mediators (histamines and others) that cause the allergic symptoms 3.

A number of possibilities exist to treat IgE mediated allergies. Most currently used therapies consist of symptomatic medications such as anti-histamines or corticosteroids. The only disease modifying therapy available is allergen-specific immunotherapy (SIT) whereby the patients are treated with multiple doses of the 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(s) 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 may 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 simply blocks binding of allergen to FcεRI-bound IgE and therefore follows the classical path of neutralizing antibodies blocking e.g. viruses from engaging their receptors. To be effective in neutralizing the allergen, IgG antibodies need to block essentially all epitopes available for IgE binding on the allergen. This mechanism therefore follows the rules of competitive inhibition 8–10.

Antibodies engaging the inhibitory receptor block cellular activation by triggering FcγRIIb, leading to the recruitment of the SH2 domain-containing inositol 5'-phosphatase (SHIP), 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 may 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 to non-competitive enzyme inhibitors 3,12,13.

The role of antibody affinity in blocking allergen-activity is poorly understood. As outlined above, however, one may expect a different impact of affinity for allergen neutralization versus engagement of the FcγRIIb, as affinity is more important for competitive versus non-competitive 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 the variable regions back to their germ-line sequence. Using *in vitro* and *in vivo* models we demonstrate that high affinity antibodies block cellular activation by allergen neutralization and engaging FcγRIIb while low affinity antibodies fail to neutralize the allergen but are still effective via the inhibitory mechanism. Hence, despite inefficient allergen-binding, low IgG affinity antibodies may still efficiently block allergic responses *in vivo*.

**Materials and 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 GL anti-Fel d 1 IgG2a**

***Cloning***

Two Fel d 1 IgG2a (A044 and F127) that recognized different epitopes on Fel d 1 were kindly provided by Dr. Franziska Zabel. The two antibodies were produced as described previously4. Briefly, Balb/c mice were immunized with 50 g of recombinant Fel d 1 coupled to VLPs derived from the bacteriophage QFel d 1–specific memory B cells were isolated by fluorescence-activated cell sorting (FACS). The sorted B cells were used to generate a random combinatorial antibody library that was screened by Sindbis-based mammalian cell display14 and allowed the isolation of the two Fel d 1 IgG2a antibodies. To clone the germline sequences the sequences of the variable regions from IgG2a A044 and F127 were aligned with the GL genomic database by <http://www.imgt.org>. The GL sequences of the variable regions from IgG2a A044 and F127 were synthesized by GeneArt (Thermo Fisher, Waltham, MA USA) with additional restriction cutting sites XhoI and ApaI for light chain as well as SacI and EcoRI for heavy chain. The variable regions of light chain from each epitope were digested with XhoI and ApaI and cloned into the light chain plasmid which contains constant region of kappa chain. Similarly, the variable regions of heavy chains were inserted in front of gamma constant region in the heavy chain plasmid via SacI and EcoRI. Afterwards the new constructed heavy and light chain plasmids were digested with NheI, PmeI, AscI and NheI and cloned into the final expression plasmid (pCB15-GL) under two independent pCMV promoters (Fig. S1).

Recombinant monomeric and dimer Fel d 1 proteins were generated as previously described4. Briefly, a cDNA that encoded a covalent dimer of chains 2 and 1 of Fel d 1, spaced by a 15 aa-linker (GGGGS)x3, was obtained by PCR. This amplicon was cloned into a modified version of pET-42a leading to the addition of the sequence LEHHHHHHGGC at the C-terminus of the covalent dimer.

***Expression***

pCB15-GL, comprising both heavy and light chain GL sequences, was transfected on HEK293T cells. Briefly, 5 x 107 cells were seeded in T75 flask in complete growth medium (Dulbecco's Modified Eagle medium (cat.11965084, Gibco) supplemented with 10% Fetal Bovine Serum and 1% Penicillin, Streptomycine) 1 day before transfection. The DEME complete growth medium was changed to serum-free DEME medium prior to transfection. 15 μg of pCB15-GL plasmid DNA was mixed with 45 μl PEI (Polyethylenimine) and incubated at RT for 15 min, which afterwards was added to the cells dropwise. The serum-free medium was changed to DEME complete growth medium 6 hours post transfection and was collected 24 hours, 48 hours and 69 hours after transfection.

***Purification***

The GL IgG2a in the supernatant from the transfected cells were purified by HiTrap Protein G HP column (Cat. 7-0402-01, GE Healthcare) with *ÄKTA pure* protein purification system (GE Healthcare). Expression and purification of GL antibodies were checked on 10% SDS-PAGE.

***ELISA of GL A044******IgG2a and F127 IgG2a***

ELISA plates were coated with 1 μg/ml mouse Fel d 1 dimer. Purified mature and GL antibodies A044 IgG2a and F127 IgG2a were first added to the wells at increasing concentrations diluted in PBS/casein. Bound antibodies were detected by horseradish peroxidase–conjugated anti-mouse IgG2a (Southern Bioscience). The reaction was stopped after 5 min with 1 M sulfuric acid and the absorbance was measured at OD 450 nm in a standard ELISA reader (BioTek Microplate Readers).

**BMMC (Bone marrow–derived mast cells)**

The method for generating bone marrow–derived mast cells (BMMCs) has been described before 4. Generally, 5\*108 bone marrow cells were cultured in complete RPMI medium supplemented with recombinant mouse stem cell factor (50 ng/ml; R&D Systems) and recombinant mouse IL-3 (30 ng/ml; R&D Systems). The final differentiation status of BMMCs was determined by measuring the expression of c-Kit (allophycocyanin-antimouse c-Kit; BD) and FcεRI (phycoerythrin-antimouse FcεRI; eBioscience) by flow cytometry 4-5 weeks after culture.

**Flow cytometry**

BMMC (Bone marrow–derived mast cells) were washed with FACS buffer (2% FBS in PBS) and blocked 1hr with mouse anti-Fel d 1 IgG2a (5 μg/ml) at 4°C. Afterward, BMMCs were washed twice with FACS buffer and stained with fluorescein isothiocyanate–anti-mouse-IgG2a (clone R19-15; BD) antibodies for 30 minutes at 4°C. Cells were then washed twice with FACS buffer and measured by flow cytometry.

**Affinity measurement using surface plasmon resonance**

Measurements were performed on a Biacore X100 instrument (GE Healthcare Europe GmbH). We used HBS-EP+ (10 mM Hepes, pH7.4, 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20) as running buffer. To determine the kinetic parameters of the mature and one GL (F127) anti-Fel d 1 antibodies a CM5 chip was immobilized on both flow cells with 2000 RU Protein A/G and then injected with either mature (A044, F127) or germline (F127) anti-Fel d 1 IgG2a at a concentration of 25 nM resulting in a coupling level between 500 – 1000 RU. Fel d 1 monomer and dimer proteins were then applied in a 2-fold serial dilution series (5 concentrations, starting for mature F127 and A044 antibodies at 100 nM and at 2500 nM for germline F127) for 2 min at constant flow rate. For the GL A044 we immobilized 200 resonance unit (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 negative control for unspecific binding to the chip surface. Samples were then injected for 3 min at different concentrations (500 nM to 31.25 nM) at a flow rate of 30 l/min. The dissociation rates were measured for another 2 min at constant buffer flow. An additional buffer control was measured and subtracted from the sample sensorgram. To determine the kinetic parameters BIAevaluation software (Biacore) was used based on the 1:1 interaction model (Langmuir).

**Epitope Mapping**

To determine whether the GL anti-Fel d 1 antibodies have kept the same epitope specificities as the mature antibodies we analyzed the binding epitopes of the GL 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 two serials injections of each parental mature antibodies until most of the epitopes were covered and the sensorgram reached a threshold value. The GL and the opposite parental antibodies were then injected once at the concentrations of 500 nM and 50 nM, respectively for 2 min at a flow rate of 30 l/min.

**BMMC degranulation**

The degree of BMMC degranulation was determined by measuring the release of β-hexosaminidase. Briefly, bone marrow–derived mast cells 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–IgG2a complexes (30 nM, 300 nM or 3000 nM) for 1 hour at 37°C followed by washing one time with Tyrode's buffer (0.1% BSA, 0.1% Glucose, 10 mmol/l HEPES, 130 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, pH 7.4). The cells were spinned down to collect the supernatants. Meanwhile the cell pellets were lysed with 0.5% Triton X-100 (Sigma) in Tyrode's buffer. Both the supernatants and lysed cells were incubated with the p-nitrophenyl-N-acetyl-p-D-Glucosamine (Sigma) in 0.1 M sodium citrate buffer (pH 4.5) for 90 minutes at 37°C. The reaction was stopped by adding 0.2 M glycine (pH 10.7). OD at 405 nm were determined as readout. The degree of degranulation was calculated as following: % degranulation = OD supernatant / (OD supernatant +OD cell lysis)

**Ear prick test**

Mice were given intravenous injections of 10 μg mouse IgE (A044 or F127 and/or 100 μg IgG2a (clone A044, A044GL, F127 or F127GL). Twenty-four hours later, each mouse was given an intravenous injection of 200 μl Evans blue dye (0.5% in PBS); DARPin against mouse FcRIIb receptor (kindly provided by Dr. Alex Eggel15) was used for blocking the receptor by local subcutaneous injection on the ears 10 min before the ear prick. 25 minutes later the administration of Evans blue dye, one drop of Fel d 1 Dimer (200 μg/ml) were placed on the outer ear skin of the anesthetized mice, following the prick of the ears with a needle. The degranulation of mast cells was determined 45 minutes after the ear prick and quantified by the degree of dye extravasation. Briefly, mice were killed and the ears collected. The dye was extracted from the collected ears by digestion with 150 μl 1 M KOH overnight at 37°C. The next day, 150 μl 5% H3PO4 in acetone was added, and the samples were centrifuged. Supernatants were collected and measured at an OD of 595 nm to quantify the extracted dye.

**Passive systemic anaphylaxis**

Mice were passively sensitized with 10μg Fel d1-specific IgE A044 by intravenous tail vain injection. The next day either 100μg IgG2as (A044, GL A044, F127 or GL F127) or 100μg IgG2as combined with AT128, a mAb directed against mouse FcRIIb 16 were injected intravenously. After 24h mice were challenged by intravenous injection of 5μg Fel d 1. Body-core temperature was measured immediately before and every 10 min after antigen-challenge for 60 min.

**Results**

**Binding properties of Fel d1-specific GL antibodies**

To investigate the role of antibody affinity in blocking allergen-activity, two low affinity Fel d1-specific GL antibodies IgG2a A044 and F127 were generated. To this end, the Fab regions of the mature IgG2a A044 and F127 7 were aligned with the germ-line sequences of both heavy and light variable chains to determine the origins of V, D and J genes before somatic recombination (Table 1). The GL(germ-line) Fabs were then cloned into mouse IgG2a expression vector (Fig. S1) and expressed in 293HEK cells. The purity and size of the GL antibodies after protein G purification was determined by SDS-PAGE and compared to the parental mature antibodies. As is shown in suppl. Fig. 2, both GL antibodies bear the same size as the mature heavy and light chains. To assess binding capacity of the GL antibodies to Fel d 1, we performed ELISA assays (Fig. S3). Using either monomer or dimeric Fel d 1 as coating antigen we found that the binding of the GL antibodies to Fel d 1 was less efficient than of the mature ones at low concentration of Fel d 1 but still reached a plateau comparable to that obtained with the mature antibodies at high concentration of Fel d 1.

Kinetic parameters of the germline (GL) antibodies (GL A044 and GL F127) were assessed by surface plasmon resonance and compared to those of mature antibodies (A044 and F127) (Table 2). The mature antibody F127 displayed higher association and lower off-rates than of GL F127 on both monomeric (Table 2A) and dimeric Fel d 1 (Table 2B) resulting in more than 100 times lower affinity for the GL antibody compared to the mature one. Similarly, compared to the mature antibody A044 the GL antibody A044 exhibits a 100 times lower affinity for monomeric (Table 2A) and dimeric Fel d 1 (Table 2B). The kinetic parameters for GL A044 could not be detected as for the others. For this reason, we used different set-up i.e direct immobilization of Fel d 1 proteins rather than captured antibodies. Comparable Kd values were obtained by using both methods validating the determination of kinetic parameters on immobilized Fel d 1 for the GL A044 antibody.

To confirm that the GL 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 GL A044 or F127 antibodies as well as for control purpose mature antibodies were injected for additional 2 min. As expected both GL 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, GL antibodies were able to bind, albeit at a low level, if Fel d 1 was presented by the other parental mature antibody.

**Fel d1-specific GL IgG2as inhibit BMMC degranulation *in vitro***

It has been previously shown 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. Since our GL IgG2a antibodies recognize the same epitopes as the mature ones but with low affinity, we next investigated the capacity of GL antibodies to block IgE-induced mast cell degranulation. For this purpose, BMMCs were incubated with IgE A044/F127 overnight and then challenged with dimeric Fel d 1 alone or with increasing concentrations of IgG2as (A044 mature, A044 GL, F127 mature and F127 GL). Interestingly, in case of BMMCs loaded with IgE A044, IgG2a F127 GL significantly inhibited mast cell degranulation whereas IgG2a A044 GL, which recognizes the same epitope as IgE A044 failed to impede the degranulation even at concentration of up to 200 µg/ml (Fig. 2A). These data suggest that the inhibitory effect of GL antibodies is not mediated through competition with IgE for binding to Feld 1 but occurs primarily through engaging the inhibitory receptor FcRIIb. The same results were obtained when mast cells were loaded with IgE F127 and incubated with either A044 GL or F127 GL antibody (Fig. 2B). Again, the GL IgG2a antibody sharing the same epitope as IgE F127 could not abrogate cell degranulation while GL A044 could.

**Inhibitory effect of GL IgG2as *in vitro* is FcRIIb dependent**

To confirm that the inhibitory effect of GL A044 is rather mediated via the inhibitory receptor FcRIIb than through competition with IgE for binding to Feld 1, a biological inhibitor (based on the DARPin technology) of mouse FcRIIb receptor was used for blocking the receptor15. As shown in Figure 3, 50 µg/ml DARPin was sufficient to abrogate the inhibitory effect of mature as well as of GL IgG2a A044 on IgE F127 loaded mast cells. In contrast, blocking FcRIIb receptor had no effect on the neutralizing activity of mature IgG2a F127. These results indicated Fel d1-specific GL IgG2as induced inhibition of BMMC degranulation is FcRIIb dependent.

**Fel d1-specific GL IgG2a blocks mast cell activation *in vivo***

Having demonstrated that low affinity GL 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, GL A044, mature F127 or GL F127 IgG2a (Fig. 4 and 5). Skin prick tests were performed with Fel d 1 dimer one day after injection of IgE and 25 minutes after injection of Evans blue dye to visualize the allergic reaction (Fig. 4A and 5A). In agreement with the *in vitro* results, co-administration of IgG2a F127 GL with IgE A044 efficiently prevented mast cell degranulation whereas no inhibition was observed with A044 GL (Fig. 4B). Using the same IgG setup but with co-injection with IgE127 confirmed these results as GL IgG2a F127 with the same epitope as the IgE did not display any inhibition of mast cell degranulation whereas GL IgG2a A044 with a different specificity successfully prevented degranulation (Fig. 5B). To further confirm the inhibitory capacity of the GL IgG2a A044 in IgE F127 induced mast cell activation, a dose response experiment was performed. To this end, mice were sensitized with IgE F127 together with different doses of IgG2a A044 GL. As is shown in Figure S5strongmast degranulation was observed in the mice injected with IgE F127 alone and inhibition by IgG2a A044GL occurred in a dose dependent manner.

To elucidate the mechanisms of GL IgG2as mediated inhibition of mast cell degranulation *in vivo*, DARPin against mouse FcRIIb receptor was used for blocking the receptor by local subcutaneous injection into the ears of mice before the prick tests. In accordance with the *in vitro* results, co-administration of IgG2as of a second specificity (no matter whether GL or mature antibodies were used) efficiently prevented mast cell degranulation in Balb/c mice (Fig. 4B and 5B), whereas no IgG-dependent inhibition of mast cell degranulation was observed in DARPin treated mice (Fig. 4C and Fig. 5C). Hence, Fel d1-specific GL IgG2as induce inhibition of mast cell activation in a FcRIIb dependent manner *in vivo*.

To better mimic the physiological situation and directly assess whether GL IgG2as mediated inhibition of systematic allergic reaction *in vivo* is FcRIIb dependent, mice were passively sensitized with Fel d 1 specific IgE A044 with or without IgG2as (A044 or GL A044or F127 or GL F127) combined with or without AT128 mAb against mouse FcIIb via intravenous injection. Fle d 1 challenging was performed 12 h after sensitization. In accordance with the *in vivo* results from passive cutaneous anaphylaxis, co-administration of IgG2as of a second specificity (both GL and mature antibodies) could dramatically alleviate the systematic anaphylaxis (core temperature dropping) in Balb/c mice (Fig. S 6B), whereas no IgG-dependent relief of systematic anaphylaxis was observed when FcIIb was blocked by AT128 mAb (Fig. S 6B). These results confirmed again Fel d1-specific GL IgG2as induce inhibition of allergic reaction is FcRIIb dependent.

In summary, when IgE-induced mast cell degranulation is blocked by engaging the inhibitory receptor, the affinity of IgGs is less important compared to IgGs compete with IgE by a classical neutralization mechanism. Thus, low affinity antibodies fail to neutralize the allergen but block mast cells activation via FcRIIb.

**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 FcRIIb 17. Here we demonstrate that the two mechanisms are governed by different affinity thresholds.

SIT has been shown to induce high-affinity blocking antibodies with somatic mutations in their complementary determining region as an indication for affinity maturation 10-18. These findings are recapitulated here as high affinity Fel d 1 specific antibodies induced by immunizing mice with Fel d 1 coupled to Q-derived VLPs indeed showed a high degree of hypermutation and affinity was dramatically lower upon back-mutation of the variable regions to germ-line sequences. Despite vastly different affinities, the GL 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, as germ-line sequence antibodies failed to block mast cell activation both *in vitro* and *in vivo*. In contrast, the inhibitory FcRIIb pathway was rather independent of the affinity as low affinity antibodies were similarly effective as high affinity antibodies. In contrast to the mouse, in humans only the presence of FcRIIb on the surface of basophils is well documented7 but FcRIIb on mast cells remains unclear19 20. Thus, the here described mechanism will likely occur in human basophils but it`s importance in human mast cells requires more detailed investigation. Furthermore, as IgA antibodies do not bind to the FcRIIb, our results may only apply to IgG and not IgA antibodies.

The here described findings support the mechanistic hypothesis that allergen neutralization is best described by a model analogous to competitive inhibition in enzymology, while the inhibitory pathway follows a non-competitive model21. From a clinical perspective, this may explain why SIT based on allergoids is remarkably effective, even though many of the allergen-epitopes are slightly denatured due to their chemical modification 22. Our results may explain these findings as 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 which 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. Our data suggest, however, that IgG antibodies triggering the inhibitory FcRIIb -pathway show an even broader cross-reactivity pattern due to the lower affinity-threshold.19 SIT may therefore generate a protective umbrella of inhibitory IgG antibodies larger than previously thought.

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**Competing interests:** The authors declare no competing financial interests.

**Figure legends:**

**Fig. 1. Epitope mapping of GL IgG2a A044 and F127 antibodies.** Surface Plasmon resonance was used to assess if GL antibodies have the same epitope specificity as the parental antibodies. The sensor chip immobilized with 1000 RU of Fel d 1 protein was twice saturated with either mature IgG2a A044 **(A)** or mature IgG2a F127 **(B),** each lasting 2 min. Subsequently, mature (50 nM) and GL (500 nM) antibodies were injected for 2 min at a constant flow rate.

**Fig. 2.** **Low affinity GL mABs inhibited IgE induced BMMC activation *in vitro* only if the epitope was different.** (**A),** BMMC were loaded with 4 μg/ml Fel d 1–specific mouse IgE A044 overnight and then incubated with Fel d 1–specific mouse IgG2a mature/GL A044 and IgG2a mature/GL F127 at different concentrations (3μg/ml, 30μg/mL and 300μg/ml) with mouse Fel d 1 dimer (3 μg/ml) for 1 hour. **(B),** BMMC were loaded with 4 μg/ml Fel d 1–specific mouse IgE F127 overnight and then incubated with Fel d 1–specific mouse IgG2a mature/GLA044 and IgG2a mature/GL F127 at different concentrations (3μg/ml, 30μg/ml and 300μg/ml) with mouse Fel d 1 dimer (3 μg/ml) for 1 hour. The naïve group BMMC which has not been treated with unspecific IgGs was taken as control. The degree of BMMC degranulation was determined by measuring the release of β-hexosaminidase. Data shown are representative for 3 independent experiments.

**Fig. 3. Low affinity GL mABs inhibited IgE induced BMMC activation could be abolished via blocking** FcγRIIb**.** BMMC were loaded with 4 μg/mL Fel d 1–specific mouse IgE F127 and FcγRIIb DARPin overnight and then incubated with Fel d 1–specific mouse IgG2a mature/GL A044 and IgG2a mature/GL F127 at the concentration of 300μg/mL with mouse Fel d 1 dimer (3 μg/ml) for 1 hour. The group which has not been treated with anti- FcγRIIb DARPin was taken as a control. The degree of BMMC degranulation was determined by measuring the release of β-hexosaminidase. Data shown are representative for 3 independent experiments.

**Fig. 4. Low affinity GL mABs inhibited IgE F127 induced mast cell activation *in vivo* via blocking FcγRIIb. (A),** Scheme of the ear prick to determine mast cell degranulation *in vivo*. Mice were given intravenous injections of 10 μg mouse IgE F127 and/or 100 μg IgG2a (A044 mature, A044GL, F127 mature or F127GL). Twenty-four hours later, each mouse was injected with 200 μL Evans blue dye via i.v. with/without receiving local FcγRIIb DARPin injection. Afterwards, the mice were challenged with 20 μg Fel d 1 Dimer on the outer ear skin 25 min later. The quantification of degranulation of mast cells was determined by the degree of dye extravasation measured at an OD of 595 nm after digestion of the ears. **(B),** severemast degranulation was observed in the mice loaded with only IgE F127 but much weaker when IgE F127 were loaded together with IgG2a A044 mature, A044GL or F127 mature. However, the combination of IgE F127 and F127 GL did not show lower degranulation ratio comparing with other groups.**(C),** mast degranulation was observed in the mice loaded with IgE F127 + DARPin and was inhibited only when loaded together with IgG2a F127 mature + DARPin. IgG2a A044 GL and A044 induced inhibition were all abolished upon local DARPin treatment. the dye was extracted from the collected ears by digestion with KOH overnight at 37°C. After fully digestion H3PO4 was added and the supernatants were collected and measured at an OD of 595 nm to for quantification. The results show the means ± SEMs of the OD595 and data shown are representative for 5 independent experiments.

**Fig.5. Low affinity GL mABs inhibited IgE A044 induced mast cell activation *in vivo* via blocking FcγRIIb. (A),** Scheme of the ear prick to determine mast cell degranulation *in vivo*. Mice were given intravenous injections of 10 μg mouse IgE A044 and/or 100 μg IgG2a (A044 mature, A044GL, F127 mature or F127GL). Twenty-four hours later, each mouse was injected with 200 μL Evans blue dye via i.v. with/without receiving local FcγRIIb DARPin injection. Afterwards, the mice were challenged with 20 μg Fel d 1 Dimer on the outer ear skin 25 min later. The quantification of degranulation of mast cells was determined by the degree of dye extravasation measured at an OD of 595 nm after digestion of the ears. **(B),** severemast degranulation was observed in the mice loaded with only IgE A044 and IgE A044+IgG2a A044GL but much weaker when IgE A044 were loaded together with IgG2a A044 mature, F127GL or F127 mature.**(C),** mast degranulation was observed in the mice loaded with IgE A044 + DARPin and was inhibited only when loaded together with IgG2a A044 mature + DARPin. IgG2a F127 and F127GL induced inhibition were all abolished upon local DARPin treatment. the dye was extracted from the collected ears by digestion with KOH overnight at 37°C. After fully digestion H3PO4 was added and the supernatants were collected and measured at an OD of 595 nm to for quantification. The results show the means ± SEMs of the OD595 and data shown are representative for 5 independent experiments.

**Fig. S 1. Cloning strategy for GL IgG2a A044 and F127.** The GL sequences of the variable regions from IgG2a A044 and F127 were synthesized by GeneArt (Thermo Fisher, Waltham, MA USA) with additional restriction cutting sites Xho I and Apa I for light chain / Sac I and EcoR I for heavy chain. Firstly, the variable regions of light chain were cloned into the light chain plasmid which comprises constant region of mouse kappa chain. The variable regions of heavy chains were inserted in front of mouse gamma constant region. Secondly, the new constructed heavy and light chain plasmids were digested with Nhe I, Pme I, Asc I and Pme I and cloned into the final expression plasmid (pCB15-GL) under two independent pCMV promoters.

**Fig. S 2. IgG2a GL mABs bear the same size as the mature ones.** GL mAb IgG2a A044 and F127 after purification showed the same size with the mature IgG2a A044 and F127. Lane1: Non-reduced A044 GL AB; Lane2: Non-reduced A044 mature mAB; Lane3: Non-reduced F127 GL mAB; Lane4: Non-reduced F127 mature mAB; Lane 5: Reduced A044 GL mAB; Lane6: Reduced A044 mature mAB; Lane7: Reduced F127 GL mAB; Lane 8: Reduced F127 mature mAB.

**Fig. S 3. Binding properties of the 2 mAbs (IgG2a A044 and F127) to monomeric Fel d 1 (A) and dimeric Fel d 1 (B) assessed by ELISA.** The germline mAbs displayed lower properties of binding to either monomeric Fel d 1 or dimeric Fel d 1, regardless of isotype.

**Fig. S 4. Biacore data of**  binding to Fel d 1 proteins of captured **IgG2a** **mature (M) and germline (GL) anti-Fel d 1 antibodies.** Panel A) show sensorgrams for binding to Fel d 1 monomer and Panel B) to Fel d 1 dimer of a series of five antibody concentrations (starting for mature antibodies at 100 nM and for germline antibodies at 2500 nM). Each curve were globally fit (black lines) in a kinetic model 1:1 binding. Panel C) shows sensorgrams of binding of A044 GL at different concentrations (500 nM to 31.25 nM) 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. S 5.** **GL mAB IgG2a A044 not F127 inhibited IgE F127 induced mast cell activation *in vivo* in a does’ dependent manner. (A),** Scheme of the ear prick to determine mast cell degranulation *in vivo*. Mice were given intravenous injections of 10 μg mouse IgE F127and/or IgG2a GL A044 (10 μg, 50 μg and 100 μg ). Twenty-four hours later, each mouse was injected with 200 μl Evans blue dye via i.v. and challenged with 20 μg Fel d 1 Dimer on the outer ear skin 25 min later. The quantification of degranulation of mast cells was determined by the degree of dye extravasation measured at an OD of 595 nm after digestion of the ears. **(B),** strongmast degranulation was observed in the mice loaded with IgE F127 and was inhibited in combination of IgG2a GL A044 in a does dependent manner. **(C),** the dye was extracted from the collected ears by digestion with KOH overnight at 37°C. After fully digestion H3PO4 was added and the supernatants were collected and measured at an OD of 595 nm to for quantification. The results show the means ± SEMs of the OD595 and data shown are representative for 5 independent experiments.

**Fig. S 6.**  **Low affinity GL mABs inhibited IgE A044 induced passive systematic anaphylaxis *in vivo* via blocking FcγRIIb. (A),** Scheme of the passive systematic anaphylaxisto determine mast cell degranulation *in vivo*. Mice were passively sensitized with 10μg Fel d1-specific IgE A044 by intravenous tail vain injection. The next day either 100μg IgG2as (A044 or A044GLor F127 or F127 GL) or 100μg IgG2as combined with AT128 mAb against mouse Fc**γ**RIIb were injected intravenously. After 24h mice were challenged by intravenous injection of 5μg Fel d 1. Body-core temperature was measured immediately before and every 10 min after antigen-challenge for 60 min. **(B),** astrongdrop of the body-core temperature was observed in the mice loaded with IgE A044 alone and IgE A044 combined with IgG2a A044 GL but was inhibited in combination of IgG2a F127 GL/F127 M/A044 M. However, the inhibition of systematic anaphylaxis induced by F127 GL/F127 M was abolished upon the administration of AT128 mAb against mouse Fc**γ**RIIb. The results show the means ± SEMs of the temperatures and data shown are representative for 5 independent experiments.