

BRIEF COMMUNICATION

Inhibition of allergen-dependent IgE activity by antibodies of the same specificity but different class

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Keywords

allergen; antibody; immunotherapy; isotype; specificity.

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Abstract

IgG₄ purified from patients undergoing specific allergen immunotherapy inhibits the activities of the serum IgE in *in vitro* assays and is thought to reduce the symptoms of the disease. However, it is not known whether this is related to an intrinsic property of this subclass or only the allergen specificity. We tested the hypothesis that allergen specificity is the critical determinant for this activity using a panel of antibodies with identical specificity but different subclasses. The different antibodies were all able to inhibit the activity of IgE to the same extent. We demonstrate that specificity is the dominant factor determining the ability of an antibody to block allergen-dependent IgE activity.

Allergy is associated with the excessive production of allergen-specific IgE. However, allergen-specific antibodies of other isotypes are produced both in allergic disease and in states of tolerance, for example in hyperimmune beekeepers and in patients treated by allergen immunotherapy (1, 2). The four human IgG subclasses, IgG₁, IgG₂, IgG₃ and IgG₄, differ mainly in the length and rigidity of the hinge region. These differences impart different functional roles, based on

their ability to activate the immune system. Similarly, IgA is represented by two subclasses: IgA₁, which predominates within the serum, and IgA₂, with a shorter hinge region and more compact structure, which predominates at mucosal surfaces (3) (Fig. 1A).

Serum from patients receiving allergen immunotherapy blocks the activity of IgE, and this inhibitory activity co-elutes with the IgG₄ fraction (4). Furthermore, depletion of IgG₄ from this serum correlates with a decrease in IgE inhibitory activity (5). However, it is unknown whether blocking activity is restricted to the IgG₄ subclasses or whether other subclasses that recognize the same epitope are equally effective in blocking IgE-mediated activity. To test this, we generated a set of recombinant monoclonal antibodies of the same specificity for the grass pollen allergen Phl p 7, with different constant region domains, representing all of the IgG and IgA subclasses. We then measured the affinity of antigen binding and ability of these antibodies to inhibit IgE-mediated activities in *in vitro* assays.

Abbreviations

BAT, basophil activation test; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FAP, facilitated antigen presentation; HBS, Hepes-buffered saline; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PIPE, polymerase incomplete primer extension; SCIT, subcutaneous immunotherapy; SPR, surface plasmon resonance; TMB, tetramethylbenzidine.

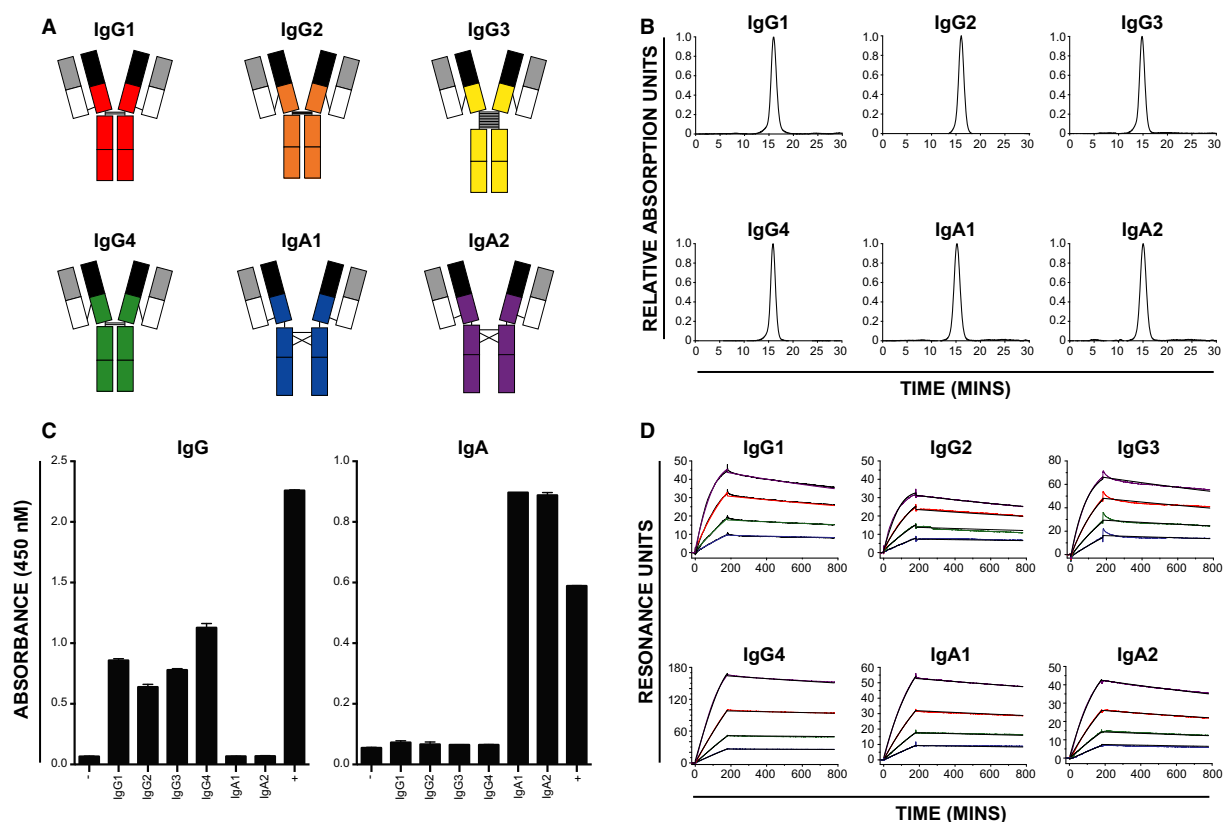


Figure 1 (A) Immunoglobulin domain structures of human IgG₁₋₄ and IgA₁₋₂. Schematic representations of the polypeptide and domain structures of human IgG₁, IgG₂, IgG₃, IgG₄, IgA₁ and IgA₂ showing the intrachain disulphide bridges (3, 13). Variable domains are shown in black and grey. (B) Size exclusion purification profiles. Constant domain-exchanged Phl p 7-specific monoclonal antibodies were purified by size exclusion chromatography (Superdex™ 200 10/300, flow rate 0.75 ml/min in PBS, pH 7.0). (C) Isotype exchange retains the antibody specificity. ELISA plates were coated with Phl p 7, and anti-

body binding was confirmed using isotype-specific detection antibodies. Assay buffer and mixed patient serum were included as negative (–) and positive (+) controls as indicated. (D) Binding kinetics and affinity for Phl p 7 are comparable between antibody isotypes. Recombinant Phl p 7-specific antibodies were captured on a CM5 sensor chip with a covalently immobilized antilambda antibody. Binding experiments were carried out with twofold serial dilutions of Phl p 7 from a starting concentration of 10 nM. Curves were fit (black lines) to derive on- and off-rates.

Methods

Detailed methods are available in the supporting information.

Antibody cloning and expression

Matched heavy- and light-chain variable antibody sequences specific to Phl p 7 allergen were previously isolated from a single B cell derived from a patient undergoing grass pollen immunotherapy (6). These sequences were subcloned into the dual antibody expression vector pVITRO1-102.1F10-IgG4/λ (7). Phl p 7-specific human IgG₁, IgG₂, IgG₃, and IgA₁ and IgA₂ expression vectors were subsequently cloned and expressed using the PIPE method (7).

Characterization of recombinant antibodies

Human 102.1F10 IgG₁ and IgG₂, and IgG₃ and IgG₄ were purified by affinity chromatography with a 5-ml HiTrap

Protein-G HP column (GE Healthcare Life Sciences, Amersham, UK). Human 102.1F10 IgA₁ and IgA₂ were purified by affinity chromatography with immobilized SSL7/Agarose (InvivoGen, Toulouse, France). The purified antibodies were analysed by size exclusion chromatography (14), and specificity was confirmed by Phl p 7 allergen ELISA using biotin-labelled isotype-specific antibodies. SPR was performed using a Biacore T200 instrument; antibodies were captured using an immobilized antilambda antibody (Life Technologies Ltd., Paisley, UK), and binding of Phl p 7 (kindly provided by Dr. Rebecca Beavil) was measured using a 3-min association phase followed by 10-min dissociation.

IgE-facilitated allergen binding (FAB) assay

IgE-facilitated allergen binding to B cells was performed as previously described (5) using serum from a grass pollen-sensitized donor (12 ISU Phl p 7-IgE), recombinant Phl p 7

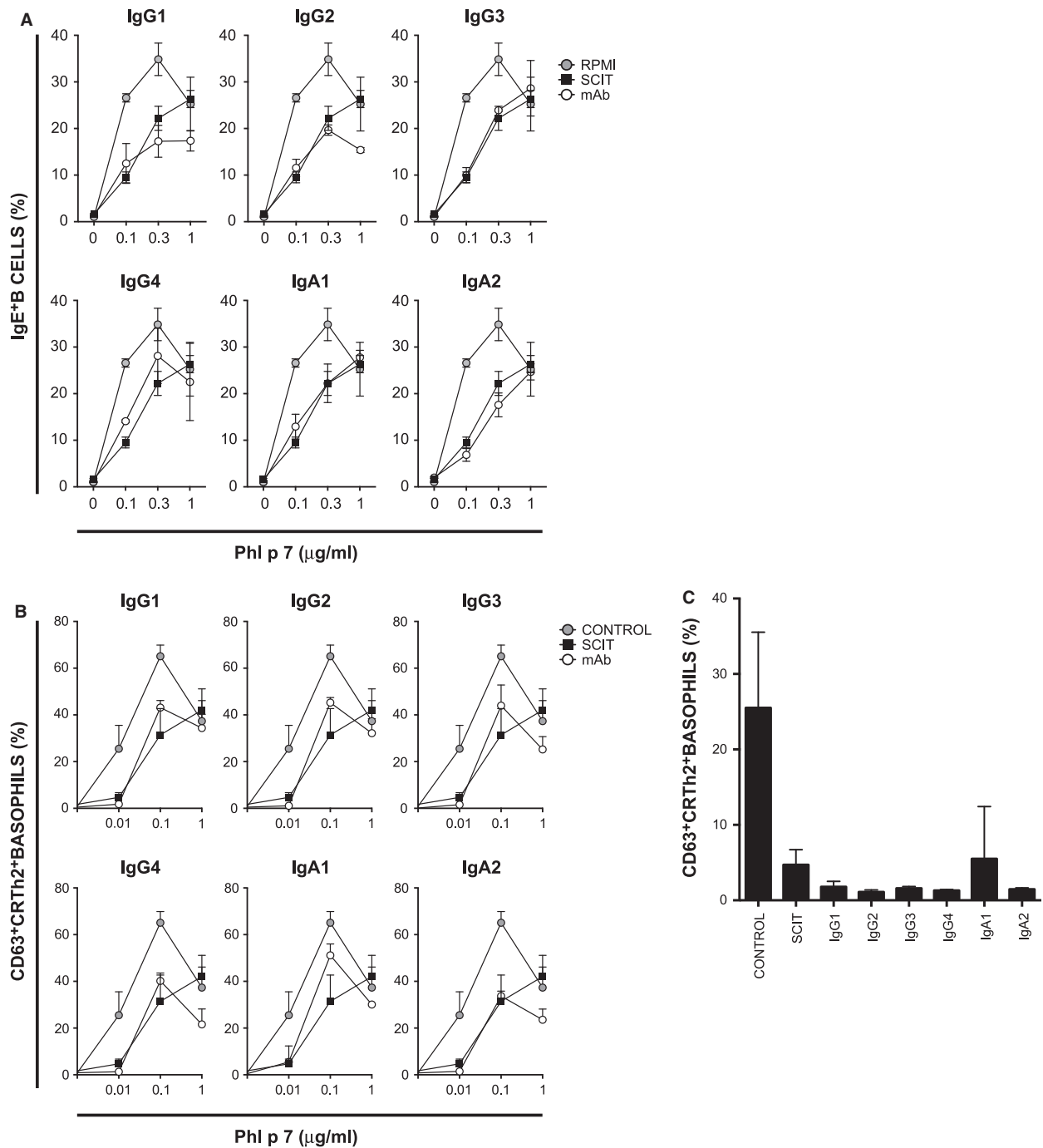


Figure 2 Inhibition of IgE-facilitated allergen presentation and basophil activation is comparable between different isotypes. Serum containing Phl p 7-specific IgE was incubated with Phl p 7 in the presence of 10 μg/ml monoclonal antibodies specific to Phl p 7 (open circles). Undiluted immunotherapy serum (SCIT, closed squares) and assay media (RPMI, grey circles) were included as positive and negative controls for IgE blocking, respectively. Binding of IgE-Phl p 7 complexes was detected by flow cytometry; data are shown as mean ± SEM. B. Basophil activation was detected by flow cytometry following incubation of whole blood from two

Phl p 7-sensitized individuals with increasing concentrations of Phl p 7 and 10 μg/ml monoclonal antibodies specific to Phl p 7 (open circles). Undiluted immunotherapy serum (SCIT, closed squares) and undiluted healthy control serum (control, grey circles) were included as positive and negative controls, respectively. C. Basophil activation following incubation at 10 ng/mL Phl p 7 in the presence of 10 μg/ml monoclonal antibodies specific to Phl p 7. Undiluted immunotherapy (SCIT) serum and healthy control serum were included as positive and negative controls, respectively.

(kindly provided by Dr. Rebecca Beavil) and purified Phl p 7-specific antibodies (10 µg/ml) (6), postimmunotherapy serum (SCIT) (patient 102) or assay media (RPMI-1640).

Basophil activation assay

Basophil (CD3⁺, CD303⁺, CD294⁺) activation (upregulation of CD63) was measured by flow cytometry following incubation of blood from a Phl p 7-sensitized donor with recombinant Phl p 7 in the additional presence of Phl p 7-specific antibodies (10 µg/ml), postsubcutaneous immunotherapy serum (SCIT) (patient 102) or control serum (human AB sera, Lonza, Verviers, Belgium).

Results

Antibody characterization

Size exclusion chromatography and ELISA confirmed the purified Phl p 7-specific IgG₁, IgG₂, IgG₃, IgG₄, IgA₁ and IgA₂ consisted of monodisperse antibodies of the expected size (Fig. 1B) and specificity (Fig. 1C). Changing the constant region had negligible effects on antibody affinities for Phl p 7 (Fig. 1D), with similar K_D values obtained for all antibody subclasses tested (Table S1).

IgE blocking activity

As IgG₄ has been previously shown to be an effective blocking antibody for IgE-mediated activity, we wished to determine whether this blocking activity was specific to the IgG₄ subclass. We therefore tested the IgG and IgA subclasses in two independent *in vitro* assays of IgE activity. Similar to IgG₄, IgG₁, IgG₂, IgG₃, IgA₁ and IgA₂ were able to inhibit binding of IgE-Phl p 7 complexes to the IgE receptor CD23 (FcεRII) on the surface of B cells (Fig. 2A). In a separate assay, all the monoclonal antibodies tested were able to inhibit IgE-dependent Phl p 7-mediated basophil activation to a similar degree (Fig. 2B and 2C).

Discussion

The inhibitory activity of non-IgE antibodies in an allergic reaction is thought to be due to their competition with IgE, by masking the epitopes on the allergen. These so-called blocking antibodies represent a potentially valuable but as yet untested therapeutic commodity for use in passive allergen immunotherapy (8). We previously isolated and cloned a monoclonal IgG₄ antibody, specific to the grass pollen allergen Phl p 7, from a single B cell isolated from the peripheral blood of a patient treated by specific allergen immunotherapy (6). This single antibody was able to inhibit Phl p 7-induced IgE activity by up to 60%. This was comparable to the blocking activity of the polyclonal postimmunotherapy serum from the same patient. To test whether this blocking activity was related solely to the specificity or to the (IgG₄) subclass of the antibody, we compared the ability of other subclasses to inhibit IgE. Although the affinities for Phl p 7 were similar

in all of the recombinant antibodies we generated, subtle but significant differences in binding rates were observed (about threefold differences in both on- and off-rate constants, in the most extreme cases), which tended to cancel out to give very similar overall affinities (ranging from 250–570 pM). These differences may reflect subtle conformational changes that constant region domains impart on the variable region, which have been reported to influence the fine specificity and affinity of isotype-swapped antibodies (9). Nevertheless, we found that the specificity for Phl p 7 was retained and, importantly, isotype exchange had no effect on IgE blocking activity; all subclasses were able to inhibit IgE to nearly the same degree in our *in vitro* assays. Of course, we cannot exclude the possibility that more subtle effects might be observed by titration of the different antibodies. However, under the conditions used here, blocking activities were dependent only on the ability to bind allergen and not on the constant region effector function.

It is almost certain that the blocking activity of an antibody is dependent on several factors such as epitope specificity, concentration and affinity for antigen (10). The affinity of IgE for allergen is an important determinant of its effector function (11), and a blocking antibody must be of approximately equal or higher affinity to prevent IgE binding. Antibody affinity will therefore be a critical factor for selecting blocking antibodies for passive immunotherapy. It is well established that IgG₄-expressing B cells secrete the most efficacious blocking antibodies *in vivo* after specific allergen immunotherapy (2). It follows that they would be the best source of heavy- and light-chain genes from which to derive recombinant blocking antibodies for passive immunotherapy. Indeed, IgG₄ has properties that may favour its use over other IgG subclasses, such as its inability to bind complement, and also the unique property of exchanging one heavy-/light-chain pair with an IgG₄ antibody of a different specificity to generate a bispecific antibody unable to form immune complexes (12).

In summary, our results demonstrate that all IgG and IgA subclasses are capable of inhibiting the activity of IgE in an allergen-specific manner. Further experiments will be required to examine whether antibodies with the same specificity but different isotype are similarly inhibitory *in vivo* or whether other mechanisms as mentioned above come into play. Phl p 7 is a relatively small allergen, and while our study provides a proof-of-concept for passive immunotherapy, it is likely that combinations of allergen-specific monoclonal antibodies directed against multiple allergens, and possibly multiple epitopes, will be required to ameliorate symptoms depending on the sensitization pattern of each individual (8).

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cal Research Centre at Guy's and St Thomas' NHS Foundation Trust and King's College London (HB & TSD). The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Author contributions

TSD, HB and HJB produced and characterized antibodies; MHS performed functional assays; JMM helped to design experiments and analyse data; LKJ, AJB, SRD and BJS helped to design experiments and write the manuscript; and LKJ performed and coordinated experiments. HJG proposed the study and helped to write the manuscript.

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Conflict of interest statements

None of the authors have any conflict of interest to declare.

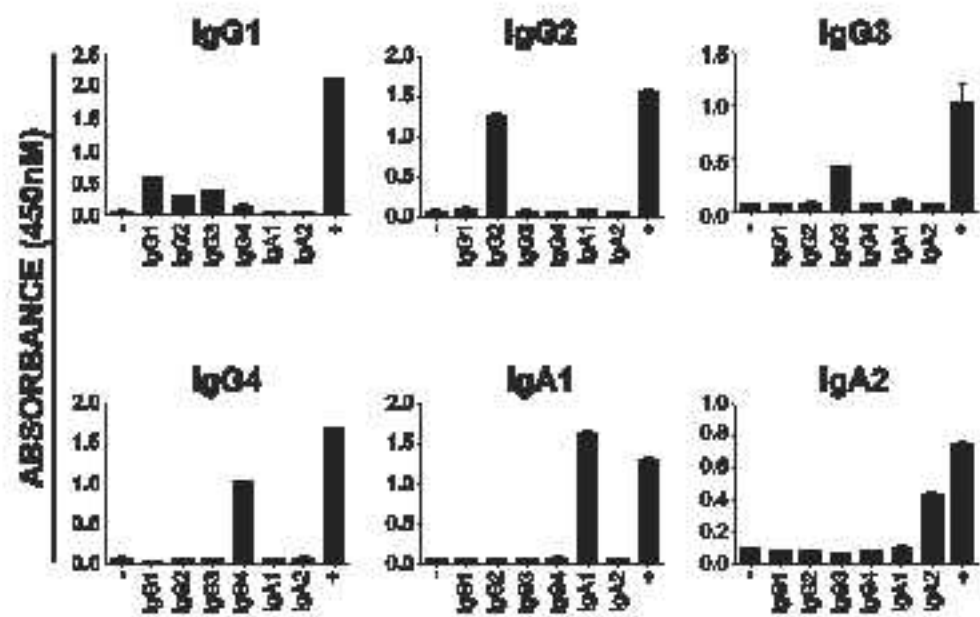
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. ELISA plates were coated with Phl p 7 and antibody binding was confirmed using subclass-specific monoclonal detection antibodies.

Appendix S1. Methods.

Table S1. SPR analysis of the interaction between Phl p 7 and recombinant antibodies (±SE) .



SUPPLEMENTARY MATERIAL

METHODS

Isotype swapping by PIPE cloning

A Phl p 7-specific IgG₄ antibody (6), sub-cloned into the dual antibody expression vector pVITRO1-102.1F10-IgG4/λ (7) was linearised by primer pair pAn_Fwd1 (CTAGCTGGCCAGACATGATAAGATACATTGATGAGTTTGG) and 102.1F10-VH_Rev (TGAGGAGACGGTGACCAGGGCTCCCTGGCCCCAGGAGTCA), flanking the C_γ₄ region, followed by *Dpn I* (New England Biolabs) treatment. Human antibody constant genes C_γ₁, C_γ₂ and C_γ₃ (Life Technologies Ltd.), were amplified using primer pair 102-Cg1-3_Fwd (CCCTGGTCACCGTCTCCTCAGCTAGCACCAAGGGCCCATC) and Cg1-3_Rev (TATCATGTCTGGCCAGCTAGTCATTTACCCGGAGACAGGG), whereas C_α₁ and C_α₂ (Life Technologies Ltd.), were amplified using primer pair 102-Ca1-2_Fwd (CCCTGGTCACCGTCTCCTCAGCTAGCCCGACCAGCCCCAAG) and Ca1-2_Rev (TATCATGTCTGGCCAGCTAGTCAGTAGCAGGTGCCGTCCA). The unpurified linearised pVITRO1-102.1F10-IgG4/λ vector was mixed in 1:1 (v/v) ratio with vector-end homologous PCR amplified C_γ₁, C_γ₂, C_γ₃, C_α₁ and C_α₂ and transformed into competent *E. coli* cells (NEB 10-beta) (7), generating Phl p 7 specific human IgG₁, IgG₂, IgG₃, IgA₁ and IgA₂ expression vectors, all available at Addgene (http://www.addgene.org/Andrew_Beavil).

Recombinant Antibody Production

Suspension serum-free adapted FreeStyle™ 293-F cells (Life Technologies Ltd.) were cultured in FreeStyle™ 293 Expression Medium supplemented with 5000 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies Ltd.) and maintained at 37°C and 5% CO₂ in humidified air. FreeStyle™ 293-F cells were transfected with the dual antibody expression vectors pVITRO1-102.1F10-IgG₁/λ, pVITRO1-102.1F10-IgG₂/λ, pVITRO1-102.1F10-IgG₃/λ, pVITRO1-102.1F10-IgG₄/λ, pVITRO1-102.1F10-IgA₁/λ and pVITRO1-102.1F10-IgA₂/λ (7) using FuGENE HD (Promega) and selected in medium containing 50 µg/ml Hygromycin (Life Technologies Ltd.). Stably transfected cells were expanded for 14 days then supernatants were harvested and filtered.

Purification of Recombinant Antibodies

Human 102.1F10 IgG₁₋₄ were purified by affinity chromatography with a 5 ml HiTrap Protein-G HP column (GE Healthcare Life Sciences) as previously described (7). Human 102.1F10 IgA₁₋₂ were purified by affinity chromatography with immobilised SSL7 / Agarose (InvivoGen).

Size exclusion chromatography

Purified antibodies were analysed by size exclusion chromatography as previously described (14). Briefly, gel filtration was performed on a Gilson HPLC system using a Superdex™ 200 10/300 GL column (GE Healthcare Life Sciences) at a flow rate of 0.75 ml/min in PBS (pH 7.0).

Grass pollen allergen and isotype-specific ELISA

Allergen specificity was confirmed by ELISA of cell-free supernatants using ELISA plates (Maxisorp, Nunc) coated with 10 µg/ml recombinant Phl p 7 allergen (kindly provided by Dr. Rebecca Beavil, MRC & Asthma UK Protein Production Facility, King's College London, UK) and detection using either Peroxidase-conjugated isotype specific polyclonal antibodies against human IgA and IgG (both Sigma) or biotin-labeled subclass specific monoclonal antibodies; IgG₁ (clone 8c/6-39, Sigma-Aldrich); IgG₂ (clone HP-6014, Sigma-Aldrich); IgG₃ (clone HP-6050, Sigma-Aldrich); IgG₄, incubated at 1 µg/ml, (clone G17-4, BD Biosciences), followed by streptavidin–horseradish peroxidase (R&D Systems). HRP-labeled anti-human antibodies were used to detect IgA₁₋₂ and IgE; IgA₁ (clone B3506B4, Abcam); IgA₂ (clone A9604D2, Abcam); IgE (Sigma-Aldrich). All antibodies were used at recommended dilutions (unless stated otherwise).

Surface plasmon resonance (SPR)

SPR was performed using a Biacore T200 instrument. A CaptureSelect™ Anti-lambda antibody (Life Technologies Ltd.) was immobilized on a CM5 chip (GE Healthcare Life Sciences) by amine coupling. Antibodies of all isotypes were captured using a 5-minute injection, and binding of Phl p 7 (kindly provided by Dr. Rebecca Beavil) was measured using a 3-minute association phase and 10-minute dissociation. Affinity and rate constants were calculated using BIAevaluation software, version 3.2 (GE Healthcare Life Sciences)

IgE-Facilitated Allergen Binding (FAB) assay

EBV-transformed B cells (1×10^5 cell per test) were incubated with serum from a grass pollen-sensitised donor (12 ISU Phl p 7-IgE), recombinant Phl p 7 (kindly provided

by Dr. Rebecca Beavil) and purified Phl p 7-specific antibodies (10 µg/ml), post-immunotherapy serum (SCIT) (patient 102) or assay media (RPMI-1640) for 1 hour at 4°C. Cells were washed and binding of Phl p 7-IgE complexes was detected using anti-IgE-PE (Miltenyi). Data were acquired using a BD FACSCanto II system (BD Bioscience) and analysed with FACS DIVA software (BD Bioscience).

Basophil Activation Assay

100 µl of heparinized blood from a Phl p 7-sensitised donor was incubated with recombinant Phl p 7 in the additional presence of Phl p 7-specific antibodies (10 µg/ml), post-immunotherapy serum (SCIT) (patient 102) or control serum (human AB sera, Lonza). Cells were stained with anti-human CD3 (PE-Cy7), CD303 (APC) and CD294 (CRTH2) (PE) to identify basophils (CD3⁻, CD303⁻, CD294⁺) and activation determined by upregulation of CD63 (FITC). Erythrocytes were lysed (FACS lysing solution, BD Biosciences) prior to acquisition and analysis as described.

Supplementary Online Results

TABLE EI. SPR analysis of the interaction between Phl p 7 and recombinant antibodies (±SE)

	$k_{on} (M^{-1} s^{-1})$	$k_{off} (s^{-1})$	$K_D (M)$
IgG₁	$1.2(\pm 0.2) \times 10^6$	$3.5(\pm 0.5) \times 10^{-4}$	2.9×10^{-10}
IgG₂	$1.5(\pm 0.3) \times 10^6$	$3.7(\pm 0.6) \times 10^{-4}$	2.5×10^{-10}
IgG₃	$1.1(\pm 0.2) \times 10^6$	$2.9(\pm 0.4) \times 10^{-4}$	2.6×10^{-10}
IgG₄	$5.2(\pm 0.5) \times 10^5$	$1.4(\pm 0.2) \times 10^{-4}$	2.7×10^{-10}
IgA₁	$6.2(\pm 0.6) \times 10^5$	$2.0(\pm 0.2) \times 10^{-4}$	3.2×10^{-10}

IgA₂	5.4(±0.4)x10 ⁵	3.1(±0.3)x10 ⁻⁴	5.7x10 ⁻¹⁰
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Supplementary Figure 1 Legend

ELISA plates were coated with Phl p 7 and antibody binding was confirmed using subclass-specific monoclonal detection antibodies. Assay buffer and mixed patient serum were included as negative (-) and positive (+) controls as indicated.