

Abbreviations used

CFA: Complete Freund adjuvant
 DC: Dendritic cell
 DTH: Delayed-type hypersensitivity
 IVIG: Intravenous immunoglobulin
 OVA: Ovalbumin
 PC: Plasma cell
 SIT: Specific immunotherapy
 TD: T cell dependent
 TNP: 2,4,6-Trinitrophenyl

The initiation and termination of immune responses require tight regulation to prevent inflammatory immune responses to non-pathogenic organisms or antigens and to minimize the risk of immune diseases, such as allergy, autoimmunity, or inflammatory bowel disease. T cell-dependent (TD) protein antigens are known to elicit strong inflammatory immune responses if recognized in the presence of inflammatory costimulatory signals, whereas lack of inflammatory costimulation leads to the induction of regulatory T cells and peripheral tolerance.¹⁻³ IgG antibodies are generated under both conditions, but whether the IgGs themselves provide different immunoregulatory effector functions is yet unknown.

The proinflammatory and anti-inflammatory effector functions of IgG subclass antibodies are mediated by their different affinities to activating Fc γ receptors (Fc γ Rs), and the expression ratio between activating Fc γ Rs and the inhibitory Fc γ RIIB on immune cells.⁴⁻⁶ However, it was recently shown that the proinflammatory and anti-inflammatory effector functions of IgG antibodies are also regulated by Fc N-linked glycosylation.⁷⁻⁹ Desialylation of serum IgG correlates with proinflammatory responses and disease activity in patients with rheumatoid arthritis.^{10,11} In contrast, sialylated IgGs mediate the immunosuppressive effects of intravenous immunoglobulin (IVIG), which is purified from pooled human plasma and administered at high doses (2 g/kg) for the treatment of autoimmunity and allergy.^{7-9,12,13} However, the inductive mechanisms for the formation of sialylated IgG antibodies are still unknown.

Here we investigated the influence of TD B-cell activation without or in the presence of an inflammatory costimulus on IgG Fc glycosylation. Our data show that tolerance induction with TD antigens induces plasma cells (PCs) expressing high levels of α 2,6-sialyltransferase and producing regulatory sialylated IgG₁ in mice. Antigen-specific sialylated murine IgG₁ antibodies thereby protect against dendritic cell (DC) maturation and pathologic TD immune reactions in an antigen-specific manner. Additionally, formation of sialylated IgG antibodies was observed in human allergic patients after successful tolerance induction by using allergen-specific immunotherapy (SIT).

METHODS**Mice**

C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Bar Harbor, Me). Fc γ RIIB^{-/-} and IL-4 receptor α ^{-/-} mice were on the C57BL/6 background.¹⁴⁻¹⁶ Mice were bred and maintained in accordance with institutional guidelines. Eight- to 12-week-old female mice were analyzed.

Reagents

Ovalbumin (OVA) was purchased from Sigma (St Louis, Mo; no. A5503). 2,4,6-Trinitrophenyl (TNP)(12)-FICOLL and TNP(12)-BSA were purchased

from Biosearch Technology (Novato, Calif). TNP(3)-OVA was prepared with TNP-e-aminocaproyl-OSu (Biosearch Technologies, no. T-1030) in house. IVIG (Intracet) was from Biotest (Boca Raton, Fla). LPS from *Escherichia coli* (no. L4524), complete Freund adjuvant (CFA; no. F5881; 1 mg mycobacterium tuberculosis/mL), and incomplete Freund adjuvant (no. F5506) were purchased from Sigma. Enriched CFA was prepared by adding heat-killed *Mycobacterium tuberculosis* H37 RA (DIFCO Laboratories, Detroit, Mich) to incomplete Freund adjuvant (5 mg mycobacterium tuberculosis/mL).

CD4⁺ cell depletion and inhibition of the CD40/CD40 ligand interaction

Mice were treated with sequential intraperitoneal injections of 250 μ g of anti-mouse CD4 (clone GK1.5) or anti-mouse CD154 (MR-1), respectively, to deplete CD4⁺ cells or to block the CD40/CD40 ligand interaction.

Synthesis of Dec-OVA in 293T human embryonic kidney cells

Dec-OVA IgG₁¹⁷ fusion antibodies were produced by means of polyethylenimine-mediated cotransfection of human embryonic kidney cells in serum-free medium containing 0.03% Primatone RL/UF (Sheffield Bio-Science, Norwich, NY) with IgH and IgL chain-encoding plasmid DNA.

OVA immunization and anti-OVA IgG antibody purification

Eight-week-old C57BL/6 mice were immunized as indicated. Serum samples were taken at day 14, and pooled serum IgG antibodies were purified with protein G-sepharose. OVA-reactive IgG was purified by using OVA coupled to cyanogen bromide-activated sepharose 4B (GE Healthcare, Fairfield, Conn) columns (in-house preparation). Enrichment of OVA-reactive IgG was verified by using ELISA.

In vitro sialylation and desialylation of IgG antibodies

The *in vitro* sialylation of the murine anti-TNP IgG₁ (clone H5)¹⁸ and anti-OVA IgG₁ hybridoma (clone 4C9, unpublished) antibodies was performed in a 2-step procedure, as described previously.⁸ Briefly, antibodies were galactosylated with human β 1,4-galactosyltransferase and uridine diphosphate galactose and subsequently sialylated with human α 2,6-sialyltransferase and CMP-sialic acid (all from Calbiochem, Nottingham, United Kingdom). Desialylation of the anti-OVA IgG₁ (4C9) was performed with a ProZyme Sialidase kit (no. GK80040; ProZyme, Hayward, Calif).

Human samples

Human blood was provided by healthy volunteers (n = 5), volunteers with untreated birch pollen allergy in or after the pollen season (n = 7), and 2 patients with birch pollen allergy after the pollen season (n = 2, SIT P1 *1998 and SIT P2 *1959) who had received complete and successful subcutaneous SIT (Allergovit; Allergopharma, Zurich, Switzerland) over 3 years (autumn 2008 to summer 2011). Serum analyses from patients were approved by local authorities, and written informed consent was obtained from the patients.

Total and anti-Bet v 1 human serum IgG purification

Human serum IgG was purified with protein G-sepharose. Bet v 1-reactive IgG was enriched by using rBet v 1 coupled to cyanogen bromide-activated sepharose 4B columns (in-house preparation). Enrichment of Bet v 1-reactive IgG was verified by means of ELISA. rBet v 1 was expressed in *E coli* BL21 by cloning the gene for Bet v 1 (accession no. X15877)¹⁹ into the pET23b vector and adding a C-terminal His-tag sequence (Novagen). Bet v 1 purification was performed by using metal affinity chromatography (Talon Superflow; Clontech, Mountain View, Calif).

Glycan analysis

IgG samples were digested with recombinantly expressed endoglycosidase S from *Streptococcus pyogenes* purified as previously described.²⁰ The resulting N-linked glycans were purified by means of solid-phase extraction with reversed-phase C18 and graphitized carbon columns (Alltech, Lexington, Ky). The samples were permethylated according to standard protocols and further analyzed by means of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in duplicate. The spectra were recorded on an Ultraflex III mass spectrometer (Bruker Daltonics, Billerica, Mass) equipped with a Smartbeam laser. Calibration was performed on a glucose ladder, and 2,5-dihydroxybenzoate was used as the matrix. The spectra were recorded in reflector positive ionization mode, and mass spectra from 3000 laser shots were accumulated.

Delayed-type hypersensitivity response

Delayed-type hypersensitivity (DTH) responses were induced by injecting 5 μ g of Dec-OVA plus 50 μ g of anti-CD40 (1C10) intraperitoneally, followed by injecting 37.5 μ g of OVA in Montanide ISA 50V (Seppic, Puteaux, France) into the right footpad 14 days later or on the indicated days. PBS in Montanide was injected into the left footpad. The differences in footpad thickness between the right and left footpads were determined by using an Oditest micrometer gauge (Kroepelin, Schluechtern, Germany) in a blind test.

Murine model of allergic airway inflammation and lung preparation and histology

Mice were injected intraperitoneally with 50 μ g of OVA in alum on days 1 and 15. On days 29 and 30, the mice were anesthetized with ketamine/xylazine, and 50 μ g of OVA in 50 μ L of PBS was applied intranasally. Alternatively, Fc γ RIIB^{-/-} mice were challenged intratracheally when indicated with 50 μ L of a 1.5% OVA solution. Mice were killed on day 36. Total cell numbers in the bronchoalveolar lavage fluid were counted, and approximately 10,000 cells underwent the cytospin protocol and placed on slides to calculate the total cell subtype numbers by using May-Grünwald-Giemsa (Sigma) staining. The right lung was strangulated, and the left lung was prepared with Tissue-Tek OCT compound or paraffin for hematoxylin and eosin histology. The right lungs were transferred to C-tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) containing 3 mL of RPMI 1640 (Gibco, Carlsbad, Calif) with 0.5% BSA, 1.5 mg of collagenase D (Roche, Mannheim, Germany), and 60 μ g of DNase I (Roche) and were sheared by using the Gentle-MACS Dissociator (Miltenyi Biotec). For restimulation, the prepared lung cells were treated in culture with 1 μ M/L OVA for 72 hours.

ELISA

OVA-, TNP-, and Bet v 1-reactive antibodies were measured by means of ELISA. OVA-, TNP(12)-BSA-, or rBet v 1-coated plates were blocked and subsequently incubated with 1:100 diluted serum or with purified antibodies in PBS. Bound antibodies were detected with anti-human IgG (clone HP-6017, mouse IgG_{2a}), IgG₁ (HP-6001, mouse IgG_{2b}), IgG₂ (HP-6014, mouse IgG₁), IgG₃ (HP-6050, mouse IgG₁), or IgG₄ (HP-6025, mouse IgG₁) and/or horseradish peroxidase-coupled polyclonal goat anti-mouse IgG₁, IgG_{2a} (IgG_{2a} haplotype a), IgG_{2c} (IgG_{2a} haplotype b), IgG_{2b}, or IgE secondary antibodies (Bethyl Laboratories, Montgomery, Tex), respectively. IL-5 and IL-13 ELISA kits were purchased from R&D Systems (Minneapolis, Minn).

Flow cytometric analysis

The following fluorochrome- or biotin-conjugated anti-mouse antibodies and reagents were used: anti-CD138 (218-2), anti-IL-4 (11B11), anti-SiglecF (E50-2440), anti-CD4 (RM4-5), streptavidin (all from BD Biosciences, San Jose, Calif), anti-CD154 (MR1; eBioscience, San Diego, Calif), anti-Mac1 (M1/70), anti-Gr-1 (RB68C5), and anti-CD11c (N418; all produced in house). Anti-mouse α 2,6-sialyltransferase polyclonal rabbit IgG (M2) was purchased from IBL (Minneapolis, Minn), total rabbit IgG (isotype control) was from R&D Systems, and Cy5-conjugated anti-rabbit IgG was from Invitrogen

(Carlsbad, Calif). Biotin-conjugated OVA was produced in house. For intracellular cytokine and antibody staining, Cytofix/Cytoperm (BD Biosciences) was used. For intracellular cytokine analysis, the cells were stimulated with phorbol 12-myristate 13-acetate/ionomycin for 4 hours; brefeldin A was added after 1 hour.

Quantitative RT-PCR

C57BL/6 mice were immunized with 100 μ g of OVA in CFA. Untreated and treated mice were killed on day 8, and CD138⁺ PCs were sorted with a FACSaria (Becton Dickinson, Franklin Lakes, NJ). cDNA (Superscript III, Invitrogen) was analyzed by means of quantitative RT-PCR for St6gal1 (forward primer, 5'-tttaccctcctcaagccccag-3'; reverse primer, 5'-tggtaggcaccatcggtg-3'; 232 bp) and β -actin (forward primer: 5'-gcaccacacctctacaatgag-3'; reverse primer, 5'-aatagcacagcctggatagcaac-3'; 180 bp) with a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, Calif). The $\Delta\Delta$ Ct method was used to calculate the relative expression.

DC culture

Bone marrow-derived DCs were generated over 9 days in Iscove Modified Dulbecco Medium (Invitrogen) containing 10% FCS, 10 ng/mL IL-4, and 20 ng/mL GM-CSF (R&D Systems). Subsequently, the cells were cultured in 96-well plates coated with 10 μ g/mL TNP-Ficoll and 0.01 μ g/mL sialylated or nonsialylated anti-TNP IgG₁ H5 antibodies or 10 mg/mL IVIG in medium containing 10% IgG-depleted FCS. After 30 minutes, cells were additionally stimulated with 500 pg/mL LPS for 48 hours. The IL-6 (BD Biosciences) and p40 (R&D Systems) concentrations were detected by using an ELISA.

Statistical analysis

Statistical analyses were performed with the Student *t* test, and the SEM was used.

RESULTS

Tolerance induction by TD antigen immunization without an inflammatory costimulus drives the formation of antigen-reactive IgG₁ in mice

To address the question of whether tolerance induction by TD immune responses without inflammatory costimuli is associated with the generation of IgG antibodies with anti-inflammatory properties, we used a fusion protein of OVA and the Dec-205 antibody with specificity for the DC surface marker CD205 (Dec-OVA).^{1-3,17} As expected, targeting DCs with 5 μ g of Dec-OVA in the absence of inflammatory costimulatory signals induced tolerance to OVA, protecting against a subsequent induced DTH pathologic immune response in C57BL/6 mice (Fig 1). DTH was induced by Dec-OVA in the presence of an agonistic anti-CD40 antibody, which induces CD40 signaling in DCs, and subsequent injection of OVA into the right footpad. OVA-specific tolerance inhibited the generation of OVA-reactive IgG_{2c} (IgG_{2a}, haplotype b), IgG_{2b}, and IgE serum antibodies (Fig 1, B, and see Fig E1, A and B, in this article's Online Repository at www.jacionline.org), as well as DTH-mediated footpad swelling (Fig 1, C). However, tolerance induction with Dec-OVA was also associated with the development of anti-OVA IgG₁ serum antibodies (Fig 1, B), which was dependent on CD4⁺ cells and CD40 ligand and IL-4 receptor α cosignaling (see Fig E2 in this article's Online Repository at www.jacionline.org).

Tolerance to OVA was also induced in C57BL/6 mice by injecting 2 mg of pure OVA without an inflammatory costimulus for priming (see Fig E1, C). Again, OVA-reactive IgG₁ serum antibodies could be observed (see Fig E1, D). In summary, our data show that tolerance induction with a TD antigen in the absence of

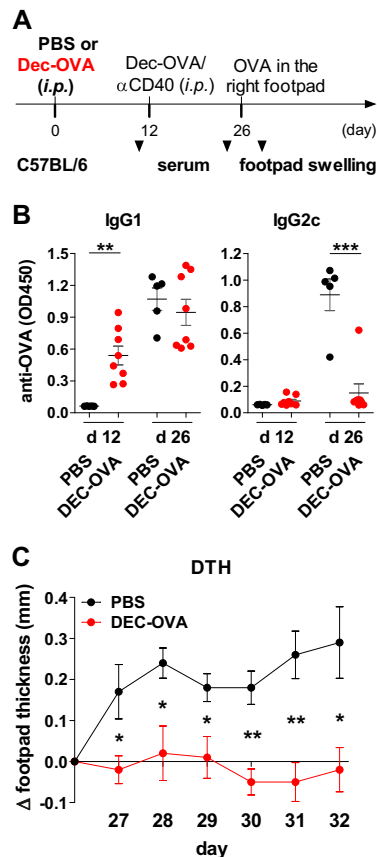


FIG 1. TD tolerance induction drives the development of antigen-reactive IgG₁. **A**, Experimental setup for Fig 1, **B** and **C**. *i.p.*, Intraperitoneal. **B**, Anti-OVA IgG subclass serum levels, as analyzed on days 12 and 26. **C**, Footpad swelling. One of 4 independent experiments is shown. **P* < .05, ***P* < .01, and ****P* < .001.

inflammatory costimulatory signals is associated with the formation of antigen-reactive serum IgG₁.

Transfer of antigen-reactive IgGs induced by TD immunization without an inflammatory costimulus protects against pathologic TD immune reactions

C57BL/6 mice were treated with an intravenous injection of 50 μ g of anti-OVA IgG purified from pooled sera of Dec-OVA (5 μ g administered intraperitoneally)-immunized C57BL/6 mice 1 day before induction of an OVA-specific pathologic DTH response to determine whether IgG antibodies induced by a TD antigen without an inflammatory costimulus are capable of inhibiting TD inflammatory immune responses (Fig 2). After transfer of OVA-reactive IgG, the generation of OVA-reactive IgG₁, IgG_{2c}, and IgG_{2b} serum antibodies on DTH induction (Fig 2, **B**) and DTH-mediated footpad swelling (Fig 2, **C**) were reduced. Therefore we conclude that TD antigen-reactive IgG molecules formed after immunization with a TD antigen in the absence of inflammatory costimulatory signals inhibit pathologic TD immune responses, including IgG₁, IgG_{2c}, and IgG_{2b} responses.

TD tolerance induction induces antigen-reactive sialylated IgGs

N-linked Fc glycosylation of IgG molecules is known to influence immunoregulatory functions of IgG molecules. In

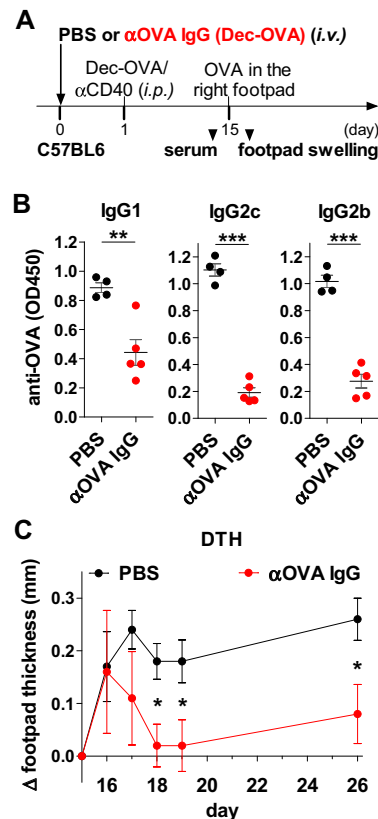


FIG 2. Antigen-reactive serum IgG from tolerized mice protects against pathologic antigen-mediated TD immune reactions. **A**, Experimental setup. On day 0, mice were injected with PBS or anti-OVA IgG purified from the pooled sera of Dec-OVA-immunized C57BL/6 mice. *i.p.*, Intraperitoneal; *i.v.*, intravenous. **B**, Anti-OVA IgG subclass serum levels. **C**, Footpad swelling. One of 2 independent experiments is shown. **P* < .05, ***P* < .01, and ****P* < .001.

particular, α 2,6-sialyltransferase-mediated terminal Fc sialylation has been associated with an immunosuppressive potential of IgG.⁷⁻⁹ The IgG Fc glycan coupled to Asn 297 is a bisecting core of 4 N-acetyl-glucosamines (GlcNAc) and 3 mannoses (Man), which can be modified by the attachment of fucose, bisecting GlcNAc, galactose (Gal) and sialic acid (murine N-glycolylneuraminic acid or human N-acetylneuraminic acid [sial]; Fig 3, **A**, and see Fig E3 in this article's Online Repository at www.jacionline.org).

To determine whether the IgG antibodies induced on TD tolerance induction exhibit different sialylation patterns than IgG antibodies formed under inflammatory conditions, we analyzed the Fc sialic acid content of purified OVA-reactive serum IgG antibodies after immunization of C57BL/6 mice with OVA alone or with an inflammatory costimulus (Fig 3, **B**, and see Fig E3). OVA-reactive serum IgGs induced under proinflammatory conditions (immunization with 5 μ g of Dec-OVA plus 50 μ g of anti-CD40 or 100 μ g of OVA in CFA) showed a significantly lower sialic acid content than steady-state total serum IgG from untreated control mice (Fig 3, **B**). In contrast, the sialic acid content of OVA-reactive IgG induced under tolerogenic conditions (immunization with 5 μ g of Dec-OVA or 2 mg of OVA without an inflammatory costimulus) was similar to the level measured in total IgG from untreated mice (Fig 3, **B**). Therefore TD tolerance induction induces the generation of antigen-reactive IgG

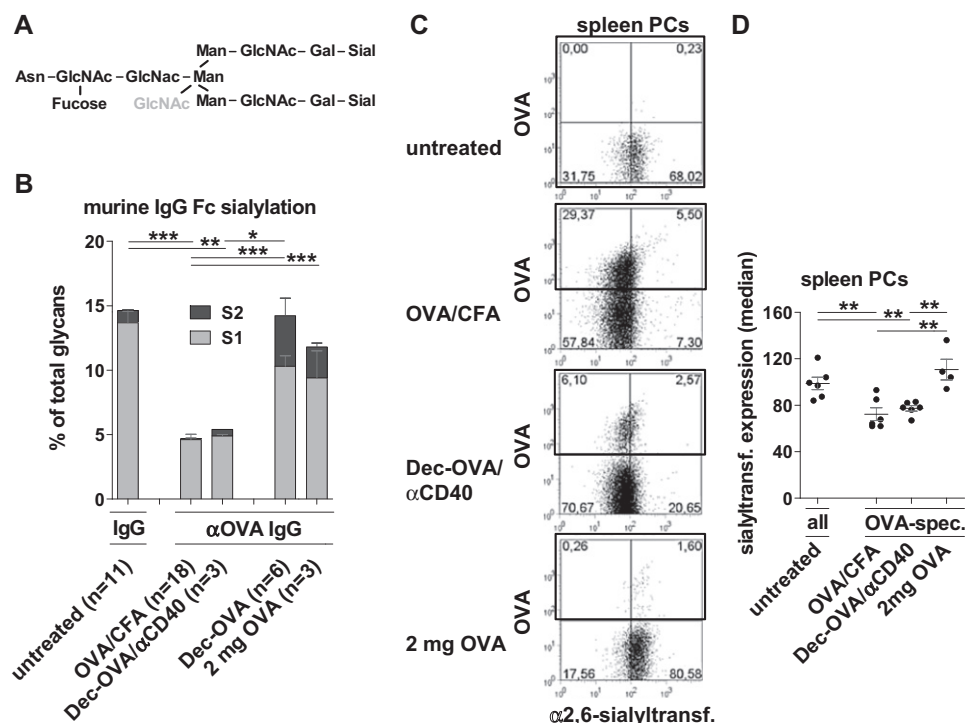


FIG 3. Tolerance induction with TD antigen induces PCs expressing high levels of $\alpha 2,6$ -sialyltransferase and secreting sialylated IgGs. **A**, Scheme of the maximal Fc glycan coupled to Asn 297. **B**, Fc sialic acid content of total serum IgG from untreated mice and anti-OVA IgG from indicated mice. Bars indicate the frequency of glycan structures with 1 or 2 sialic acid residues (S1 and S2). **C** and **D**, Splenic PCs from indicated mice were analyzed for intracellular OVA reactivity and $\alpha 2,6$ -sialyltransferase expression 9 days after immunization. Fig 3, D, Median of $\alpha 2,6$ -sialyltransferase expression (gated total PCs from untreated mice and OVA-reactive PCs from immunized mice, as indicated in Fig 3, C). One of 3 independent experiments is shown. * $P < .05$, ** $P < .01$, and *** $P < .001$.

serum antibodies with a higher degree of Fc sialylation compared with that seen in IgG molecules formed during inflammation.

TD tolerance induction drives the formation of antigen-reactive PCs producing high levels of $\alpha 2,6$ -sialyltransferase

To determine whether the differences in the Fc sialylation content of TD antigen-reactive serum IgGs are associated with differences in $\alpha 2,6$ -sialyltransferase expression in antibody-secreting B cells, we measured $\alpha 2,6$ -sialyltransferase protein levels in antigen-reactive CD138⁺ splenic PCs using flow cytometry (Fig 3, C and D). Antigen-reactive PCs induced under proinflammatory conditions (immunization of C57BL/6 mice with 5 μ g of Dec-OVA plus 50 μ g of anti-CD40 or 100 μ g of OVA in CFA) expressed lower $\alpha 2,6$ -sialyltransferase levels than steady-state PCs from untreated mice. The expression level of $\alpha 2,6$ -sialyltransferase further decreased in antigen-reactive PCs after immunization with OVA in enriched CFA containing additional heat-killed *Mycobacterium tuberculosis* (see Fig E4 in this article's Online Repository at www.jacionline.org). In contrast, antigen-reactive PCs formed after tolerance induction (immunization with 2 mg of OVA alone) did not downregulate $\alpha 2,6$ -sialyltransferase expression, showing $\alpha 2,6$ -sialyltransferase expression levels comparable with those in steady-state PCs of untreated mice (Fig 3, C and D). Accordingly, TD inflammation leads to reduction of transcription of the St6gal1

gene encoding $\alpha 2,6$ -sialyltransferase in splenic PCs (see Fig E5 in this article's Online Repository at www.jacionline.org). Thus TD immune responses induced without an inflammatory costimulus do not downregulate $\alpha 2,6$ -sialyltransferase expression in antigen-reactive PCs and thereby mediate secretion of sialylated IgGs.

Antigen-specific sialylated monoclonal IgG₁ protects against TD pathologic immune responses in an antigen-specific manner

C57BL/6 mice were injected intravenously with anti-OVA IgG₁ molecules (murine hybridoma antibodies, clone 4C9) with different Fc sialylation contents 1 day before DTH induction to further investigate whether Fc sialylation is involved in antibody-mediated blocking of inflammatory TD immune reactions (Fig 4, A). Highly sialylated IgG₁ antibodies (*in vitro* sialylated to 80% sialylation [sial]), desialylated IgG₁ antibodies (sialidase-treated to exhibit <2% sialylation [de-sial]), and mixtures of both antibodies, resulting in 5% or 20% sialylated IgG₁ antibodies, were used for transfer (Fig 4, A and B). Sialylation of the 4C9 antibodies did not influence OVA binding (see Fig E6 in this article's Online Repository at www.jacionline.org). Only 20% and 80% sialylated but not 5% sialylated and desialylated anti-OVA IgG₁ 4C9 antibodies blocked OVA-induced DTH responses (Fig 4, C), which is consistent with the sialylation frequencies observed in antigen-specific serum IgG after TD tolerance induction (Fig 3, B).

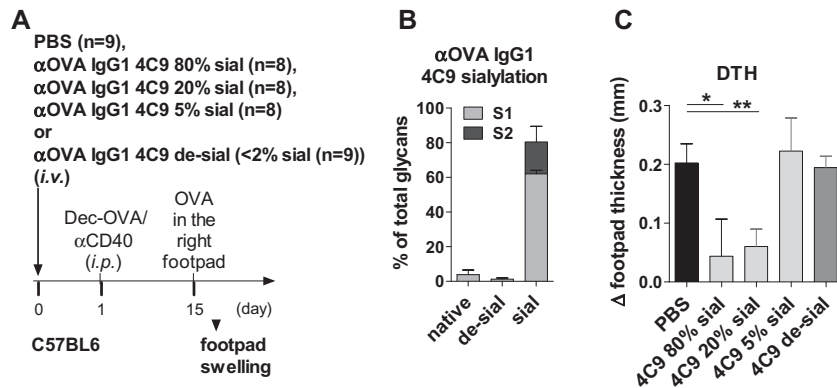


FIG 4. IgG₁-mediated protection from OVA-induced DTH requires Fc sialylation. **A**, Experimental setup. On day 0, mice were injected intravenously with 100 µg of the indicated antibodies. *i.v.*, Intravenous. **B**, Frequency of Fc sialic acid modifications with 1 or 2 sialic acid residues (S1 and S2) on native (native, 4% sialylation), sialidase-treated (*de-sial*, <2% sialylation) and *in vitro* sialylated (*sial*, 80% sialylation) OVA-specific IgG₁ (4C9). **C**, Footpad swelling 5 days after local DTH induction. One of 2 independent experiments is shown. *P < .05 and **P < .01.

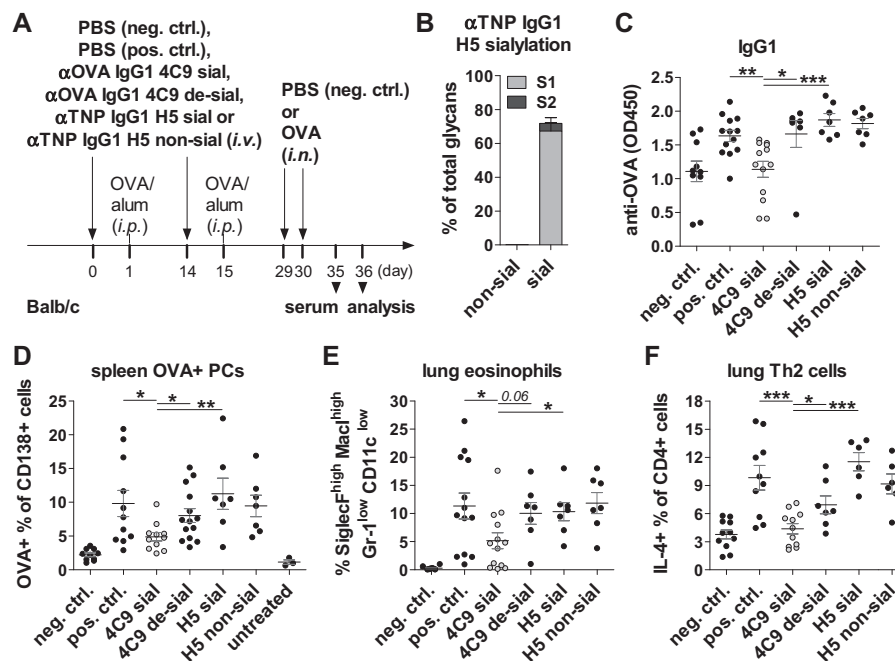


FIG 5. Only OVA-reactive sialylated IgG₁ protects against OVA-induced allergic airway inflammation. **A**, Experimental setup. On days 0 and 14, mice were injected intravenously with 200 µg of *in vitro* sialylated (4C9 *sial*, 80% sialylation) or sialidase-treated (4C9 *de-sial*, <2% sialylation) OVA-specific IgG₁ (4C9) or *in vitro* sialylated (H5 *sial*, 70% sialylation) or native nonsialylated (H5 *non-sial*, <1% sialylation) TNP-specific IgG₁ (H5) antibodies. *i.n.*, Intranasal; *i.p.*, intraperitoneal; *i.v.*, intravenous. **B**, Frequency of Fc sialic acid modifications on native (non-sial) or sialylated (*sial*) anti-TNP IgG₁ (H5). **C**, Anti-OVA IgG₁ levels on day 35. **D-F**, Frequencies of OVA-reactive PCs and lung SiglecF^{high}Mac1^{high}Gr-1^{low}CD11c^{low} eosinophils and Th2 cells. The results summarize 2 independent experiments. *P < .05, **P < .01, and ***P < .001.

To determine whether sialylated IgG₁ can also inhibit the development of allergic airway inflammation and whether the tolerogenic effect of sialylated IgG₁ was antigen specific, we investigated the anti-inflammatory effect of sialylated monoclonal IgG₁ in an OVA-induced allergic airway inflammation model in BALB/c mice (Fig 5 and see Fig E7 in this article's Online Repository at www.jacionline.org). Allergic airway inflammation was induced by means of intraperitoneal sensitization 2 times with 50 µg of OVA in alum and 2 subsequent intranasal challenges with OVA that induced formation of desialylated anti-OVA serum

IgG (see Fig E7). To investigate the tolerogenic potential of sialylated OVA-reactive IgG₁, mice were injected intravenously with OVA-reactive IgG₁ (4C9) with enhanced Fc sialylation (80% sialylation as described above [*sial*] or low Fc sialylation (<2% sialylation, as described above [*de-sial*] or, as an unspecific control, with TNP-reactive IgG₁ (clone H5) with enhanced Fc sialylation (70% sialylation after *in vitro* sialylation [*sial*] or low Fc sialylation (native hybridoma antibody with <1% sialylation [*non-sial*]) 1 day before each of the OVA sensitizations (Fig 5, A and B, and see Fig E7). Only *in vitro* sialylated anti-OVA

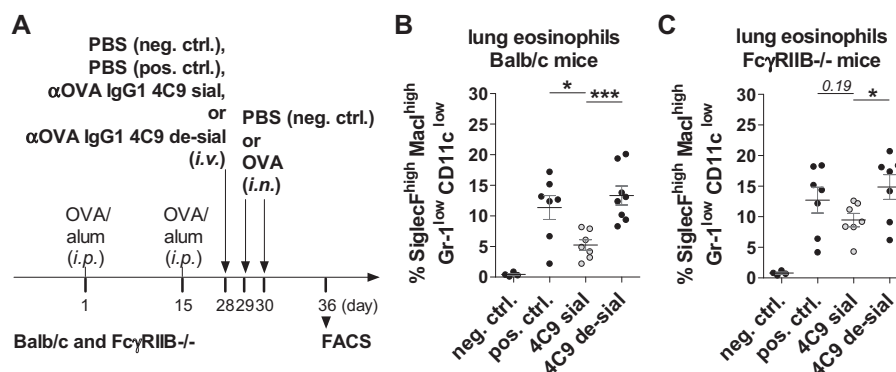


FIG 6. Therapeutic application of antigen-specific sialylated monoclonal IgG₁ after sensitization protects against allergic airway inflammation. **A**, Experimental setup in BALB/c and FcγRIIB^{-/-} mice. On day 28, the mice were injected intravenously (i.v.) with 200 μg of *in vitro* sialylated (4C9 sial, 80% sialylation) or sialidase-treated (4C9 de-sial, <2% sialylation) OVA-specific IgG₁ (4C9) antibodies. i.n., Intranasal; i.v., intravenous. **B** and **C**, Frequency of lung SiglecF^{high}Mac^{high}Gr-1^{low}CD11c^{low} eosinophils in BALB/c and FcγRIIB^{-/-} mice, as measured by means of flow cytometry. One of 2 independent experiments is shown for each strain. **P* < .05 and ****P* < .001.

IgG₁ 4C9 antibodies blocked anti-OVA serum IgG₁, IgG_{2a}, and IgG_{2b} responses; OVA-reactive splenic PC development; and eosinophilic and IL-4⁺ T_H2 cell infiltration in the lung (Fig 5, C-F, and see Fig E7).

It is known that Fc sialylation reduces the affinity of IgG antibodies to the IgG inhibitory receptor FcγRIIB and that the sialylated subfraction of IVIG mediates immunosuppression through the C-type lectin, SIGN-R1, (specific ICAM-3 grabbing nonintegrin-related 1) expressed on regulatory marginal zone macrophages primarily independent of FcγRIIB.^{8,12,13} Antibody transfer before induction of allergic airway inflammation was performed in FcγRIIB-deficient mice to understand whether the protective effect of OVA-reactive sialylated IgG₁ is independent of FcγRIIB (see Fig E8 in this article's Online Repository at www.jacionline.org). Only *in vitro* sialylated (sial) but not desialylated (de-sial) anti-OVA IgG₁ 4C9 antibodies inhibited anti-OVA IgG₁, IgG_{2c}, IgG_{2b}, and IgE serum antibody responses; OVA-reactive splenic CD138⁺ PC development; eosinophilic infiltration in the lung; total numbers of eosinophils, lymphocytes, monocytes, and neutrophils in the bronchoalveolar lavage fluid; and IL-5 and IL-13 in the supernatants of restimulated lung cells (see Fig E8).

The data show that prophylactic application of sialylated but not desialylated OVA-reactive IgG₁ protects against TD inflammatory immune responses, including IgG₁, IgG_{2a}, IgG_{2b}, and IgE responses, independent of the inhibitory FcγRIIB in an antigen-specific manner. The therapeutic application of *in vitro* sialylated anti-OVA IgG₁ 4C9 antibodies after sensitization was sufficient to block ongoing inflammatory DTH and allergic immune responses, although this effect might be at least partially dependent on FcγRIIB (Fig 6 and see Fig E9 in this article's Online Repository at www.jacionline.org).

Immune complexes containing sialylated IgG₁ inhibit the maturation of DCs independent of the IgG inhibitory receptor FcγRIIB

We showed that prophylactic treatment with antigen-specific sialylated IgG₁ inhibited the induction of antigen-specific inflammatory T- and B-cell responses. DC maturation is a prerequisite

for the induction of inflammatory T- and B-cell responses. The proinflammatory IL-6 and IL-12/IL-23 p40 response by LPS-stimulated bone marrow-derived DCs from C57BL/6 wild-type and FcγRIIB-deficient mice was studied in the presence of IVIG or immune complexes (ICs) containing sialylated or nonsialylated IgG₁ to examine the role of sialylated IgG₁ in DC maturation (Fig 7 and see Fig E10 in this article's Online Repository at www.jacionline.org). Only IVIG²¹ and ICs containing sialylated IgGs were able to inhibit LPS-stimulated DC maturation in both strains. Therefore our data suggest that ICs containing sialylated IgG₁ inhibit LPS-stimulated DC maturation independent of FcγRIIB.

Successful tolerance induction with SIT induces sialylated antigen-specific IgGs in allergic patients

To investigate whether tolerance induction in human allergic patients is also associated with the generation of sialylated serum IgGs, we examined sialylation of anti-Bet v 1 (the major birch pollen allergen) serum IgG after successful SIT. Patients with birch pollen allergy are characterized by allergic rhinoconjunctivitis and anti-Bet v 1 serum IgE antibodies. Allergen-reactive serum IgG levels are low in and after the pollen season (see Fig E11 in this article's Online Repository at www.jacionline.org).²²⁻²⁷ SIT is associated with the induction of IL-10-producing regulatory T cells and a 10- to 100-fold increase in allergen-reactive human serum IgG antibodies, mainly protecting IgG₄ (the counterpart of murine IgG₁) and IgG₁.²²⁻²⁷ Here we investigated 2 patients with birch pollen allergy after a complete and successful SIT with birch pollen extract for 3 years (2008-2011: Allergovit, Allergopharma). In both patients SIT resulted in decreased anti-Bet v 1-reactive serum IgE levels (patient 1: 09/2008, 19 kU/L; 08/2011, 4 kU/L; patient 2: 2008, 60 kU/L; 08/2011, 29 kU/L), reduced allergic rhinoconjunctivitis, and high anti-Bet v 1 serum IgG antibody titers compared with the low titers of volunteers with untreated birch pollen allergy in or after the pollen season (see Fig E11).²²⁻²⁷ The sialylation level of Bet v 1-reactive serum IgG from both treated patients were comparable with anti-inflammatory commercial IVIG and total serum IgG levels from healthy volunteers (Fig 8). The low amounts of Bet

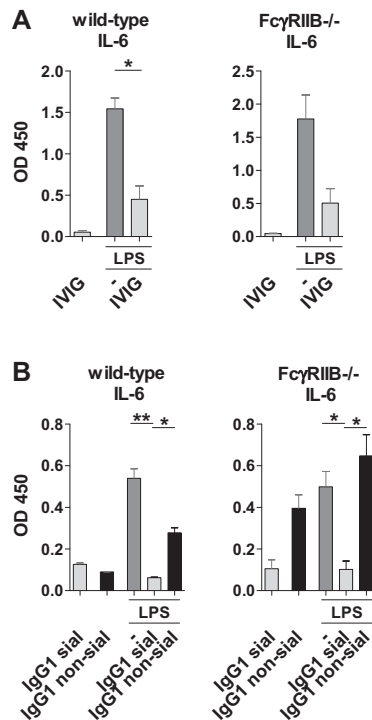


FIG 7. ICs containing sialylated IgGs inhibit LPS-induced DC maturation independent of Fc γ RIIB. Bone marrow–derived DCs from C57BL/6 wild-type and Fc γ RIIB^{-/-} mice were preincubated with IVIG (**A**) or ICs containing TNP-Ficoll and *in vitro* sialylated (IgG1 sial, 70% sialylation) or native non-sialylated (IgG1 non-sial, <1% sialylation) TNP-specific IgG₁ (H5). (**B**) for 30 minutes before adding LPS for 48 hours. IL-6 concentrations in the supernatants were analyzed by using ELISA. One of 3 independent experiments is shown for Fig 7, A and B. **P* < .05 and ***P* < .01.

v 1–reactive serum IgG from volunteers with untreated birch pollen allergy were not sufficient for Fc glycosylation analysis. Thus successful tolerance induction in allergic patients by using SIT also induces sialylated antigen-specific IgGs.

DISCUSSION

Tolerance induction with TD antigens induces regulatory T cells, whereas TD immune responses in an inflammatory environment induce proinflammatory T-cell responses. However, both immune reactions induce IgG antibodies. Our results show that tolerance induction with a TD antigen leads to the formation of sialylated anti-inflammatory IgG molecules, whereas TD immunization with inflammatory costimuli induces desialylated inflammatory IgG. Therefore it is not sufficient to study the presence of antigen-reactive serum IgG alone to predict the inflammatory quality of an immune response. The consideration of Fc glycosylation of the antigen-reactive IgG, particularly Fc sialylation, is crucial for differentiation between a tolerogenic and pathogenic immune status.

The immunoregulatory and anti-inflammatory properties of sialylated IgG have been described before and underlie the immunosuppressive effect of IVIG,^{7-9,12,13} but the origin of sialylated versus nonsialylated antibodies has not been described. Our data show that stimulation with TD antigens under inflammatory conditions leads to downregulation of α 2,6-sialyltransferase and production of desialylated IgGs by the formed PCs. In contrast, PCs formed on tolerance induction with a TD antigen do not

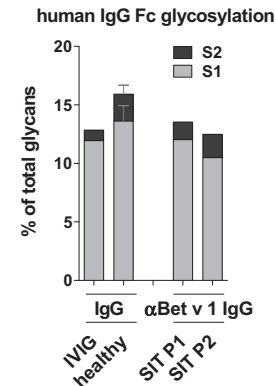


FIG 8. Tolerance induction against birch pollen allergy by SIT leads to formation of sialylated anti-Bet v 1 serum IgGs in human patients. Fc N-linked sialic acid content of commercial IVIG and total serum IgG from healthy volunteers (*n* = 5) or Bet v 1–reactive serum IgG from 2 patients with birch pollen allergy who had received a successful SIT over 3 years (SIT P1 and SIT P2) is shown. Bars indicate the frequency of glycan structures with 1 or 2 sialic acids (S1 and S2).

downregulate α 2,6-sialyltransferase expression. Thereby these PCs secrete immunosuppressive sialylated IgGs with a sialylation pattern comparable with that of PCs present under steady-state conditions in nonimmunized mice. The antigen reactivity of steady-state PCs in untreated mice is not known, but normal serum contains autoreactive and polyreactive IgGs, which might play an important role in maintaining self-tolerance.^{28,29} The induction of tolerance and sialylated IgGs by TD antigens might thereby be dependent on regulatory T cells.

Because TD tolerance induction primarily induced the non-complement-binding murine IgG₁ subclass, we investigated the inhibitory effect of sialylated monoclonal IgG₁. Our data show that antigen-specific sialylated murine IgG₁ antibodies are sufficient to inhibit pathologic immune responses in an antigen-specific manner. In contrast to the high amounts of IgG (2 g/kg) that are required for IVIG therapy, low doses of sialylated antigen-specific IgG₁ (2.5 mg/kg) were sufficient to mediate the immunosuppressive functions. Potential suppressive roles for sialylated IgG_{2a}, IgG_{2b}, and IgG₃ have to be investigated in the future.

Sialylated anti-OVA IgG₁ administered prophylactically before sensitization with OVA inhibited TD inflammatory responses by the inhibition of inflammatory T- and B-cell responses. Furthermore, ICs containing sialylated IgG₁ blocked LPS-induced DC maturation. Both effects were independent of Fc γ RIIB. Because Fc γ RIIB is the only IgG inhibitory receptor on murine DCs known thus far, our data suggest a yet unidentified inhibitory receptor for sialylated IgGs on DCs, which regulates DC maturation and thereby the development of antigen-specific proinflammatory T- and B-cell responses.

Therapeutic application of sialylated IgG₁ administered after sensitization with OVA blocked the late phases of allergic airway inflammation. However, the therapeutic effect was at least partially dependent on Fc γ RIIB. Previously, it was shown that the sialylated subfraction of IVIG inhibits innate immune responses through binding to SIGN-R1 on regulatory marginal zone macrophages, eventually upregulating Fc γ RIIB expression on other innate effector cells.^{12,13} Taking into account the data provided in this study, it is likely that blockage of allergic airway

inflammation through therapeutic application of sialylated IgG is mediated by inhibiting innate effector cells, indirectly depending on FcγRIIB.

Our results further show that successful tolerance induction in patients with birch pollen allergy by using allergen SIT also leads to the induction of sialylated allergen-specific serum IgGs. This finding supports the idea that the development of anti-inflammatory sialylated IgG in response to TD tolerance induction not only applies to the murine but also to the human system.

In summary, our data describe a novel antigen-specific immunoregulatory mechanism through the generation of regulatory sialylated IgGs after TD tolerance induction. Antigen-specific sialylated IgGs might have evolved as a physiologic control mechanism of inflammation. Such antibodies might serve as novel antigen-specific immunotherapeutics to suppress deleterious inflammatory responses in patients with allergic and autoimmune diseases.

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Key messages

- Tolerance induction with protein antigens induces sialylated IgG antibodies in mice and after allergen SIT in allergic patients.
- Antigen-specific sialylated IgGs indicate a novel antigen-specific immunoregulatory mechanism and might be a promising tool to treat allergy in an antigen-specific manner.

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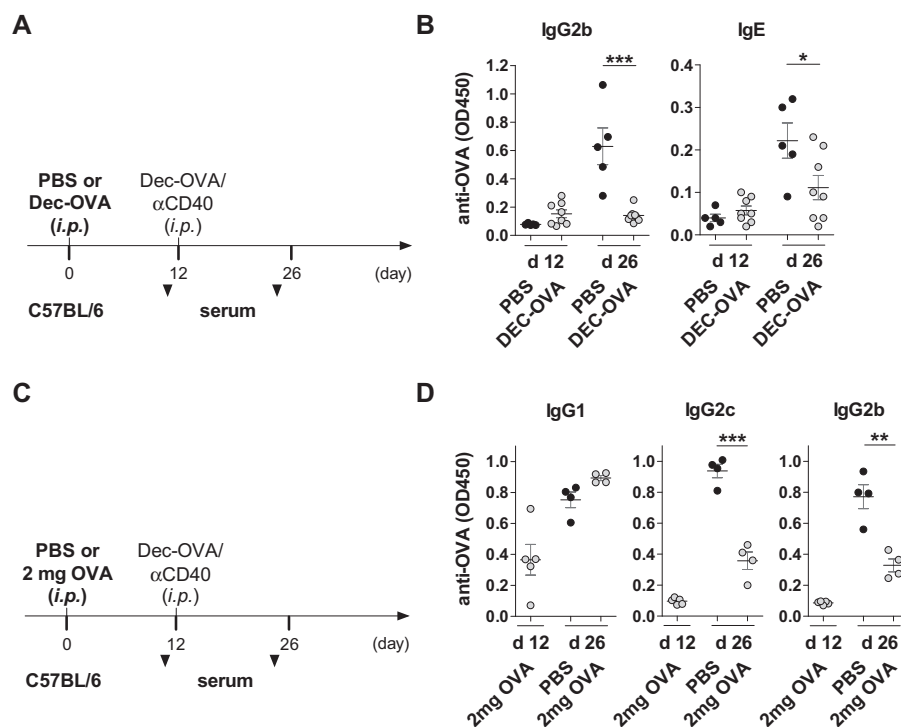


FIG E1. TD tolerance induction drives the formation of antigen-reactive IgG₁. **A**, Experimental setup for Fig E1, **B**, as described in Fig 1. **B**, Anti-OVA IgG_{2b} and IgE serum levels were analyzed on days 12 and 26. One of 3 independent experiments is shown. **C**, Experimental setup for Fig E1, **D**. **D**, Anti-OVA IgG subclass serum levels on days 12 and 26. One of 3 independent experiments is shown. *i.p.*, Intraperitoneal. **P* < .05, ***P* < .01, and ****P* < .001.

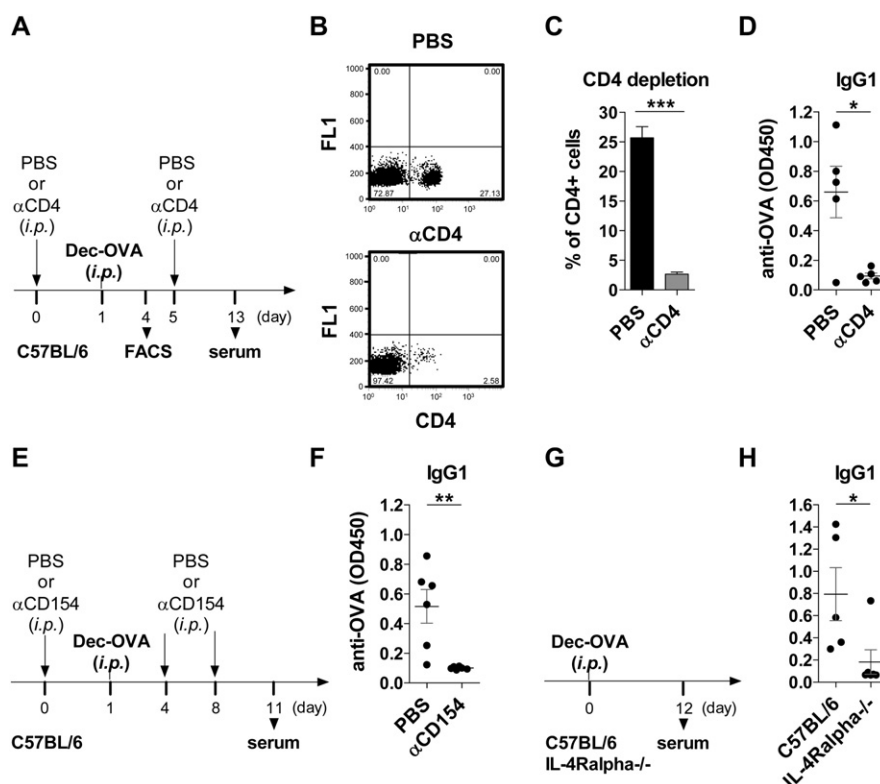


FIG E2. Antigen-reactive IgG₁ induced by TD tolerance induction is dependent on CD4⁺ T-cell help, CD40/CD40 ligand interaction, and IL-4 receptor α signaling. **A**, Experimental setup for Fig E2, B-D. **B**, Efficacy of CD4⁺ cell depletion in blood samples. **C**, Bar graph indicates the frequency of CD4⁺ cells for all analyzed mice. **E** and **G**, Experimental setups for Fig E2, F and H, respectively. **D**, **F**, and **H**, Anti-OVA IgG₁ serum levels. One of 2 independent experiments is shown for Fig E2, D, F, and H. **P* < .05, ***P* < .01, and ****P* < .001.



FIG E3. IgG N-linked Fc glycostructures. **A**, Scheme of the maximal IgG Fc glycan coupled to Asn 297; the cleavage site of endoglycosidase S (*EndoS*) is indicated. **B**, Possible human and murine oligosaccharide compositions of IgG Fc glycans coupled to Asn297. G, Galactose; S, sialic acid. The numbers show the m/z values of the distinct Fc glycan structures released on EndoS treatment.

Human and murine IgG Fc glycan structures	mass (m/z)	abbreviation
Neu5Ac — Gal — GlcNAc — Man Man — GlcNAc	1982	G1 S1
Neu5Gc — Gal — GlcNAc — Man Man — GlcNAc	2012	G1 S1
Neu5Ac — Gal — GlcNAc — Man GlcNAc — Man	1982	G1 S1
Neu5Gc — Gal — GlcNAc — Man GlcNAc — Man	2012	G1 S1
Neu5Ac — Gal — GlcNAc — Man Man — GlcNAc	2227	G1 S1
Neu5Gc — Gal — GlcNAc — Man Man — GlcNAc	2257	G1 S1
Neu5Ac — Gal — GlcNAc — Man Gal — GlcNAc — Man	2186	G2 S1
Neu5Gc — Gal — GlcNAc — Man Gal — GlcNAc — Man	2216	G2 S1
Neu5Ac — Gal — GlcNAc — Man Gal — GlcNAc — Man	2431	G2 S1
Neu5Gc — Gal — GlcNAc — Man Gal — GlcNAc — Man	2461	G2 S1
Neu5Ac — Gal — GlcNAc — Man Man — GlcNAc	2547	G2 S2
Neu5Gc — Gal — GlcNAc — Man Man — GlcNAc	2607	G2 S2
Neu5Ac — Gal — GlcNAc — Man Man — GlcNAc	2792	G2 S2
Neu5Gc — Gal — GlcNAc — Man Man — GlcNAc	2852	G2 S2

FIG E3. (Continued)

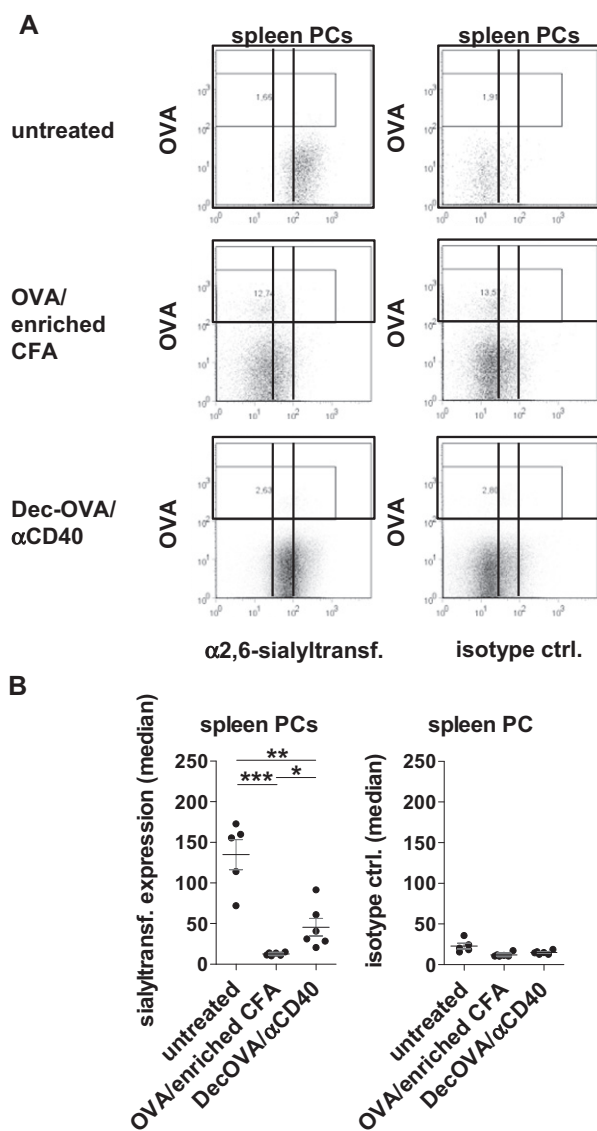


FIG E4. α2,6-sialyltransferase protein expression. **A** and **B**, PCs from indicated mice were analyzed for intracellular OVA reactivity and binding of an anti-α2,6-sialyltransferase or isotype control rabbit IgG 9 days after immunization. Fig E4, **B**, Median of α2,6-sialyltransferase expression for both rabbit IgG stainings (gated total PCs from untreated mice and OVA-reactive PCs from immunized mice, as indicated in Fig E4, **A**). One of 2 independent experiments is shown. * $P < .05$, ** $P < .01$, and *** $P < .001$.

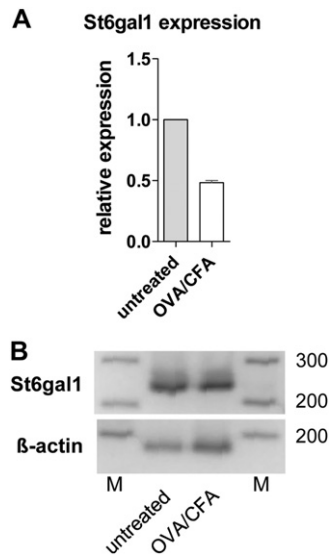


FIG E5. St6gal1 transcription. **A** and **B**, PCs from untreated ($n = 3$) and OVA in CFA-treated ($n = 3$) mice were sorted on day 8 and analyzed by means of RT-PCR. Fig E5, **A**, Relative transcription of St6gal1. Fig E5, **B**, Amplified St6gal1 and β -actin DNA after 55 RT-PCR cycles indicating specificity. *M*, Marker.

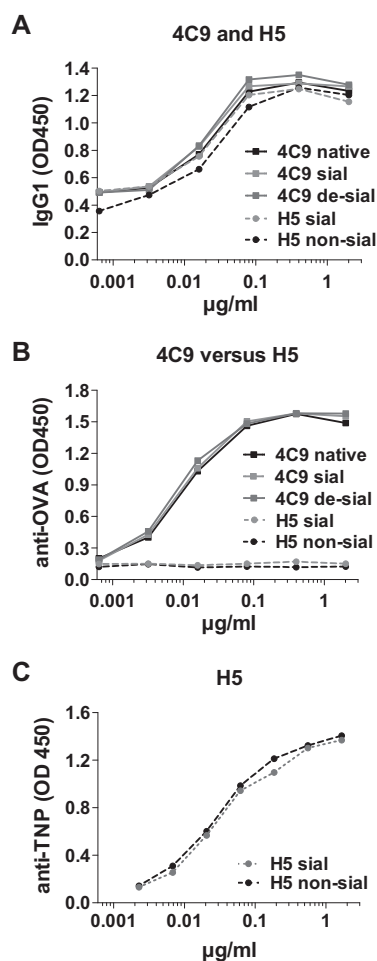


FIG E6. IgG sialylation does not influence antigen binding. **A-C**, IgG₁ (Fig E6, A), OVA reactivity (Fig E6, B), and TNP reactivity (Fig E6, C) ELISA of native (4C9 native, 4% sialylation), *in vitro* sialylated (4C9 sial, 80% sialylation), and sialidase-treated (4C9 de-sial, <2% sialylation) OVA-specific IgG₁ (4C9) or of *in vitro* sialylated (H5 sial, 70% sialylation) and native nonsialylated (H5 non-sial, <1% sialylation) TNP-specific IgG₁ (H5). One of 2 independent experiments is shown for each image.

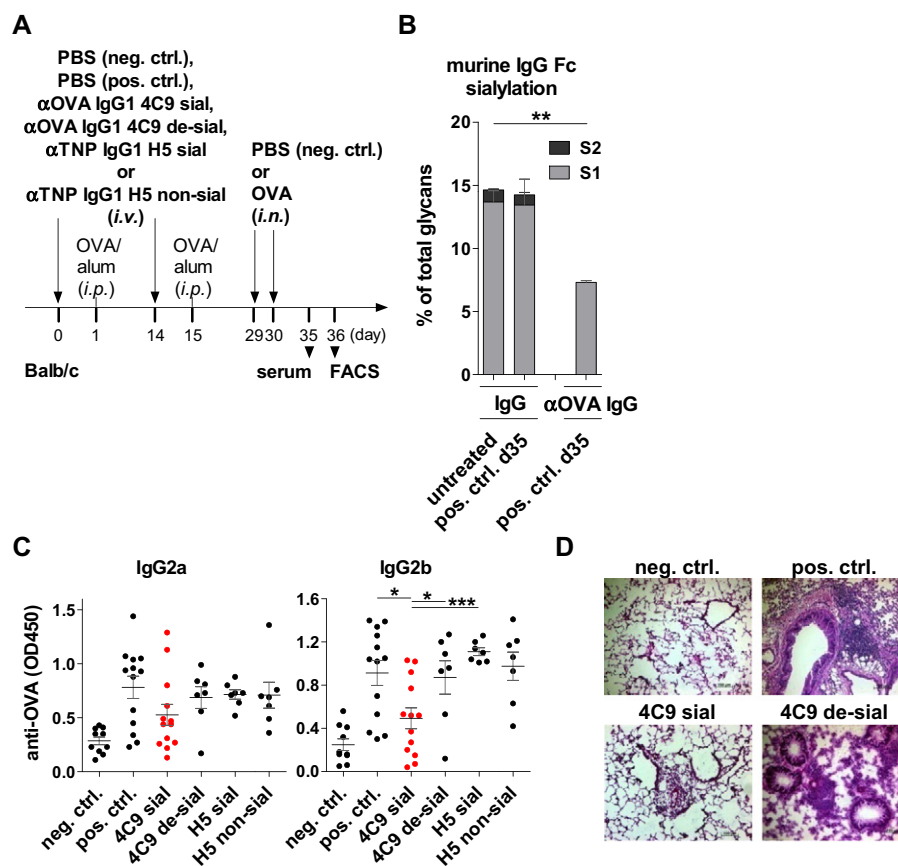


FIG E7. OVA-reactive sialylated IgG₁ protects against OVA-induced allergic airway inflammation. **A**, Experimental setup in BALB/c mice, as described in Fig 5. On days 0 and 14, mice were injected intravenously with 200 μ g of *in vitro* sialylated (4C9 sial, 80% sialylation) or sialidase-treated (4C9 de-sial, <2% sialylation) OVA-specific IgG₁ (4C9) or *in vitro* sialylated (H5 sial, 70% sialylation) or native nonsialylated (H5 non-sial, <1% sialylation) TNP-specific IgG₁ (H5) antibodies. *i.n.*, Intranasal; *i.p.*, intraperitoneal; *i.v.*, intravenous. **B**, Fc sialic acid content of total serum IgG from untreated and positive control mice (day 35) or OVA-reactive IgG from positive control mice (day 35). Bars indicate the frequency of glycan structures with 1 or 2 sialic acid residues (S1 and S2). **C**, Anti-OVA IgG subclass serum levels (day 35). **D**, Hematoxylin and eosin-stained lung sections. The results summarize 2 independent experiments. * P < .05, ** P < .01, and *** P < .001.

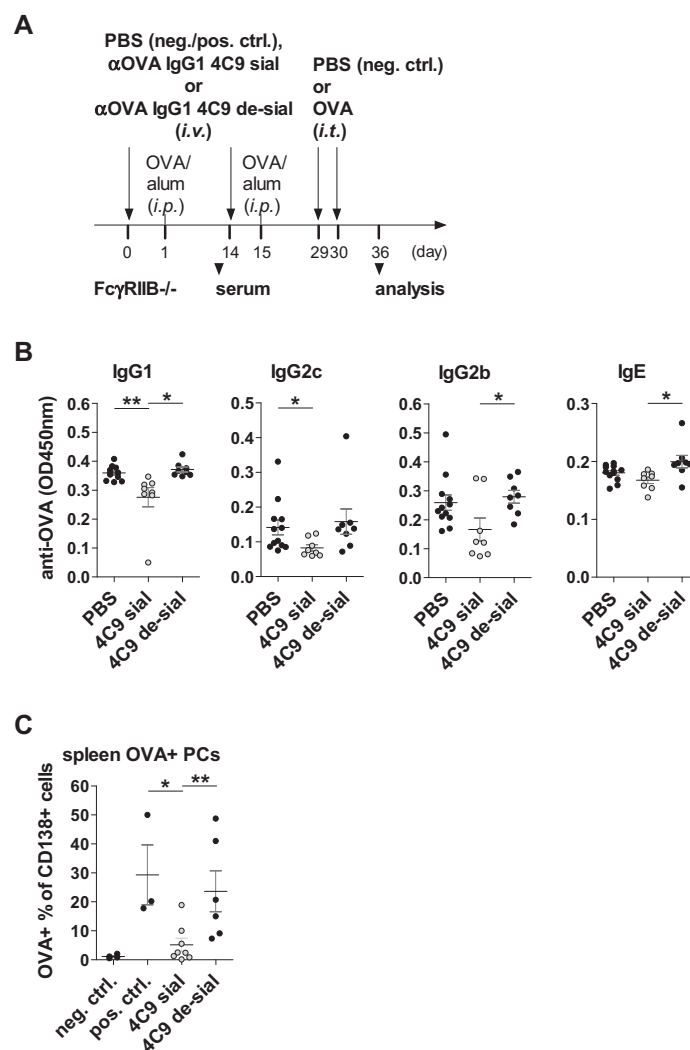


FIG E8. Antigen-specific sialylated monoclonal IgG₁ protects against allergic airway inflammation independent of FcγRIIB. **A**, Experimental setup in FcγRIIB^{-/-} mice. On days 0 and 14, mice were injected intravenously with 200 μg of *in vitro* sialylated (4C9 sial, 80% sialylation) or sialidase-treated (4C9 de-sial, <2% sialylation) OVA-specific IgG₁ (4C9). *i.p.*, Intraperitoneal; *i.t.*, intratracheal; *i.v.*, intravenous. **B**, Anti-OVA antibody levels on day 13. **C** and **D**, Frequency of OVA-reactive PCs (Fig E8, C) and lung SiglecF^{high}Macl^{high}Gr-1^{low}CD11c^{low} eosinophils (Fig E8, D). **E**, Numbers of total cells and eosinophils, lymphocytes, monocytes, and neutrophils in the bronchoalveolar lavage fluid. **F**, IL-5 and IL-13 in the supernatant of restimulated lung cells. **G**, Hematoxylin and eosin-stained lung sections. One of 2 independent experiments is shown. **P* < .05, ***P* < .01, and ****P* < .001.

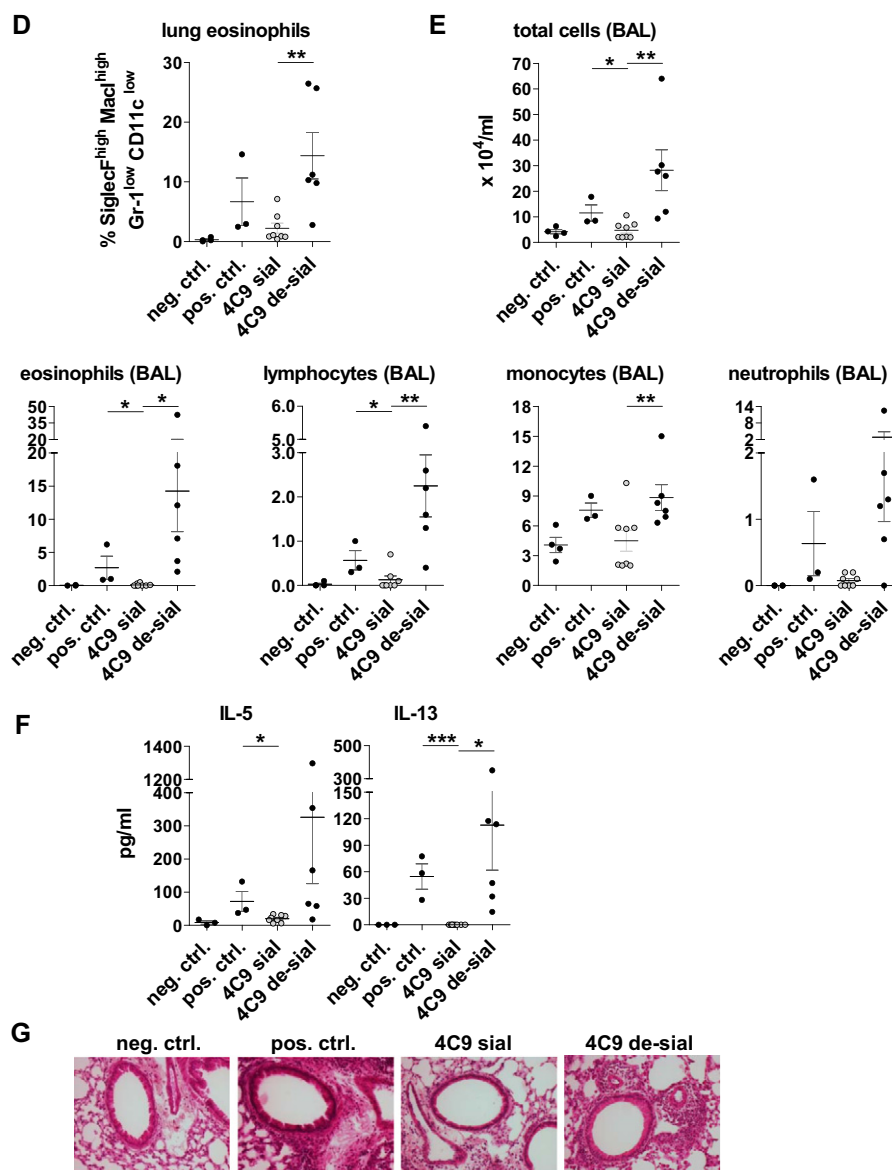


FIG E8. (Continued)

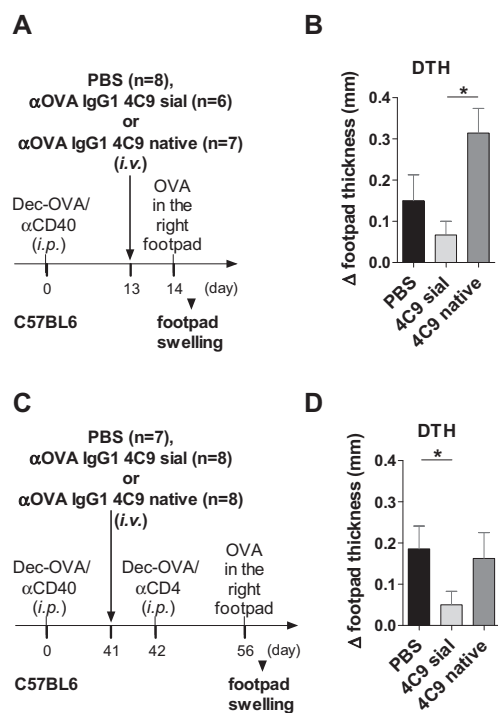


FIG E9. Antigen-specific sialylated monoclonal IgG₁ protects against ongoing inflammatory DTH responses. **A** and **C**, Experimental setup for Fig E9, **B** and **D**, respectively. On days 13 or 41, mice were injected intravenously (*i.v.*) with 100 μ g of *in vitro* sialylated (4C9 sial, 80% sialylation) or sialidase-treated (4C9 de-sial, <2% sialylation) OVA-specific IgG₁ (4C9) antibodies, respectively. *i.p.*, Intraperitoneal. **B** and **D**, Footpad swelling 5 or 3 days after local DTH induction, respectively. One of 2 independent experiments is shown for Fig E9, **B** and **D**. **P* < .05.

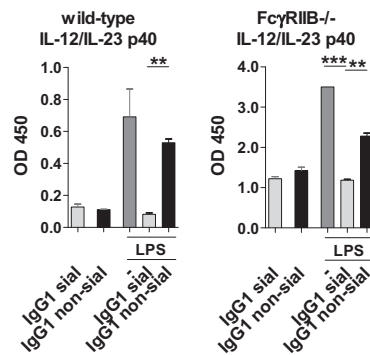


FIG E10. ICs containing sialylated IgGs inhibit LPS-induced DC maturation independent of FcγRIIB. Bone marrow-derived DCs from C57BL/6 wild-type and FcγRIIB^{-/-} mice were preincubated with ICs containing TNP-Ficoll and *in vitro* sialylated (*IgG1 sial*, 70% sialylation) or native nonsialylated (*IgG1 non-sial*, <1% sialylation) TNP-specific IgG₁ (H5), for 30 minutes before adding LPS for 48 hours, as described in Fig 7. IL-12/IL-23 p40 concentrations in the supernatants were analyzed by using ELISA. One of 3 independent experiments is shown. ** $P < .01$ and **** $P < .001$.

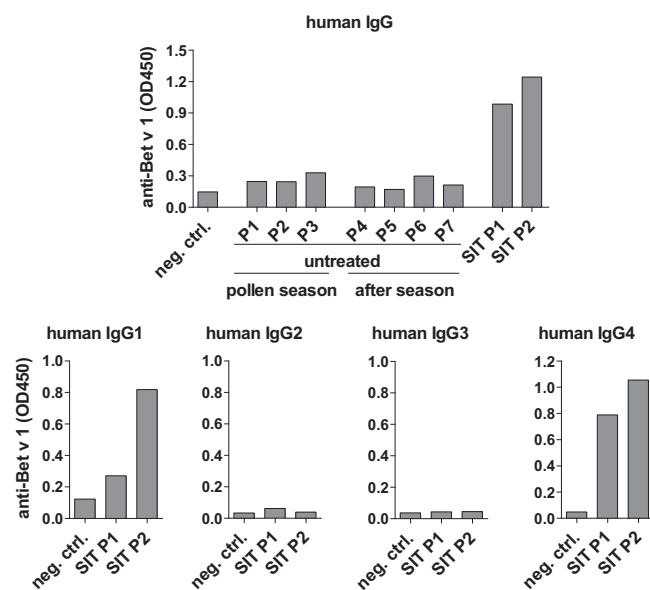


FIG E11. SIT against birch pollen allergy induces anti-Bet v 1 serum IgGs in patients. Anti-Bet v 1 human total or subclass IgG serum levels from volunteers with untreated birch pollen allergy in or after the pollen season (*untreated P1-P7*) or from 2 patients after the pollen season who had received a successful SIT over 3 years (*SIT P1* and *SIT P2*) are shown. The average values of 1 ELISA experiment of 3 independent experiments are shown. *neg. ctrl.*, Anti-Bet v 1 IgG-negative serum.