

Allergen specificity of IgG₄-expressing B cells in patients with grass pollen allergy undergoing immunotherapy

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Background: Serum IgG₄ responses to allergen immunotherapy are well documented as blocking allergen binding to receptor-bound IgE on antigen-presenting cells and effector cells, but the molecular characteristics of treatment-induced IgG₄, particularly in relation to expressed antibody, are poorly defined.

Objectives: We aimed to clone and express recombinant IgG₄ from patients receiving grass pollen immunotherapy using single B cells to obtain matched heavy- and light-chain pairs. **Methods:** IgG₄⁺ B cells were enriched from blood samples taken from 5 patients receiving grass pollen immunotherapy. Matched heavy- and light-chain variable-region sequences were amplified from single IgG₄⁺ B cells. Variable regions were cloned and expressed as recombinant IgG₄. Binding analysis of grass pollen-specific IgG₄ was performed by using surface plasmon resonance. Functional assays were used to determine IgE blocking activity. In a separate experiment grass pollen-specific antibodies were depleted from serum samples to determine the proportion of grass pollen-specific IgG₄ within total IgG₄.

Results: Depletion of grass pollen-specific antibodies from serum led to a modest reduction in total IgG₄ levels. Matched heavy- and light-chain sequences were cloned from single IgG₄⁺ B cells and expressed as recombinant IgG₄. We identified an IgG₄ that binds with extremely high affinity to the grass pollen allergen Phl p 7. Furthermore, we found that a single specific

mAb can block IgE-mediated facilitated allergen presentation, as well as IgE-mediated basophil activation.

Conclusion: Although increases in IgG₄ levels cannot be wholly accounted for within the allergen-specific fraction, allergen immunotherapy might result in the production of high-affinity allergen-specific blocking IgG₄. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Immunotherapy, IgG₄, Phl p 7, blocking antibody

Sensitization to grass pollen affects up to 20% of the general population, causing symptoms that include rhinitis and asthma. IgE antibodies specific for timothy grass (*Phleum pratense*) might be directed toward any of 9 allergenic proteins contained in pollen grains but are most commonly specific to the major allergens Phl p 1 and Phl p 5.¹

Allergen immunotherapy is the only form of treatment that modulates the immune response to allergen with well-documented effects on both T- and B-cell responses.² Treatment with immunotherapy results in long-term reductions in symptoms that are associated with the induction and persistence of blocking antibodies, particularly IgG₄.³ Blocking antibodies have been shown to inhibit IgE-mediated responses, such as facilitated allergen presentation and basophil histamine release.⁴

Analysis of rearranged immunoglobulin variable-region genes in B cells from atopic subjects has been useful in identifying molecular signatures of allergic disease. For example, a biased use of VH5 genes by IgE⁺ B cells has been reported in several studies.⁵⁻⁸ Although serum antibody responses to allergen immunotherapy have been well characterized, the molecular characteristics of immunotherapy-induced IgG₄ have not been examined. With the exception of a small panel of birch pollen (Bet v 1)-specific IgG₄-producing EBV lines,⁹ few monoclonal IgG₄ antibodies have been cloned from samples from patients undergoing immunotherapy. Single-cell RT-PCR has made possible the reconstruction of recombinant antibodies with natural pairings of heavy and light chains. These techniques provide sequence information that can be directly related to the antigen and epitope specificity of the expressed mAbs. These techniques have been applied to infectious diseases and human autoimmune diseases¹⁰⁻¹⁴ but not previously to allergic diseases. Here we report on the construction and expression of recombinant human IgG₄ antibodies obtained from peripheral blood B cells of patients receiving allergen immunotherapy. We have identified a monoclonal IgG₄ antibody specific for the grass pollen allergen Phl p 7 and have defined the binding kinetics of this antibody using surface plasmon resonance (SPR). We have shown that this

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Abbreviations used

CDR: Complementarity-determining region
 FWR: Framework region
 HBS: HEPES-buffered saline
 ISU: ISAC standardized units
 SPR: Surface plasmon resonance

antibody binds with high affinity and blocks polyclonal IgE responses to grass pollen allergen in functional assays.

METHODS**Patient data**

Five patients (Table 1) receiving subcutaneous immunotherapy with an alum-absorbed 6-grass mix (Alutard; ALK-Abelló, Hørsholm, Denmark) were recruited at the Royal Brompton Hospital (London, United Kingdom). The study was approved by the London and City Research Ethics Committee, and blood samples were obtained after obtaining written informed consent. Levels of serum IgE and IgG₄ specific for the grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 7 were determined by using ELISA (see Fig E1 in this article's Online Repository at www.jacionline.org) with 10 µg/mL allergen, as described below.

Enrichment of IgG₄ memory B cells

PBMCs were isolated from blood by mean of density gradient centrifugation over Ficoll-Paque Plus medium (GE Healthcare, Fairfield, Conn). Switched memory B cells were isolated from PBMCs by means of negative selection (switched memory B-cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany). IgG₄ memory B cells were enriched by using magnetic depletion of non-IgG₄ isotypes with biotin-labeled antibodies specific for IgG₁ (clone 8c/6-39), IgG₂ (clone HP-6014), IgG₃ (clone HP-6050; all from Sigma-Aldrich, St Louis, Mo), and IgA (clone IS11-8E10, Miltenyi Biotec) and anti-biotin microbeads (Miltenyi Biotec). Enriched IgG₄ memory B cells were diluted to 1 cell per microliter in PBS, and single cells were identified by means of light microscopy. In one sample (SCIT105) single cells were isolated by means of a cell sorter (FACSaria II; BD Biosciences, San Jose, Calif) after enrichment of IgG₄ B cells, as described. Cells were stored frozen at -80°C.

Single-cell RT-PCR

Single IgG₄-enriched B cells were thawed on ice and lysed for 15 minutes in buffer containing 0.4% Igepal (Sigma-Aldrich), 60 µmol/L deoxyribonucleotide triphosphates (Bioline, London, United Kingdom), 2.5 µmol/L dithiothreitol, and 8 units of RNase OUT (Invitrogen, Carlsbad, Calif). cDNA was generated with Superscript III reverse transcriptase by using 5 µg/mL each of oligo (dT) and random primers (both Invitrogen) for 10 minutes at 37°C, 45 minutes at 42°C, and 10 minutes at 50°C.

Heavy- and light-chain variable regions (V-regions) were amplified from single-cell cDNA by means of nested PCR (see Fig E2 in this article's Online Repository at www.jacionline.org) in a 25-µL reaction by using Platinum Taq polymerase (Invitrogen) and 2.5 µL of cDNA (first round) or 2.5 µL of first-round PCR product (second round). A mixture of forward primers annealing in the leader region (round 1) or framework region (FWR; round 2) were used to cover all known V-region genes (see Table E1 in this article's Online Repository at www.jacionline.org). Reverse primers were specific for IgG₄, Igκ, or Igλ constant regions (see Table E1). PCR products were purified (PCR purification kit; Qiagen, Hilden, Germany) and sequenced by using the appropriate reverse primer (Eurofins MWG, Ebersberg, Germany). Sequences (excluding primer annealing regions) were analyzed with IMGT V-Quest.¹⁵ The percentage frequency of replacement mutations in both the complementarity-determining regions (CDRs) and FWRs were compared with the expected frequency (based on germline sequences) by using the focused binomial test by Hershberg et al.¹⁶

Antibody cloning

First-round PCR products were used as a template for a second PCR by using V-region- or J-region-specific primers (see Table E1) containing restriction enzyme target sequences. PCR products were digested with the corresponding restriction enzymes (New England Biolabs, Ipswich, Mass) and purified by using agarose gel (1%) electrophoresis and gel extraction (Gel Purification Kit, Qiagen). Purified, restriction site-modified PCR products were ligated into modified pTT3 vectors¹⁷ containing a human V-region leader sequence (VH1-02, VκA26, Vλ8a) and either IgG₄ (pIgγ4), Igκ (pIgκ), or Igλ (pIgλ) constant regions (see Fig E2).¹⁸ Ligated vectors were transformed into competent *Escherichia coli* (XL-2 Blue; Stratagene, La Jolla, Calif), and LB-broth containing 0.1 mg/mL ampicillin (Sigma-Aldrich) was inoculated with single colonies and incubated overnight at 37°C. Plasmid DNA was purified (Qiagen) and sequenced with vector-specific primers (see Table E1). Sequences were aligned to the original nested PCR product (ClustalW, www.ebi.ac.uk) to ensure no base mutations had been introduced.

Human embryonic kidney cells (HEK293E; ATCC no. CRL-1537) were transfected with a 4:1 ratio of polyethylenimine (Sigma-Aldrich) to DNA and a 2:1 ratio of pIgκ/λ to pIgγ4. Cells were cultured for 10 days in Dulbecco modified Eagle medium supplemented with 10% FCS, 2 mmol/L L-glutamine, 10 U of penicillin/streptomycin, and 250 µg/mL Geneticin (all from Invitrogen). Culture supernatants were harvested, centrifuged to remove cell debris, and filtered through 0.22 µm syringe filters.

IgG₄ ELISA and microarray immunoassay

Antibody expression was confirmed by means of ELISA of cell-free supernatants. ELISA plates (Maxisorp, Nunc) were coated with 2 µg/mL anti-human-IgG₄ (clone JDC-14, BD Biosciences). Plates were blocked with PBS and 1% BSA, and 50-µL supernatants were incubated in duplicates for 16 hours at 4°C. Plates were incubated with 1 µg/mL biotin-conjugated anti-human IgG₄ (clone G17-4, BD Biosciences), followed by streptavidin-horseradish peroxidase (R&D Systems, Minneapolis, Minn). IgG₄ was detected with TMB (R&D Systems) by measuring absorbance at 450 nm with human IgG₄ (Sigma-Aldrich) to generate a standard curve. All antibodies were expressed at 0.5 to 12 µg/mL.

Antibody specificity was tested by using a commercially available allergen microarray immunoassay (ISAC; Phadia, Uppsala, Sweden). Allergen chips were washed and incubated with 20 µL of undiluted cell-free supernatant for 2 hours at room temperature. Specific IgG₄ was detected with a fluorescently labeled anti-human IgG₄ antibody. Slides were analyzed with a biochip scanner, and results were evaluated with MIA software (Phadia).

Depletion of allergen-specific IgG₄

Phleum pratense (0.5 mg; ALK-Abelló) or BSA (Sigma-Aldrich) were immobilized with amine-reactive Sepharose beads (GE Healthcare UK, Hertfordshire, United Kingdom) by means of overnight incubation at 4°C. Unreacted sites were blocked with 0.5 mol/L ethanolamine, followed by extensive washing in PBS to remove unbound allergen. Serum samples were diluted in PBS to normalize the total IgG₄ concentrations to approximately 500 µg/mL. Diluted sera were incubated with 50 µL of *P pratense*- or BSA-coupled beads for 4 hours at room temperature with constant gentle rotation. Specific antibodies were depleted from serum by means of centrifugation. Total IgG₄ levels were measured with ELISA, as described, and specific IgG₄ levels were measured by using a modified protocol in which ELISA plates were coated with 10 µg/mL grass pollen extract (*P pratense*, ALK-Abelló) instead of anti-IgG₄. Specific depletion of grass pollen-specific antibody was confirmed by using serial dilutions of a monoclonal grass pollen-specific IgG₄ antibody incubated with either grass pollen or BSA-coupled Sepharose beads (data not shown).

SPR

Recombinant IgG₄ (102.1F10) was purified on a protein G column (GE Healthcare) according to the manufacturer's instructions. Purified IgG₄ was dialyzed into HEPES-buffered saline (HBS) and concentrated to 0.5 mg/mL.

TABLE I. Patients' characteristics

Patient ID	Age (y)	Sensitizations*	Symptoms*	Duration of SCIT (mo)
101	49	Grass pollen, HDM, tree pollen, dog, cat	Seasonal allergic rhinitis, cough, asthma	25
102	31	Grass pollen, HDM, tree pollen, dog, cat	Seasonal allergic rhinitis	41
103	43	Grass pollen, HDM, dog, cat	Seasonal allergic rhinitis	14
104	49	Grass pollen, HDM, tree pollen	Seasonal allergic rhinitis	59
105	32	Grass pollen, HDM, dog, cat, horse	Seasonal allergic rhinitis	51

HDM, House dust mite; SCIT, subcutaneous immunotherapy.

*Sensitizations (determined by means of skin prick tests) and symptoms were reported before starting immunotherapy.

TABLE II. Identity with germline immunoglobulin genes

Patient ID	Clone	Closest germline gene			Identity (%)*	Closest germline gene		Identity (%)*
		IGHV	IGHD	IGHJ		IGLV	IGLJ	
101	101.C6	5-a	5-5	6	99.28	IGK2-24	3	99.15
102	102.1D3	5-51	3-9	4	92.81	IGK1-33	2	90.13
102	102.1E10	4-39	4-23	4	95.02	IGL2-23	3	96.42
102	102.1E2	3-23	3-3	4	89.47	IGK3-20	1	90.04
102	102.1F10	1-3	3-10	4	91.37	IGL1-40	3	96.68
102	102.1F9	1-2	6-13	5	97.15	IGK4-1	1	95.42
102	102.2E11	3.73	5-24	3	93.17	IGK3-20	1	95.67
102	102.2F4	4-4	2-15	4	86.40	IGK2D-29	2	91.93
103	103.1F6	4-39	3-16	3	99.27	IGL2-8	2	94.57
104	104.1D6	3-74	7-27	2	95.96	IGK3-15	1	90.79
104	104.1F3	5-51	3-22	6	96.42	IGK1-5	1	97.37
105	105.2H5	1-69	6-13	6	88.85	IGK1-5	2	95.65
105	105.3F4	1-3	2-15	6	88.54	IGK3-15	2	94.35
105	105.3G6	3-11	4-23	4	96.32	IGK4-1	2	98.76

*Percentage identity excludes the 5' primer sequence.

(Amicon-Ultra; Millipore, Temecula, Calif). SPR assays were performed with a Biacore T100 instrument (Biacore, Piscataway, NJ). IgG₄ was immobilized on a CM5 chip (Biacore) by using an amine coupling kit (Biacore). A second surface was prepared with purified polyclonal human IgG₄ (Sigma-Aldrich) as a reference control. Recombinant Phl p 7 allergen (Biomay, Vienna, Austria) was diluted in HBS, 2 mmol/L CaCl₂, and 2 mmol/L β-mercaptoethanol (9.7×10^{-10} to 1.56×10^{-8} mol/L) or HBS, 10 mmol/L EDTA, and 2 mmol/L β-mercaptoethanol (1.25×10^{-7} to 1.6×10^{-5} mol/L) for calcium-free conditions. Allergen was injected onto the flow cell for 5 minutes with a dissociation time of 15 minutes at 5 μL/min. Regeneration of the sensor surface was performed with a 60-second injection of glycine-HCl (pH 2.5). Affinity and rate constants were calculated by using BIAevaluation software (Biacore).

IgE-facilitated allergen binding assay

IgE-facilitated allergen binding to B cells was performed as previously reported.¹⁹ Serum from a grass pollen-sensitized donor (12 ISAC standardized units [ISU] Phl p 7-IgE) was incubated with 0, 0.1, 1, and 3 μg/mL Phl p 7 (Biomay) in the presence of purified IgG₄ (10 μg/mL 102.1F10 or 101.C6), postimmunotherapy serum (patient 102), or medium (RPMI-1640). Binding of Phl p 7-IgE complexes to EBV-transformed B cells was detected by using fluorescein isothiocyanate-labeled anti-IgE antibody (0.8 μg/mL; DakoCytomation, Glostrup, Denmark). Data were acquired with a BD FACSCanto II system (BD Biosciences) and analyzed with FACS DIVA software (BD Biosciences).

Basophil activation assay

Heparinized whole blood (different subject to IgE-facilitated allergen binding assay; 100 μL per test) was incubated with 0, 1, 10, 100, and 1000 ng/mL of recombinant Phl p 7 in the additional presence of monoclonal IgG₄ antibody (102.1F10 or 101.C6, both 10 μg/mL), postimmunotherapy serum (patient 102), or control serum (human AB sera; Lonza, Basel, Switzerland).

Cells were immunostained with anti-human CD3 (phycoerythrin-Cy7), CD303 (allophycocyanin), and CD294 (CRTH2; phycoerythrin) to identify basophils (CD3⁺CD303⁺CD294⁺), and activation was determined based on up-regulation of CD63 (fluorescein isothiocyanate). Erythrocytes were lysed with FACS lysing solution (BD Biosciences) for 10 minutes at room temperature in the dark before acquisition on the BD FACSCanto II flow cytometer (BD Biosciences). Data were analyzed with BD FACSDiva software (BD Biosciences).

RESULTS

Molecular characteristics of IgG₄

Immunoglobulin heavy- and light-chain V-region sequences were amplified from 14 individual IgG₄⁺ memory B cells isolated from 5 patients (Table I) receiving grass pollen immunotherapy. Variable (V), diversity (D) and joining (J) IgH gene and V and J IGL gene sequences were compared with known immunoglobulin germline sequences by using IMGT/V-quest (Table II). IgH V gene use in the 14 IgG₄ antibodies consisted of VH1 (n = 4), VH3 (n = 4), VH4 (n = 3), and VH5 (n = 3) families. The ratio of κ to λ light chains was 11:3. V-gene analysis revealed a higher frequency of mutations in the CDRs compared with the FWRs of 11 (79%) of 14 heavy-chain V genes and 13 (93%) of 14 light-chain V genes (Fig 1). CDR3 lengths ranged from 5 to 21 (IgH) and 9 to 11 (IGL) amino acids (Table III). The focused binomial test¹⁶ was used to calculate whether the frequency of mutations was significantly different from the expected frequency (based on germline sequences). Increased frequencies of mutation indicate positive selection, whereas fewer than expected mutations indicate negative selection. Four of 14 IgH sequences showed evidence of significant negative selection in FWRs (Table IV), whereas no positive or negative selection was observed in the

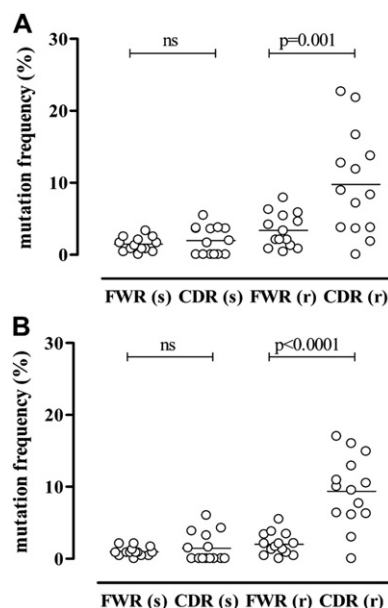


FIG 1. V gene mutation frequency. Heavy-chain (**A**) and light-chain (**B**) V gene sequences (excluding primer annealing regions) were analyzed with IMGT/V-Quest. The percentage of mutations in the FWRs and CDRs were determined from the total number of nucleotides. Comparisons were made by using a paired *t* test. *ns*, Not significant; *r*, replacement mutation; *s*, silent mutation.

remaining 10 sequences. Heavy-chain CDRs showed no evidence of significant selection. A similar pattern was observed for light-chain sequences, with 6 of 14 sequences showing evidence of significant negative selection in FWRs (with no selection in the remaining eight). Notably 1 light-chain sequence (102.1F10) showed evidence of significant positive selection in the CDR ($P = .01$, Table IV).

Expression and specificity of recombinant IgG₄

Eleven of 14 matched IgG₄ heavy- and light-chain V-region sequences were successfully cloned and expressed. Expression levels ranged from 0.5 to 12 $\mu\text{g/mL}$. Supernatants were used to test for the specificity to 103 common allergens by using a commercially available microarray immunoassay that included the timothy grass allergens Phl p 1, 2, 4, 5, 6, 7, 11, and 12. Initially, we found that 2 of 11 antibodies were specific for the grass pollen allergen Phl p 7 (102.1F10; 9.9 ISU-G4, 102.1E10; 0.2 ISU-G4). 102.1F10 also bound to the homologous birch pollen allergen Bet v 4 (3.2 ISU-G4). Both antibodies were derived from the same subject but were not clonally related (Tables II and III). Subsequent analysis with SPR confirmed that 102.1F10 is specific for Phl p 7, whereas 102.1E10 did not bind to Phl p 7.

Depletion of grass pollen-specific IgG₄

We next assessed the contribution of serum grass pollen-specific IgG₄ levels to total IgG₄ levels in sera from all 5 patients undergoing immunotherapy. Grass pollen-specific immunoglobulin was depleted from serum by using *P pratense*-coupled Sepharose beads. The remaining IgG₄ was measured by means of ELISA compared with a control depletion with BSA-coupled Sepharose beads. *P pratense*-specific IgG₄ was undetectable in serum incubated with grass pollen-coupled Sepharose beads but

present in control samples incubated with BSA-coupled beads (Fig 2, A). In contrast, total IgG₄ was detectable in both depleted and control samples (Fig 2, B). Depletion of *P pratense*-specific immunoglobulin resulted in a 1% to 40% reduction in total IgG₄ levels when compared with control samples (Fig 2, C).

Determination of recombinant IgG₄ affinity for Phl p 7

We determined the binding affinities and kinetics of recombinant Phl p 7 to monoclonal Phl p 7-specific IgG₄ antibody (102.1F10) by using SPR. Recombinant Phl p 7 bound to 102.1F10 with a fast association rate ($k_{\text{on}} = 2.17 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) and slow dissociation rate ($k_{\text{off}} = 5.73 \times 10^{-4} \text{ s}^{-1}$), resulting in an overall high-affinity interaction ($K_D = 2.6 \times 10^{-10} \text{ M}$, Fig 3). Because Phl p 7 is a calcium-binding protein and IgE binding has been shown to depend on calcium,²⁰ we repeated the experiment in calcium-free buffer. This resulted in a reduction in affinity by approximately 10,000-fold ($K_D = 2.5 \times 10^{-6} \text{ M}$, Fig 3).

Inhibitory activity of recombinant IgG₄

The ability of Phl p 7-specific recombinant IgG₄ to block IgE activity was determined by using 2 separate assays. Similar to the effects of postimmunotherapy serum, addition of 102.1F10 resulted in a 40% reduction in binding of IgE-Phl p 7 complexes, whereas a different IgG₄ that does not bind to Phl p 7 (101.C6) had no effect on IgE-Phl p 7 complex binding (1 $\mu\text{g/mL}$ Phl p 7; Fig 4, A). Similar results were obtained in a separate assay measuring IgE-allergen-dependent activation of blood basophils. 102.1F10 inhibited Phl p 7-induced upregulation of CD63 by 50% to 60%, whereas 101.C6 had no effect (10 and 100 $\mu\text{g/mL}$ Phl p 7; Fig 4, B).

DISCUSSION

We have described the use of single-cell RT-PCR for the molecular analysis and expression of IgG₄ antibodies derived from 5 patients treated with grass pollen immunotherapy. Although we did not formally assess the clinical response to treatment, all participants reported an improvement in their hay fever symptoms. IgG₄ is the least abundant subclass in adult human serum, ranging from 0.2 to 1 g/L compared with 5 to 12 g/L for IgG₁.²¹ Allergen immunotherapy results in the production of high levels of allergen-specific IgG₄ antibodies; in our cohort of patients, all had serum IgG₄ specific for the major allergens Phl p 1, 2, and 5 (see Fig E1). We therefore had anticipated that most or all of the recombinant IgG₄ antibodies we generated would be specific for grass pollen allergen. In fact, despite using a microarray containing 103 common allergens (including Phl p 1, 2, 4, 5, 6, 7, 11, and 12), only 1 of the 11 recombinant IgG₄ antibodies was identified as allergen specific.

Total serum IgE and IgG₄ concentrations have been shown to correlate with numbers of IgE/IgG₄-secreting cells isolated from peripheral blood²²⁻²⁴; however, the same relationship was not found between serum titers of allergen-specific IgG and numbers of allergen-specific IgG⁺ memory B cells.²⁵ In our study we found that grass pollen-specific IgG₄ contributed between 0% and 40% to total IgG₄ levels. Although we were unable to measure the frequency of allergen-specific IgG₄⁺ B cells in peripheral blood, the specific clone that we went on to identify was derived from the patient who generated the highest number of single

TABLE III. IgH and IgL CDR3 sequence and antibody specificity

Clone	IgH CDR3 sequence	IgL CDR3 sequence	Antigen specificity
101.C6	VRRNSYGYGYGMDV (16)	MQATQFPFT (9)	Undetermined
102.1D3	ARLRVLTGSDLGFFDY (16)	QQYDDLPT (9)	Undetermined
102.1E10	ARHRWEGTWSFDY (13)	CSYAGTTTFGV (11)	Phl p 7
102.1E2	AKLPEYSLIRLYFKS (15)	QQYGNPPAIT (10)	NT
102.1F10	ARAFYSSGVMFDS (14)	QSFDTSLSGWI (11)	Phl p 7, Bet v 4
102.1F9	ARAGPGIAAIGNWLD (16)	HQYLSILST (9)	Undetermined
102.2E11	TRTSELAAFDI (11)	QQYSTSPRT (9)	NT
102.2F4	ARDVAAFDS (9)	MQSLQLPYT (9)	Undetermined
103.1F6	ARHAETYYDYVWAPGGSADF (21)	SSYAGSHSIL (10)	Undetermined
104.1D6	ASWVL (5)	QQYKNWPWT (9)	Undetermined
104.1F3	ARQAKPYYSYPM (14)	QHYNFSFGT (9)	Undetermined
105.2H5	ARGPAAGIYGMDV (13)	QQYNIDPPMYT (11)	Undetermined
105.3F4	AWVVVEATHYKGM (15)	QQYNNWPRT (10)	Undetermined
105.3G6	ARDLRVIGTVTPSDH (16)	QQYYRSPRS (9)	NT

CDR3 length is shown in parentheses.

NT, Not tested.

TABLE IV. Replacement/Silent mutation analysis

Clone	IgH CDR	IgH FWR	IgL CDR	IgL FWR
101.C6	0.42	-0.24	-0.11	-0.09
102.1D3	0.28	-0.15	0.19	-0.27
102.1E10	0.09	-0.39	0.22	-0.13
102.1E2	0.40	-0.15	0.44	-0.01
102.1F10	-0.07	-0.20	0.01	0.06
102.1F9	0.32	0.48	0.13	-0.03
102.2E11	0.46	-0.19	-0.38	-0.01
102.2F4	-0.44	-0.02	0.18	-0.02
103.1F6	-0.19	-0.30	0.07	-0.40
104.1D6	0.48	-0.21	0.18	-0.16
104.1F3	-0.31	-0.01	-0.49	-0.01
105.2H5	0.28	-0.01	0.23	-0.22
105.3F4	-0.19	-0.04	0.46	-0.21
105.3G6	0.07	-0.20	0.45	-0.02

P values are derived from the focused binomial test. *P* values of less than zero indicate negative selection. Significant *P* values (<0.05) are shown in boldface.

IgG₄⁺ B cells. Only 1 of 11 of the cloned IgG₄ antibodies was specific for grass pollen, suggesting that allergen-specific IgG₄⁺ B cells might represent a modest fraction of IgG₄⁺ B cells. This varied considerably from patient to patient but raises the possibility that increases in allergen-specific IgG₄ levels after immunotherapy might be accompanied by increased total IgG₄ levels that are either not specific for allergen or are of too low affinity to be detected by using standard methods. IgG₄ production by B cells is induced by IL-10,²⁶ which is increased both locally and in the periphery after allergen immunotherapy.²⁷ IL-10 diverts the T_H2 response from IgE to IgG₄ by promoting cell differentiation at the expense of proliferation and subsequent switching to IgE,²⁸ thereby increasing antibody production by all B cells expressing IgG₄. After the termination of immunotherapy, IgG₄ levels decrease to near-baseline (before immunotherapy) levels,³ but the IgG₄ fraction of serum is still protective. Whether this accounts for the long-term clinical effects of allergen immunotherapy remains to be determined, and there might be other potential mechanisms responsible, although it suggests that a beneficial process of affinity maturation of allergen-specific IgG₄-producing B cells might prevail.

To estimate the extent to which the various clones had previously undergone affinity maturation *in vivo*, we examined

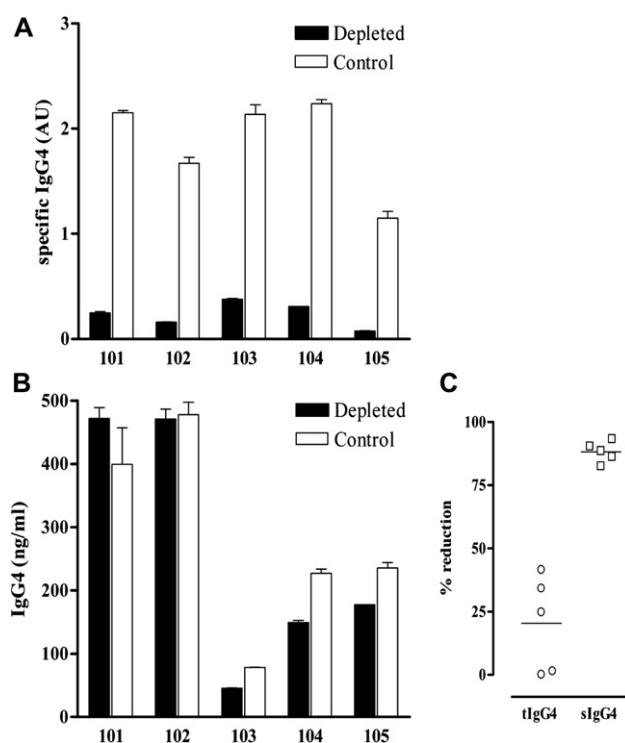


FIG 2. Depletion of allergen-specific IgG₄ from serum has a modest effect on total IgG₄ levels. Allergen-specific immunoglobulins were removed from diluted serum by means of incubation with *P. pratense*-coupled Sepharose beads for 4 hours at room temperature. *P. pratense*-specific (A) and total (B) IgG₄ levels were measured in the depleted serum by means of ELISA. The percentage reduction (C) in total (tlgG₄) and specific (slgG₄) IgG₄ levels was determined from a control depletion by using BSA-coupled Sepharose beads.

the somatic mutations and distribution of replacement and silent mutations between the CDRs and FWRs. The accumulation of replacement mutations in CDRs is thought to signify positive (antigen-mediated) selection that results in increased binding affinity. Conversely, a lower than expected number of replacement mutations and more frequent silent mutations in the FWRs indicate a process of negative selection that is thought to maintain B-cell receptor structure.²⁹ Although a limited number of sequences

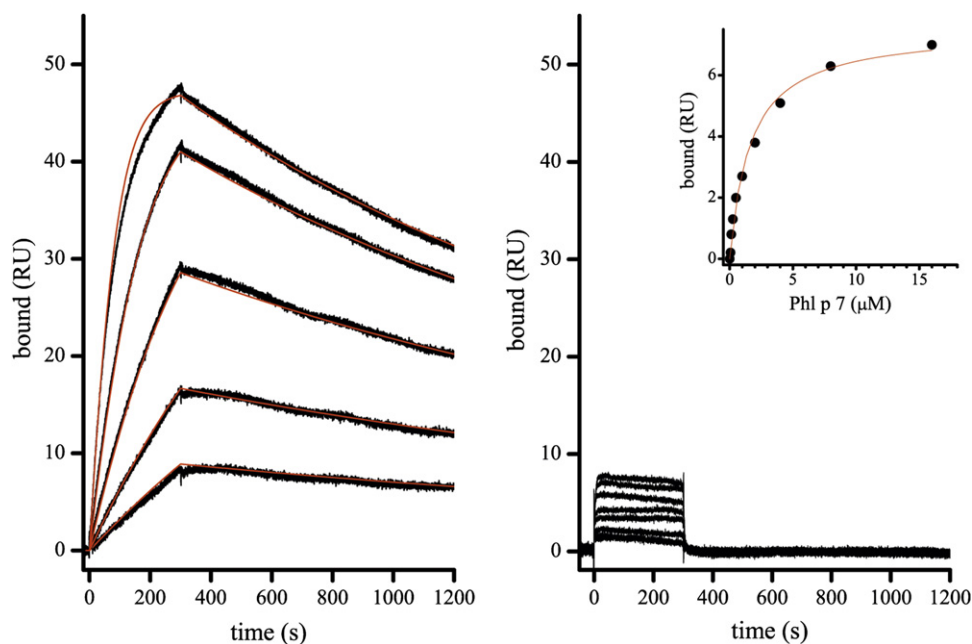


FIG 3. Binding analysis of Phl p 7 to recombinant IgG₄. Recombinant IgG₄ (102.1F10) was covalently immobilized on a sensor chip. Experiments were carried out in either buffer containing 2 mmol/L calcium chloride (*left panel*) with 2-fold serial dilutions of Phl p 7 from 16 nmol/L or in buffer containing 10 mmol/L EDTA (*right panel*) with 2-fold serial dilutions of Phl p 7 from 16 μmol/L. In the presence of calcium, high-affinity binding is observed. Curves were fit (*red lines*) to derive on and off rates. In the absence of calcium, the binding affinity is about 10,000 times weaker, and the interaction displays fast-on/fast-off kinetics. Consequently, affinities were estimated by using equilibrium methods (*right panel inset*).

were analyzed in the present study, we found that the frequency of replacement mutations was higher in the CDRs compared with the FWRs. Using a recently described sequence analysis program,¹⁶ we identified 4 of 14 heavy-chain and 6 of 14 light-chain sequences with evidence of significant negative selection in the FWRs. The only sequence that showed evidence of positive selection in the CDRs was 102.1F10, which was found to bind to the grass pollen allergen Phl p 7 with high affinity. Interestingly, this selection appeared to be restricted to the (λ) light chain and not the heavy chain. Both the heavy- and light-chain sequences contain mutations that result in very different (compared with germline) amino acids within the antigen-binding loop structures of the CDRs (eg, from neutral glycine to polar aspartic acid), thus implying a significant role for both chains in antigen binding. It would therefore be of interest to determine the crystal structure of this antibody (Fab) in complex with Phl p 7 to investigate this further. The frequency of VH gene use was consistent with previous reports on human V-region gene use,³⁰ with VH1 and VH3 being most common, followed by VH4 and VH5. No IgG₄ was detected that used genes of the VH2, VH6, or VH7 families. Previous studies have found an overexpression of VH5 genes by allergen-specific IgE.⁵⁻⁸ We found that 3 (20%) of 14 heavy chains expressed VH5 genes; however, the number of sequences analyzed is too small to comment on the significance of this finding.

Recombinant human antibodies with specificity for the grass pollen allergens Phl p 2 and Phl p 5³¹⁻³³ have previously been constructed by using combinatorial libraries from allergic patients. A disadvantage of this technique is that it relies on a random pairing of heavy and light chains. Use of immortalized B-cell lines can overcome this and has previously been used to generate birch

pollen-specific IgG₄.⁹ Nevertheless, despite the recent improvements,³⁴ immortalization is generally inefficient for the purpose of isolating rare antigen-specific cells. Furthermore, recombinant antibody production is eventually required to generate sufficient protein for functional assays or use in human subjects. This is the first report of a human mAb specific for Phl p 7 isolated by means of single-cell RT-PCR. Similar methods could also be applied to isolate single B cells from mucosal tissue, which would reveal whether blocking antibodies or other allergen-specific antibodies are located within the target organ. SPR studies demonstrate that this antibody binds to recombinant Phl p 7 with very high affinity and in a calcium-dependent manner. Serum IgE responses to timothy grass pollen (*P. pratense*) are usually dominated by the major allergens Phl p 1 and Phl p 5, which are recognized by more than 90% of patients allergic to grass pollen. In contrast, only approximately 10% of patients with grass pollen allergy have Phl p 7-specific IgE.¹ Of the 5 patients included in this study, only patient 102 (from whom the Phl p 7-specific IgG₄ antibody was derived) was found to have Phl p 7-specific IgE in serum (see Fig E1).

Phl p 7 is a stable 8-kDa protein that belongs to a novel family of highly cross-reactive 2 EF-hand calcium-binding proteins or polcalcins.^{35,36} Phl p 7 has been shown to be highly allergenic, eliciting potent immediate responses in sensitized subjects.²⁰ Patients with IgE specific for Phl p 7 react to a wide range of allergens from other tree and grass pollen species to the extent that Phl p 7-specific IgE can be regarded as a marker of polysensitization.³⁷ In fact, Phl p 7 has been proposed as a suitable candidate target for vaccine design because it contains most of the IgE epitopes found in calcium-binding proteins.³⁸ Phl p 7-specific IgG antibodies raised in rabbits have been shown to block basophil

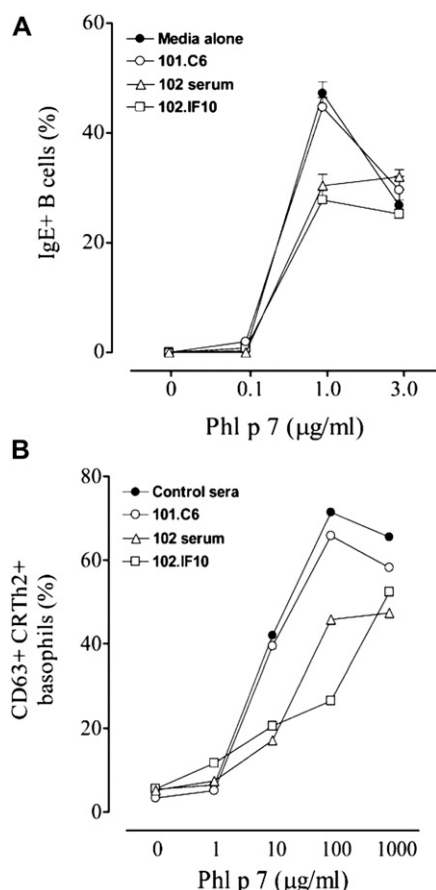


FIG 4. Inhibition of IgE-facilitated allergen presentation and basophil activation by monoclonal IgG₄. **A**, Serum containing Phl p 7-specific IgE was incubated with Phl p 7 in the additional presence of either Phl p 7-specific IgG₄ (102.1F10) or an isotype control (101.C6). Postimmunotherapy serum from patient 102 and assay media were included as positive and negative controls, respectively. Binding of IgE–Phl p 7 complexes were detected by using flow cytometry. Data are shown as means $\pm$ SEMs. **B**, Basophil activation was detected by using flow cytometry after incubation of whole blood with Phl p 7 and either Phl p 7-specific IgG₄ (102.1F10) or an isotype control (101.C6). Postimmunotherapy serum from patient 102 and assay media were included as positive and negative controls, respectively.

degranulation induced with (polyclonal) patient serum exposed to Phl p 7.³⁸ This raises the possibility that blocking antibodies targeted to Phl p 7 might be of therapeutic value in allergic patients who are sensitized to calcium-binding proteins.

A recent study demonstrated that inhibition of house dust mite-induced responses by blocking antibodies depended on the complexity of the IgE repertoire and required more than 1 specificity of blocking antibody.^{39,40} In contrast, separate studies demonstrate that single allergen-specific IgG antibodies or Fab fragments can block histamine release triggered by polyclonal IgE serum and the pollen allergens Phl p 2 and Bet v 1.^{9,32} We found that a single monoclonal IgG₄ antibody was able to inhibit both IgE-facilitated allergen presentation and basophil activation induced by Phl p 7 to the same extent as postimmunotherapy serum. Thus the possibility of preventing IgE-mediated inflammation by means of passive immunotherapy with tailored combinations of high-affinity blocking antibodies or Fab fragments becomes a realistic prospect. Previous forms of passive

immunotherapy have relied on isolation of serum from hyperimmune subjects, such as beekeepers.⁴¹ Although these early studies demonstrated some degree of efficacy in terms of reducing symptom severity, the process is impractical as a form of therapy because it requires large volumes of serum from highly selected donors. As the present study demonstrates, the generation of monoclonal IgG₄ blocking antibodies specific for particular allergen components is now possible. Furthermore, the advent of component-resolved diagnostics means that tailored treatments are becoming more accepted in treatment regimens. We have described techniques that enable the cloning and expression of fully human allergen-specific antibodies from single B cells, which permits the generation of high-affinity blocking antibodies of potential therapeutic value.

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Key messages

- Grass pollen immunotherapy results in increased production of allergen-specific IgG₄, but this does not fully account for serum IgG₄ levels.
- Monoclonal allergen-specific IgG₄ antibodies can be produced that bind with high affinity to grass pollen and block IgE-mediated functional responses.

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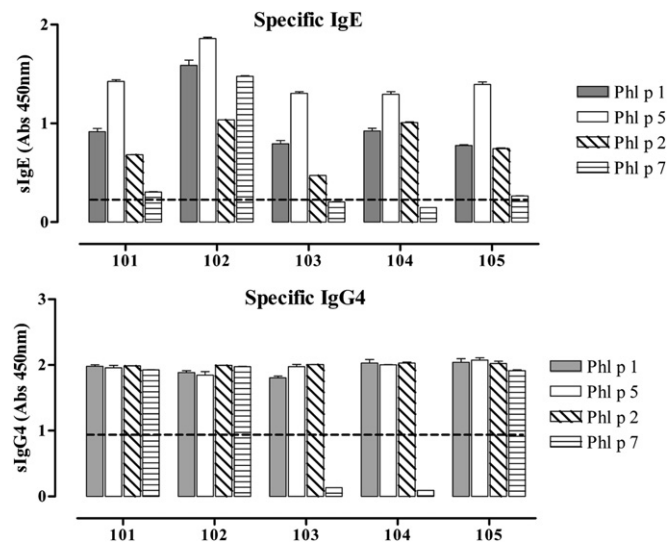


FIG E1. Serum IgE and IgG₄ levels specific for grass pollen allergens. Serum samples taken at the same time as blood collection were tested for the presence of IgE and IgG₄ specific for the grass pollen allergens Phl p 1, 2, 5, and 7 by means of ELISA. The dotted line represents the limit of detection.

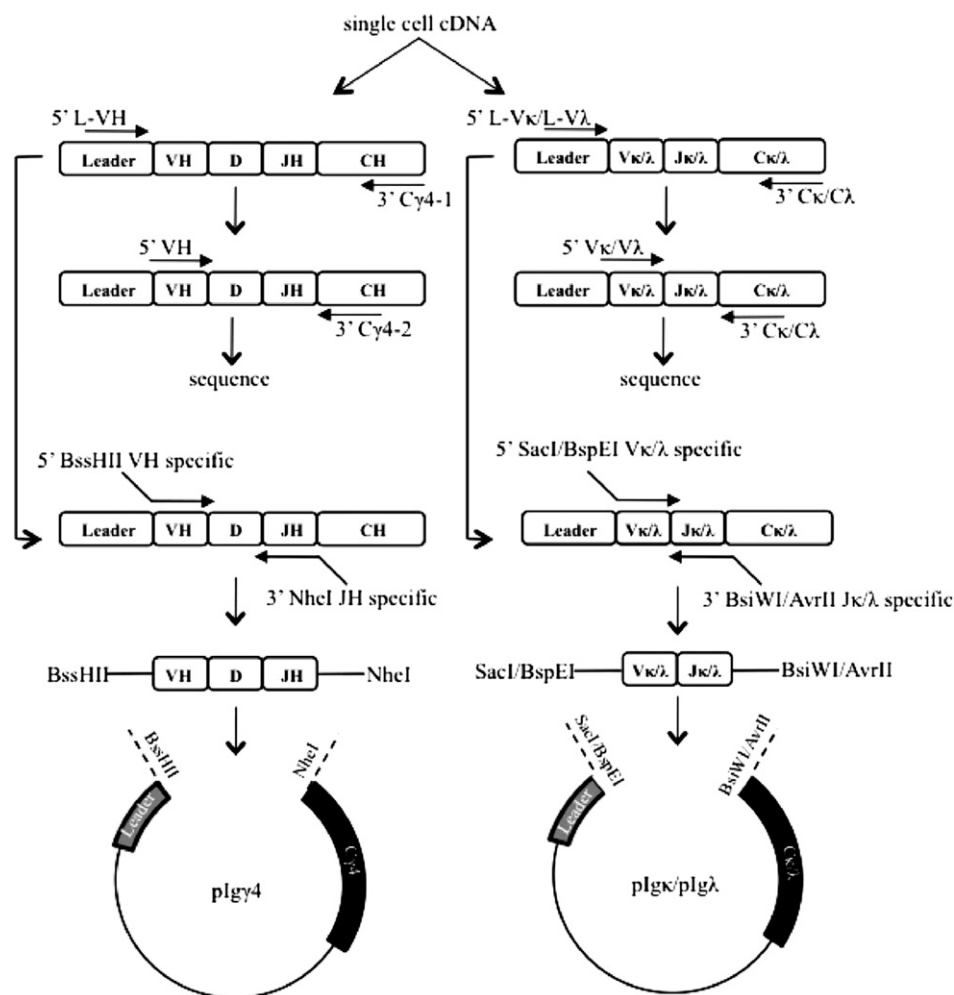


FIG E2. Cloning strategy for the generation of recombinant IgG₄. Paired heavy- and light-chain variable regions from single IgG₄⁺ memory B cells were amplified by means of RT-PCR and sequenced. Restriction enzyme sites were introduced by using PCR to permit cloning into modified pTT3 vectors containing human leader and constant region genes.

TABLE E1. Primers

Primer	Sequence
IgH first-round PCR	
VH1L	CCA TGG ACT GGA CCT GGA
VH2L	CAG ATG GAC ATA CTT TGT TCC AC
VH3L	CCA TGG AGT TTG GGC TGA GC
VH4L	CGA TGA AAC ACC TGT GGT TCT T
VH5L	ATG GGG TCA ACC GCC ATC CT
VH6L	GAT GTC TGT CTC CTT CCT CAT
γ4-1	GGG GGA CCA TAT TTG GAC TC
IgH second-round PCR	
VH1F	CAG GTG CAG CTG GTG CAG TCT G
VH2F	GTC TTG TCC CAG GTC AAC TTA AGG GAG TCT T
VH3F	GAG GTG CAG CTG GTG GAG TCT G
VH4F	CAG GTG CAG CTG CAG GAG TCG G
VH5F	GAG GTG CAG CTG CTG CAG TCT G
VH6F	CTG TCA CAG GTA CAG CTG CAG CAG TCA G
γ4-2	GAT CTA CGT TGC AGG TGT AGG TCT TCG TG
Igκ first round	
L-Vk-1/2	ATG AGG GTC CCC GCT CAG CTG CTG G
L-Vk3	CTC TTC CTC CTG CTA CTC TGG CTC CCA G
L-Vk4	ATT TCT CTG TTG CTC TGG ATC TCT G
Ck543-566	GTT TCT CGT AGT CTG CTT TGC TCA
Igκ second round	
Pan-Vk	ATG ACC CAG ACT CCA TCC ACC CTG
Ck 494-516	GTG CTG TCT TGC TGT CCT GCT
Igλ first round	
L-lambda 1 forward	GGT CCT GGG CCC AGT CTG TGC TG
L-lambda 2 forward	GGT CCT GGG CCC AGT CTG CCC TG
L-lambda 3 forward	GCT CTG TGA CCT CCT ATG AGC TG
L-lambda 4/5 forward	GGT CTC TCT CGC AGC CTG TGC TG
L-lambda 6 forward	GTT CTT GGG CCA ATT TTA TGC TG
L-lambda 7 forward	GGT CCA ATT CCC AGG CTG TGG TG
L-lambda 8 forward	GAG TGG ATT CTC AGA CTG TGG TG
C-Lambda reverse	CAC CAG TGT GGC CTT GTT GGC TTG
Igλ second round	
F-lambda 1	CAG TCT GTG TTG ACG CAG CC
F-lambda 2	CAG TCT GCC CTG ACT CAG CC
F-lambda 3	TCT TAT GAG CTG ACA CAG CCA C
F-lambda 3l	TCT TCT GAG CTG ACT CAG GAC CC
F-lambda 4ab	CAG CTT GTG CTG ACT CAA TC
F-lambda 4c	CTG CCT GTG CTG ACT CAG C
F-lambda 5/9	CAG CCT GTG CTG ACT CAG C
F-lambda 6	AAT TTT ATG CTG ACT CAG CCC CAC T
F-lambda 7/8	CAG ACT GTG GTG ACC CAG GAG
F-lambda 10	CAG GCA GGG CTG ACT CAG
C-lambda-2	GGG TGG GAA CAG AGT GAC C
IgH cloning PCR	
VH1-3/1-5- <i>Bss</i> HII	c aca ggc gcg cac tcc CAG GTC CAG CTT GTG CAG TCT
VH3-23- <i>Bss</i> HII	c aca ggc gcg cac tcc GAG GTG CAG CTG TTG GAG TCT
VH1-2/1-69- <i>Bss</i> HII	c aca ggc gcg cac tcc CAG GTG CAG CTG GTG CAG T
VH3-73- <i>Bss</i> HII	c aca ggc gcg cac tcc GAG GTG CAG CTG GTG GAG T
VH3-74- <i>Bss</i> HII	c aca ggc gcg cac tcc GAG GTG CAG CTG GTG GAG

TABLE E1. (Continued)

Primer	Sequence
VH4-39- <i>Bss</i> HII	c aca ggc gcg cac tcc CAG CTG CAG CTG CAG GAG
VH5-51- <i>Bss</i> HII	c aca ggc gcg cac tcc GAG GTG CAG CTG GTG CAG T
VH4-04- <i>Bss</i> HII	c aca ggc gcg cac tcc CAG GTG CAG CTG CAG GAG T
VH3-11- <i>Bss</i> HII	c aca ggc gcg cac tcc CAG GTG CAG CTG GTG GAG T
VH5a- <i>Bss</i> HII	c aca ggc gcg cac tcc GAA GTG CAG CTG GTG CAG TCT
JH1/4/5- <i>Nhe</i> I	ctg tgt gct agC TGA GGA GAC GGT GAC CAG
JH2- <i>Nhe</i> I	ctg tgt gct agC TGA GGA GAC AGT GAC CAG G
JH3- <i>Nhe</i> I	ctg tgt gct agC TGA AGA GAC GGT GAC CAT TGT
JH6- <i>Nhe</i> I	ctg tgt gct agC TGA GGA GAC GGT GAC CGT
Igκ cloning PCR	
Vκ1-33- <i>Sac</i> II	gct agc cgc ggt GAC ATC CAG ATG ACC CAG
Vκ2-24/2D-29- <i>Sac</i> II	gct agc cgc ggt GAT ATT GTG ATG ACC CAG ACT
Vκ3-15- <i>Sac</i> II	gct agc cgc ggt GAA ATA GTG ATG ACG CAG TCT
Vκ3-20- <i>Sac</i> II	gct agc cgc ggt GGT GCA TCC AGC AGG G
Vκ4-1- <i>Sac</i> II	gct agc cgc ggt GAC ATC GTG ATG ACC CAG
Vκ1-5- <i>Sac</i> II	gct agc cgc ggt GAC ATC CAG ATG ACC CAG
Jκ1- <i>Bsi</i> WI	cgc cac cgt acG TTT GAT TTC CAC CTT GGT CC
Jκ2- <i>Bsi</i> WI	cgc cac cgt acG TTT GAT CTC CAG CTT GGT C
Jκ3- <i>Bsi</i> WI	cgc cac cgt acG TTT AAT CTC CAG TCG TGT CC
Jκ4- <i>Bsi</i> WI	cgc cac cgt acG TTT GAT CTC CAC CTT GGT C
Jκ5- <i>Bsi</i> WI	cgc cac cgt acG TTT AAT CTC CAG TCG TGT CC
Igλ cloning PCR	
Vλ1-40- <i>Bsp</i> EI	gga tcc gga gtc gac tct CAG TCT GTG CTG ACG CAG C
Vλ1-47- <i>Bsp</i> EI	gga tcc gga gtc gac tct CAG TCT GTG CTG ACT CAG C
Vλ1-51- <i>Bsp</i> EI	gga tcc gga gtc gac tct CAG TCT GTG TTG ACG CAG C
Vλ2-8/2-23- <i>Bsp</i> EI	gga tcc gga gtc gac tct CAG TCT GCC CTG ACT CAG C
Jλ- <i>Avr</i> II	tt ggg ctg acC TAG GAC GGT CA
Vector sequencing	
pTT3-seq-F	CCA AAA ACG AGG AGG ATT TGA TAT
pTT3-seq-R	TCT CCG AGG GAT CTC GAC CAA ATG A

Restriction enzyme sites are displayed in lower case.

(Continued)