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Grass Pollen Immunotherapy Induces an Allergen-Specific IgA2 Antibody Response Associated with Mucosal TGF-β Expression[1]

Charles Pilette,[2]*† Kayhan T. Nouri-Aria,[2]* Mikila R. Jacobson,* Louisa K. Wilcock,* Bruno Detry,† Samantha M. Walker,* James N. Francis,* and Stephen R. Durham[3]*

Allergen immunotherapy (IT) has long-term efficacy in IgE-mediated allergic rhinitis and asthma. IT has been shown to modify lymphocyte responses to allergen, inducing IL-10 production and IgG Abs. In contrast, a putative role for IgA and local TGF-β-producing cells remains to be determined. In 44 patients with seasonal rhinitis/asthma, serum IgA1, IgA2, and polymeric (J chain-containing) Abs to the major allergen Phl p 5 were determined by ELISA before and after a 2-year double-blind trial of grass pollen (*Phleum pratense*) injection IT. Nasal TGF-β expression was assessed by in situ hybridization. Sera from five IT patients were fractionated for functional analysis of the effects of IgA and IgG Abs on IL-10 production by blood monocytes and allergen-IgE binding to B cells. Serum Phl p 5-specific IgA2 Abs increased after a 2-year treatment (~8-fold increase, $p = 0.002$) in contrast to IgA1. Increases in polymeric Abs to Phl p 5 (~2-fold increase, $p = 0.02$) and in nasal TGF-β mRNA ($p = 0.05$) were also observed, and TGF-β mRNA correlated with serum Phl p 5 IgA2 ($r = 0.61$, $p = 0.009$). Post-IT IgA fractions triggered IL-10 secretion by monocytes while not inhibiting allergen-IgE binding to B cells as observed with IgG fractions. This study shows for the first time that the IgA response to IT is selective for IgA2, correlates with increased local TGF-β expression, and induces monocyte IL-10 expression, suggesting that IgA Abs could thereby contribute to the tolerance developed in IT-treated allergic patients. *The Journal of Immunology,* **2007, 178: 4658–4666.**

Allergen-specific immunotherapy (IT)[4] represents the only curative treatment of allergic diseases currently available and is highly effective in venom anaphylaxis and in seasonal rhinitis and asthma due to pollen allergy (1). Different immunological changes, putatively underlying clinical tolerance to further allergen exposure, have been observed following IT. These include increases in allergen-specific IgG, particularly of the IgG4 subclass (2), and modest reductions in specific IgE Abs (3). Effector inflammatory cells such as eosinophils, mast cells, and basophils are reduced in allergic mucosal tissues (4). Some studies (5–8) have shown decreased peripheral blood T cell responses to allergen and/or immune deviation following treatment. Enhanced IFN-γ responses to allergen have been observed after grass pollen IT, and parallel decreased skin and nasal late-phase responses to allergen challenge (9), observations that suggest that IT may induce in mucosal T cells a preferential deviation of Th2 in favor of Th1 responses.

Pioneering studies indicated that IgA production is associated with oral tolerance (10), and transient IgA deficiency was proposed as a risk factor for IgE sensitization in early life (11). Low total IgA and IgG4 levels were observed in sensitized children and allergic individuals as compared with healthy controls, and relative deficiency in allergen-specific IgA Abs has been described in the serum (12, 13) but not in the nasal secretions (14) of these patients. Increased specific IgA concentrations have been observed after IT in some (15–17) but not all studies (18, 19). IgA, the predominant Ab isotype in mucosal tissues and secretions (reviewed in Refs. 20 and 21), contributes to frontline defense mechanisms at mucosal sites by neutralizing inhaled and ingested Ags. In contrast to serum IgA, which is mainly monomeric (m-IgA), mucosal plasma cells produce polymeric IgA (p-IgA) associated with a polypeptide called the joining (J) chain. J chain-containing p-IgA binds to the epithelial p-Ig receptor, which assumes its transport across the epithelium into mucosal secretions where p-IgA remains linked to the extracellular part of the receptor, called the secretory component, to form secretory IgA (S-IgA). S-IgA can act as a scavenger that binds Ags in the mucosal lumen before they can trigger proinflammatory signals. Other unique properties of IgA have been identified, such as a poor complement activation capacity and inhibition of monocyte/macrophage activation (22), suggesting that IgA may, like IgG4, represent a noninflammatory isotype.

The inhibitory cytokine TGF-β is the main cytokine responsible for inducing the switching of B lymphocytes in favor of IgA synthesis and represents a key factor in tolerogenic regulatory responses (for a review, see Ref. 23). The induction of TGF-β expression has been observed in peripheral mononuclear cells following IT (17) and is at least partly related to the development

*Upper Respiratory Medicine, Section of Allergy and Clinical Immunology, Imperial College, National Heart and Lung Institute, London, United Kingdom; and †Unit of Pneumology, University of Louvain, Brussels, Belgium

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[2] C.P. and K.T.N.-A. contributed equally to the present study.

[3] Address correspondence and reprint requests to Prof. S. R. Durham, Upper Respiratory Medicine, Section of Allergy and Clinical Immunology, National Heart and Lung Institute, Imperial College, Dovehouse Street, London, U.K. E-mail address: s.durham@imperial.ac.uk

[4] Abbreviations used in this paper: IT, immunotherapy; a.u. arbitrary unit; J chain, joining chain; m-IgA, monomeric IgA; Phl p, *Phleum pratense*; p-IgA, polymeric IgA; S-IgA, secretory IgA.

Table I. *Clinical response to immunotherapy*[a]

	Post-IT	Placebo	Point Estimate Median (95% Confidence Interval)	*p* Value[b]
No. of patients	17	20		
Mean age (range)	32 (22–64)	32 (23–59)		
Symptoms score	1277 (135–2588)	1386 (941–3899)	1186.5 (241.5–1928.6)	0.01
Medication score	357 (49–2236)	1851 (476–3947)	1043.0 (332.0–2667.1)	0.007
Early skin response to grass pollen (mm^2)	275.5 (231.5–313.1)	385.5 (302.0–576.2)	184.7 (58.5–365.0)	0.007
Late skin response to grass pollen (mm^2)	246 (175–490)	3193 (1985–4483)	3542.0 (2647.0–4412.6)	0.000
Methacholine PC_{20} (mg/ml)	32.0 (6.5–32.0)	4.5 (3.1–24.0)	2.6 (0.001–9.6)	0.04

[a] Values represent medians (interquartile range).
[b] Mann-Whitney *U* test was used for between-group comparisons (IT versus placebo) of posttreatment (May/June 1998) minus baseline (May/June 1996) values; pre-treatment baseline data have been reported previously (24).

of regulatory T cells, whereas TGF-β expression has not been evaluated after treatment in the target tissues of mucosal allergic inflammation. The relationship between changes in Ab and cell responses following IT remains also unclear and, in contrast to IgG and IL-10, the IgA response to IT has not been characterized with regard to subclass and production site, its relationship to the expression of TGF-β, and its functional relevance to immunological tolerance.

In a double-blind, placebo-controlled trial of grass pollen IT (24) serum and nasal biopsy specimens were collected before and after 2 years of treatment. Allergen-specific IgA subclasses and polymeric Ig (containing the J chain) concentrations were determined in preseasonal and peak seasonal sera, before and after treatment. Biopsies were examined for TGF-β-expressing cells by in situ hybridization and for the presence of allergen-binding cells expressing IgA. The findings on IgA and TGF-β responses were related to clinical improvement and, to investigate the contribution of IgA to immunological tolerance sera from IT-treated patients, were fractionated and tested for the presence of IgA Abs with "blocking" activity on allergen-IgE binding to B cells (25) and with IL-10-inducing capacity in monocytes.

Materials and Methods

Subjects

All subjects had severe seasonal rhinitis and a positive skin reaction (wheal $>$ 5 mm) to *Phleum pratense* (Phl p) (Aquagen; ALK Abelló) as previously described (24). Thirty-seven of the 44 participants consented to nasal biopsy before and after treatment. Control subjects comprised nine nonatopic, healthy subjects. The study was approved by the Ethics Committee of the Royal Brompton Hospital (London, U.K.), and all subjects gave written informed consent.

Study design

The details of the IT protocol have been previously reported (24) and the clinical response to treatment is summarized in Table I. Briefly, active treatment involved a modified "cluster" regimen of injections with a standardized aluminum hydroxide-adsorbed extract of Phl p (Alutard SQ; ALK Abelló). Up-dosing injections were given twice a week for 4 wk during the winter followed by monthly depot maintenance injections for 3 years. Maintenance injections of 1-ml contained 100,000 standard quality units that included 20 μg of the major grass pollen allergen Phl p 5. Placebo injections contained 0.01 mg/ml histamine acid phosphate (in PBS) in allergen diluent.

Serum and nasal biopsy specimens

Serum samples were taken before and at the height of the pollen season before IT and during the peak pollen season and out of season after 2 years of treatment. Nasal biopsies were taken at baseline before the season and, after 2 years of treatment, during the peak pollen season and at the end of the study (out of season). Local anesthesia of the inferior turbinate was achieved using 1 ml of 10% cocaine solution, and a 2.5-mm biopsy was taken later using Gerritsma forceps.

Serum allergen-specific IgA Ab assays

Allergen-specific IgA1 and IgA2 concentrations were determined by sandwich ELISA. Microplates were coated with 10 μg/ml Phl p 5 major allergen, Phl p 1, or grass extract (ALK Abelló), blocked with 1% BSA, and incubated with 10-fold diluted (in PBS) serum samples. Mouse mAbs to IgA1 (clone B3506) or IgA2 (clone A9603) (26) were used at 1 μg/ml for detection and revealed by HRP-conjugated anti-mouse IgG (1/10,000; Sigma-Aldrich) followed by incubation with hydrogen peroxide and tetramethylbenzidine in phosphate-citrate buffer. Specificities of the mAbs to IgA subclasses were checked by ELISA. Allergen-specific IgM Abs were assessed by using a mAb to IgM (1/5,000) from Sigma-Aldrich.

To detect allergen-specific polymeric Abs, anti-J chain rabbit polyclonal IgG Ab (1/500; gift from P. Brandtzaeg, University of Oslo, Oslo, Norway) was used, revealed by HRP-conjugated anti-rabbit IgG (1/10,000; Sigma-Aldrich). The results were expressed as arbitrary units by comparison with dilutions of a reference (post-IT) serum.

In situ hybridization for TGF-β mRNA

Biopsies were fixed in 4% formaldehyde, dehydrated in 15% sucrose, and then snap frozen and stored for in situ hybridization studies as described (27). Riboprobes, both antisense (complementary to mRNA) and sense (identical sequence to mRNA), were prepared from cDNA encoding TGF-β, inserted into different pGEM vectors, and linearized with restriction enzymes. Transcription was performed in the presence of ^{35}S-uridine triphosphate and the appropriate T7 or SP6 RNA polymerase. Controls included sense probes and antisense probes (following pretreatment of sections with RNase). Specific hybridization was recognized as clear dense deposits of silver grains in the photographic emulsion overlaying the tissue sections. Counts were performed at $\times$200 magnification along the entire length of the basement membrane at one-grid depth (0.45 mm) with an Olympus BH2 microscope (Olympus Optica). The results, obtained on average in four fields (equivalent to 0.8 mm^2, 0.2–1.8 mm^2) of subepithelial tissue per biopsy specimen, were expressed as the number of positive cells per square millimeter.

Colocalization of TGF-β mRNA to leukocytes

The phenotype of TGF-β-expressing cells in nasal biopsy tissues was assessed by sequential immunohistochemistry/in situ hybridization. Colocalization of TGF-β mRNA to $CD3^+$ T lymphocytes and $CD68^+$ macrophages was performed on paraformaldehyde-fixed sections using consecutive immunohistochemistry with the phenotype-specific markers CD3 (T cells), CD68 (macrophages) (DakoCytomation), and alkaline phosphatase anti-alkaline phosphatase (DakoCytomation), and developed using FAST red (Sigma-Aldrich). The staining was followed by in situ hybridization using a ^{35}S-labeled TGF-β antisense probe. Only double-positive cells were counted.

Colocalization of grass allergen to nasal IgA-expressing cells

Dual staining was conducted using the alkaline phosphatase anti-alkaline phosphatase mouse monoclonal system (DakoCytomation). The endogenous peroxidase of sections was blocked using peroxidase-blocking reagent (DakoCytomation), followed by the blocking of avidin-biotin (Vector Laboratories). Sections were then incubated with anti-IgA2 mAb (clone A9603) or anti-IgA1 mAb (clone B3506) and biotinylated Phl p 5 (ALK Abelló) for 1 h. The sections were washed extensively using PBS and incubated for 45 min with rabbit anti-mouse IgG (DakoCytomation) and streptavidin-FITC (Molecular Probes) and, after washes, the sections were incubated with alkaline phosphatase anti-alkaline phosphatase and HRP anti-FITC (DakoCytomation). Reactions were revealed by using hydrogen peroxide and diaminobenzidine (Phl p5) followed by Fast Blue substrate (IgA2 or IgA1).

Table II. *Serum specific IgA1, IgA2, polymeric and IgM antibodies to grass pollen allergens and nasal TGF-β mRNA expression*[a]

	IT		Placebo		
	Before (preseason)	After 2 years (peak season)	Before (preseason)	After 2 years (peak season)	*p* Value[b]
Serum IgA1					
Grass extract	33.6 (5.7, 80.4)	37.2 (26.1, 68.7)	25.3 (14.9, 51.9)	16.4 (8.9, 40.9)	0.05
Phl p 1	48.7 (16.0, 181.5)	56.9 (25.5, 131.4)	26.6 (21.5, 41.5)	25.6 (19.4, 36.1)	0.03
Phl p 5	17.6 (11.3, 29.9)	20.2 (16.7, 24.4)	13.3 (10.9, 19.1)	10.1 (9.5, 14.1)	0.002
Serum IgA2					
Grass extract	5.4 (1.0, 20.9)	35.7 (29.4, 49.9)**	13.7 (2.7, 20.4)	8.2 (1.0, 17.8)	<0.0001
Phl p 1	29.3 (13.9, 38.0)	64.3 (29.3, 76.1)*	33.0 (24.9, 57.4)	11.8 (0.4, 22.1)	0.01
Phl p 5	12.4 (2.9, 18.8)	79.6 (60.3, 100.9)**	9.6 (4.9, 21.5)	6.2 (0.2, 11.5)	<0.0001
Serum pIg					
Grass extract	9.5 (8.2, 11.6)	9.2 (5.3, 17.7)	9.9 (9.7, 10.4)	10.4 (5.0, 12.6)	0.58
Phl p 1	18.5 (17.7, 19.3)	19.4 (18.8, 20.1)	19.8 (18.7, 20.2)	20.1 (19.4, 21.5)	0.5
Phl p 5	46.3 (31.6, 96.8)	108.0 (94.1, 151.3)*	58.3 (46.1, 83.6)	80.2 (54.4, 116.5)*	0.02
Serum IgM					
Phl p 5	18.9 (14.7, 22.9)	28.5 (22.5, 33.7)	28.3 (21.6, 31.9)	33.6 (17.2, 38.4)	0.06
Nasal TGF-β mRNA/mm^2	0 (0, 2.1)	2.9 (0, 9.1)*	0.35 (0, 2.5)	0 (0, 1.3)	0.05

[a] Values represent medians (interquartile range), in arbitrary units for serum measurements. Asterisks refer to within-group comparisons, posttreatment (peak season) versus baseline (peak season) (Wilcoxon matched pairs test). (*), $p = 0.06$; *, $p = 0.02$; **, $p = 0.002$.
[b] Values refer to between-group differences (posttreatment minus baseline), IT versus placebo (Mann-Whitney *U* test).

Serum fractionation

Serum (~30 ml) from five grass pollen IT-treated patients were separated into fractions containing subclasses of p-IgA and m-IgA by gel filtration followed by affinity chromatography. Briefly, sera were run (~20 cc/h) through a 2% acrylamide, 2% agarose (home-made) column (100 × 5 cm of gel, LKB Pharmacia) and the 10 ml-fractions collected were analyzed for their content by Ouchterlony double immunodiffusion (Igs, α2-macroglobulin) and ELISA (J chain-containing IgA) techniques. Accordingly, three series of fractions presumably containing IgA polymers, IgA monomers, and IgG, respectively, were pooled (pool fractions 1, 2, and 3). Pool fractions 1 and 2 were subjected to jacalin-based affinity chromatography to purify p-IgA1 and m-IgA1, because jacalin binds selectively to IgA1. Run-through fractions were passed on a protein G column (to eliminate IgG) to obtain fractions enriched in p-IgA2 and m-IgA2. Pool-fraction 3 was run on a protein G column to obtain control (flow-through) and IgG (eluate) fractions. The six final fractions obtained were concentrated by ultrafiltration (Amicon), resuspended in PBS, and filtered on 0.22-μm pore filters (Millipore) before use in functional assays.

The presence in the fractions of Phl p-specific IgA1, IgA2, and polymeric (J chain-containing) Abs was measured by ELISA as described above, as well as Phl p-specific IgG Abs (using 1 μg/ml anti-human IgG mAb; gift from M. E. Conley, University of Tennessee, Memphis, TN) and total protein concentrations by using the BCA assay (Pierce). In selected experiments, fractions were corrected for Phl p Ab levels to assess the intrinsic Ab activity.

IL-10 release assay in monocytes

Peripheral monocytes were isolated from blood obtained from healthy donors. After red cell sedimentation on dextran 500, leukocyte-rich plasma was centrifuged over Nycoprep 1.068 (Nycomed) and mononuclear cells were washed twice in RPMI 1640 (Cambrex) culture medium (supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin). Monocytes were purified from PBMC by negative selection using magnetic cell sorting (Miltenyi Biotec monocyte isolation kit II) following the manufacturer's instructions. After washing in RPMI 1640, monocytes were distributed at 0.25×10^6 cells/ml in 96-well plates.

Monocytes were then incubated in triplicate conditions for 1 h on ice with 20 μl of post-IT IgA serum fractions in 100 μl (dilution 1/5 of each fraction in RPMI 1640). After a gentle wash, cross-linking was performed by incubation for 1 h on ice with either Phl p extract (20 μg/ml) or anti-Ig Ab (affinity-purified goat ACP17 anti-human IgA at 0.5 mg/ml or anti-human IgG mAb at 0.1 mg/ml), and culture was conducted at 37°C with 5%CO_2 for 20 h. Supernatants were then collected and assayed for IL-10 by ELISA using paired Abs (BD Pharmingen) with a detection limit of ~1 pg/ml.

Purified human monoclonal (myeloma) IgA1 and IgA2, as well as polyclonal milk S-IgA (prepared in our laboratory), were also used as pure IgA preparations, followed by anti-IgA cross-linking. My43 (gift from Dr. R. Monteiro, Faculté Necker, Université Paris-V, Paris, France) was used as a blocking mouse IgM mAb to FcαRI (CD89).

Real time quantitative PCR for IL-10 mRNA

After stimulation with purified human IgA/anti-IgA cross-linking, monocytes were cultured for 20 h and total RNA was extracted from 0.5×10^6 cells using the RNeasy Protect mini kit (Qiagen), and 2 μg of total RNA was used to generate first-strand cDNA synthesis using Transcriptor reverse-transcriptase enzyme (Roche). The reaction mix containing of 2 μg of RNA and poly(dT) was heated to 65°C for 5 min and chilled on ice for 1 min. First-stand synthesis was then performed in a 200-μl total reaction volume by adding 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dNTP-mix, 40U/μl RNaseOUT (Invitrogen Life Technologies), and 400 U of Transcriptor reverse-transcriptase enzyme (Roche) at 55°C for 2 h. The reaction was inactivated by heating at 85°C for 5 min. cDNA was stored at −20°C until amplification. Real-time PCR was performed on a LightCycler system (Roche) using predeveloped primers for human IL-10 (Search-LC) following the manufacturer's instructions. The reaction mix contained 5 μl of cDNA, 1 mM primers, and Master mix (FastStart DNA Master plus SYBR Green I; Roche) in a final volume of 20 μl. β-Actin primers were designed following sequence published in GenBank (accession number M10277) in two different exons synthesized by Invitrogen Life Technologies: sense, 5′-GTGACACCAAGGAGAAGCTGTGCTA-3′ (position: 2294–2317); and antisense, 5′-CTTCATGATGGAGTTGAAGG TAGTT-3′ (position: 2588–2612). PCR conditions were 95°C for 10 s (denaturation), 60°C for 5 s (hybridization), and 72°C for 7 s (elongation). After amplification, a melting curve was performed to ensure that only one product had been amplified. Moreover, separation of the products on 2% agarose gel confirmed the size of the amplicon.

IL-10 intracellular staining

Monocytes were treated with 2 μM monensin (to inhibit cytokine secretion; Sigma-Aldrich) for 30 min before stimulation by human m-IgA or 1 μg/ml LPS (Sigma-Aldrich) as a positive control. At the end of the 20-h culture, monocytes were fixed and permeabilized using the IntraPrep reagent (Immunotech Coulter). Immunostaining was performed by using FITC-conjugated rat anti-human IL-10 mAb (BD Biosciences) following the manufacturer's instructions. After washing, monocyte-associated fluorescence was analyzed by using a FACScan flow cytometer (BD Biosciences).

Allergen-IgE binding to B cells assay

IgA fractions were tested in facilitated Ag presentation assay, as previously described (28). B cells derived from PBMC of an atopic donor were transformed by EBV infection and enriched for $CD23^+$ cells. Serum from an atopic grass pollen-allergic patient (containing high concentrations of allergen-specific IgE) was preincubated with Phl p allergen extract (3 μg/ml) in the presence of post-IT serum fractions (as described above) or medium alone (control) at 37°C for 1 h. In selected experiments, fractions were

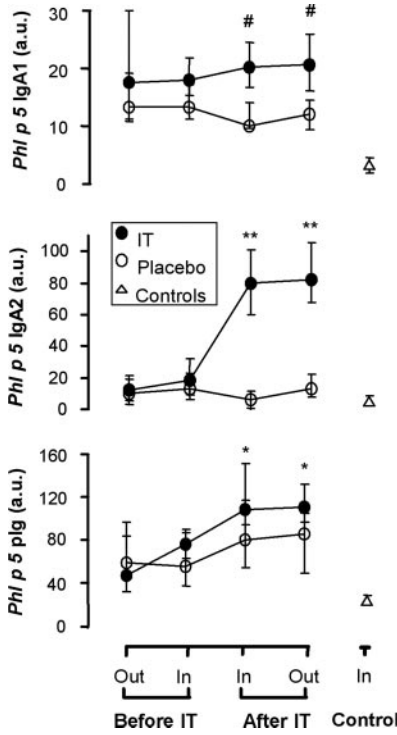

FIGURE 1. Serum IgA response to immunotherapy. Serum Phl p5-specific IgA1, IgA2, and p-Ig concentrations were determined in grass pollen allergic patients before and after IT, both during the peak (In) and outside (Out) of the pollen season, as well as in healthy nonatopic controls. Values are medians and interquartile ranges. #, $p = 0.002$, placebo vs IT (Mann-Whitney U test); ∗, $p < 0.05$ or ∗∗, $p < 0.001$, after IT vs baseline (Wilcoxon matched pairs test).

corrected for Phl p Ab levels. EBV-transformed B cells (1×10^5) were added and further incubated for 1 h at 4°C. Cells were washed and the surface binding of allergen-specific IgE complexes was detected using a FITC-labeled, anti-IgE polyclonal Ab (DakoCytomation) and analyzed with a FACSCalibur flow cytometer (BD Biosciences).

To assess whether inhibition by IgA would be restricted to mucosal S-IgA, we generated in vitro S-IgA Abs. Exogenous human free secretory component was added (1.3-fold, molar excess) to p-IgA fractions as previously described (29), and the resulting S-IgA fractions were tested for facilitated Ag presentation inhibition.

Statistical analysis

Within-group comparisons were performed using the Wilcoxon matched-pairs signed-ranks test. Between-group comparisons were performed using the Mann-Whitney U test. Correlation coefficients were obtained using Spearman's rank method. All analyses were performed using a statistics software package (Minitab). All tests were two-tailed and $p < 0.05$ was considered statistically significant.

Results

Increase in allergen-specific IgA2 after immunotherapy

Two years of treatment with a depot grass pollen extract was highly effective in reducing seasonal symptoms, rescue medication use, and bronchial hyperresponsiveness during the pollen season (Table I). A highly significant 8-fold increase in IgA2 Ab levels to Phl p 5 was observed ($p = 0.002$; Table II and Fig. 1). In contrast, no significant increase in IgA1 Abs to Phl p 5 was observed after IT as compared with baseline (Fig. 1 and Table II); Phl p 5 IgA1 Ab levels were, however, significantly higher in IT-treated patients when compared with placebo-treated patients ($p = 0.002$). Similar results were obtained for specific IgA responses to the whole grass pollen extract used for IT and to the major grass allergen Phl p 1 (Table II).

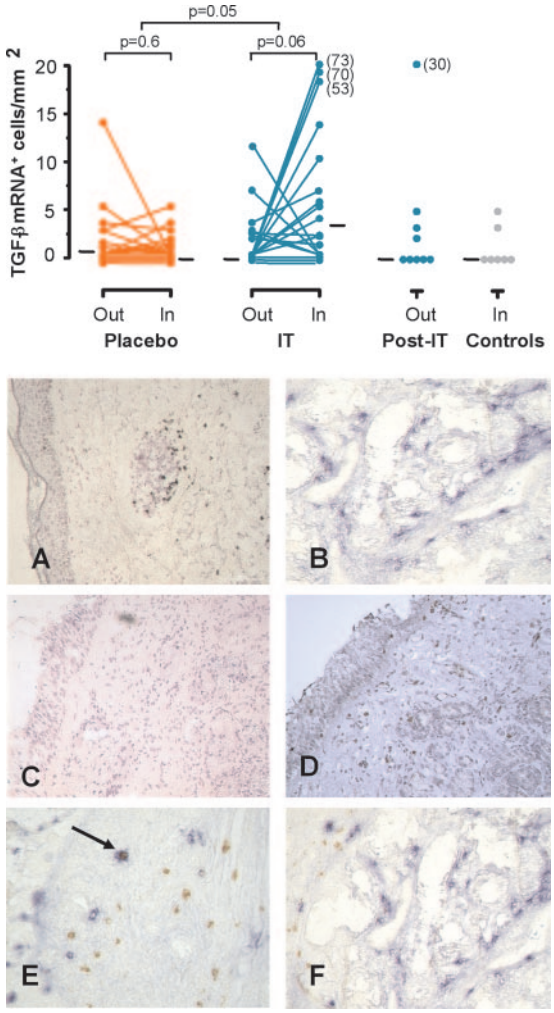

FIGURE 2. Nasal TGF-β mRNA expression after immunotherapy and colocalization of nasal IgA1/2-expressing cells to Phl p5. TGF-β expression was assessed by in situ hybridization in nasal biopsies obtained from grass pollen allergic patients before/after IT, during the peak (In) and outside (Out) the pollen season. Biopsies were also obtained 2 years after completion of IT (Post-IT) as well as in healthy nonatopic controls. Bars represent median values. Pictures (×200 original magnification) show a representative TGF-β mRNA in situ hybridization signal (*A*) and staining of IgA^+ (blue) and/or Phl $p5^+$ cells (brown) cells in nasal biopsy sections from a IT-treated patient: IgA2 (*B*), isotypic control (*C*), Phl p5 (*D*), Phl p5/IgA2 dual staining (*E*, arrow), and Phl p5/IgA1 dual staining (*F*).

To assess the specificity of the response, IgA Ab concentrations to nonrelevant allergens (house dust mite (Der p) and birch pollen (Bet v)) were determined. No increase in IgA2 concentrations to Der p (76.5 (34.0, 206.5) median (range) vs 61.5 (39.0, 149.6) arbitrary units (a.u.), IT vs placebo treatment, $p = 0.9$) and Bet v (14.4 (5.4, 40.4) vs 25.4 (9.9, 40.4) a.u., $p = 0.5$) was observed after grass pollen IT. More particularly, no increase in Der p-IgA2 was observed in the 9/20 patients who were sensitized to mite (data not shown), confirming the Ag specificity of treatment.

Increase in specific polymeric Abs after immunotherapy

Because IgA produced within mucosal tissues is associated with the J chain, serum concentrations of J chain-containing polymeric Abs were evaluated to assess the contribution of the nasal mucosa to the rise in serum IgA Abs following IT. A significant increase in concentration of p-Ig to Phl p 5 was observed at the peak of the second pollen season in both groups ($p = 0.02$) but was more

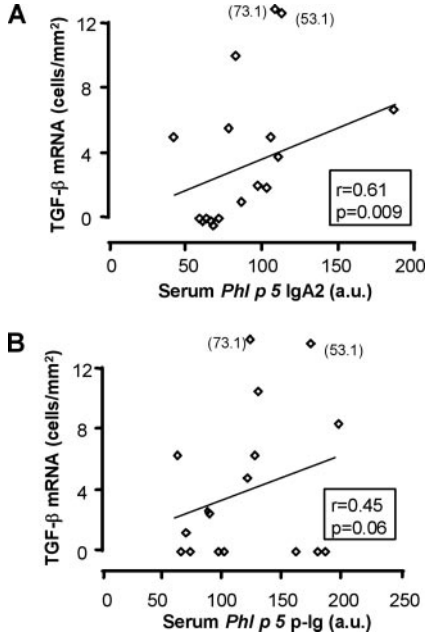

FIGURE 3. Correlation between serum-specific IgA and nasal TGF-β responses after immunotherapy. Nasal TGF-β mRNA was plotted against serum IgA2 (*A*) and p-Ig (*B*) Ab levels to Phl p 5 in IT-treated patients (n = 17), and correlation tested by the Spearman's method.

pronounced in IT-treated patients as compared with placebo-treated patients (~2-fold increase from baseline, $p = 0.02$) (Fig. 1 and Table II). The increase in polymeric Abs was not due to a rise in specific IgM Ab levels to Phl p 5, which did not change significantly during treatment (Table II).

Because IgA is thought to be part of the normal immune response to mucosal allergens, we determined serum Phl p-specific IgA responses in healthy nonatopic controls. Peak-seasonal concentrations of Phl p 5 IgA2 were similar to those observed in placebo-treated atopics (2.5 (0.5, 7.1) vs 6.2 (0.2, 11.5) a.u., p = 0.7), whereas Phl p 5 IgA1 concentration was lower (1.8 (0, 3.2) vs 10.1 (9.5, 14.1) a.u., $p = 0.01$) (Fig. 1).

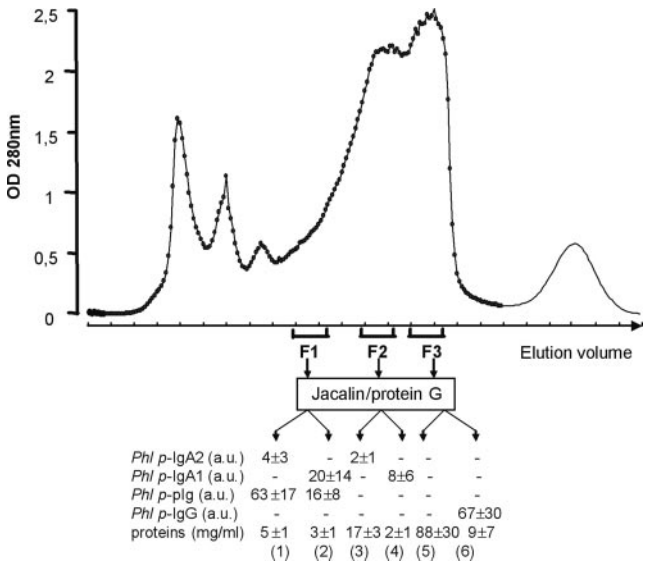

FIGURE 4. Fractionation of post-IT serum. The absorbance (OD_{280}) curve following gel filtration of one IT serum is shown. Fractions of interest were pooled (pool fractions F1, F2, and F3) and subjected to affinity chromatography using jacalin (IgA1) and protein G (IgG). Six final fractions were obtained, enriched in p-IgA2 (1), p-IgA1 (2), m-IgA2 (3), m-IgA1 (4), various serum proteins (5) and IgG (6), respectively, as confirmed by measurements of allergen-specific Ab levels (a.u.) and total protein concentrations. Values are means ± SD (n = 5 IT patients).

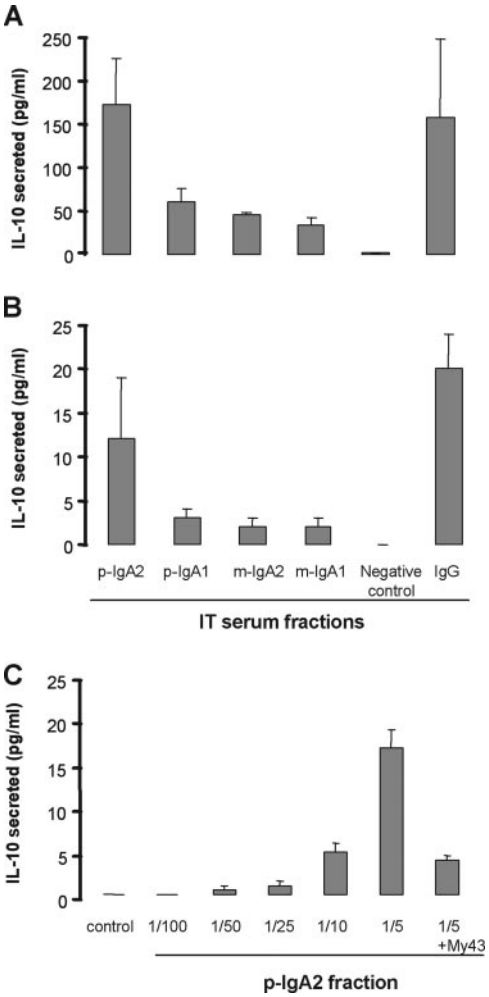

FIGURE 5. Induction by post-IT serum fractions of IL-10 secretion by monocytes. IL-10 was assayed by ELISA in the supernatants of peripheral blood monocytes incubated with post-IT serum fractions followed by cross-linking with anti-Ig Ab (*A*) or Phl p allergen (*B*). Negative control consisted of fraction 5 (see Fig. 4) followed by allergen (*A*) or anti-IgA plus anti-IgG Abs (*B*). Data are means ± SEM (experiments in triplicates, n = 5 IT patients). Dose-response curve to dilutions of the p-IgA2 fraction, followed by allergen cross-linking, is shown, with the effect of the blocking mAb My43 (*C*).

Induction of nasal TGF-β expression after immunotherapy

The numbers of TGF-β mRNA expressing cells were significantly increased in nasal tissues following IT as compared with placebo ($p = 0.05$; Fig. 2 and Table II). In contrast, low numbers of TGF-βmRNA$^+$ cells were observed in nasal biopsies obtained from IT-treated patients 2 years following treatment outside of the pollen season (in the absence of allergen stimulation) and were also low in biopsies taken during the peak pollen season in control nonatopic subjects (Fig. 2). Colocalization stainings showed that the contribution to TGF-β was mainly from CD68$^+$ macrophages (35.1% of TGF-β$^+$ cells) and secondly from CD3$^+$ T cells (19.8%), with 9.7% of macrophages and 2.2% of T cells producing this cytokine.

Most IgA (IgA1 and IgA2)-expressing cells were located around submucosal glands, and most Phl p5-positive cells were within the

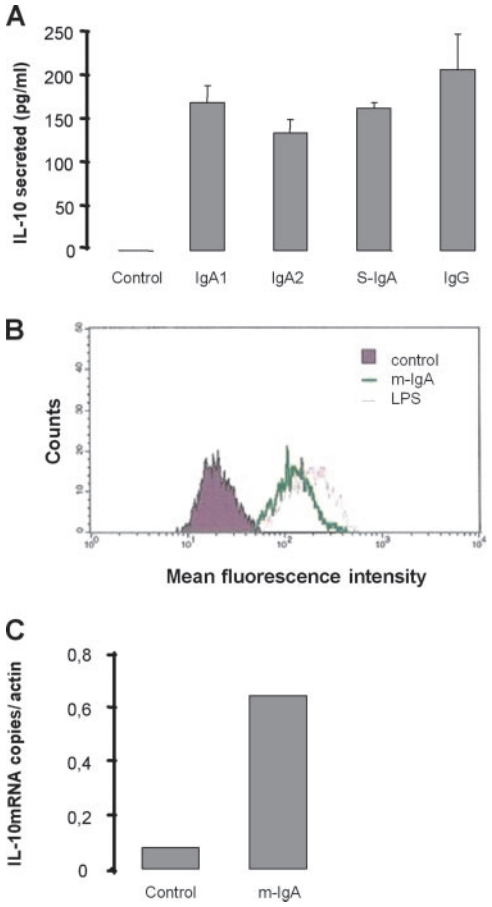

FIGURE 6. Effect of pure preparations of IgA on IL-10 expression. *A*, IL-10 expression was assessed by ELISA in supernatants of monocytes incubated with human monoclonal (myeloma) IgA1 and IgA2, milk S-IgA, or human IgG. *B*, Intracellular staining of IL-10 protein is shown by the shift of the fluorescence curve of monensin-treated monocytes stimulated by m-IgA or LPS (as a positive control) as compared with control unstimulated monocytes. *C*, Effect of m-IgA on IL-10mRNA expression in monocytes as assessed by real-time PCR.

nasal epithelium and subepithelial tissue (Fig. 2). Although no significant difference was observed between IT and placebo groups for nasal IgA1 and IgA2 cell numbers (data not shown), some Phl p 5-specific, IgA2-expressing (double positive) cells could also be detected locally in the lamina propria of nasal tissues from IT-treated patients (Fig. 2*E*, arrow), although in very low numbers.

Serum IgA2 response to immunotherapy is associated with nasal TGF-β expression

Although TGF-β mRNA was not detected in the nasal biopsies from some IT-treated patients, nasal TGF-β mRNA correlated significantly with serum Phl p 5-specific IgA2 ($r = 0.61$, $p = 0.009$) (Fig. 3). A trend for correlation was also observed between nasal TGF-β mRNA and serum Phl p 5 polymeric Abs ($r = 0.45$, $p = 0.06$) (Fig. 3).

Postimmunotherapy serum IgA induces IL-10 secretion by monocytes

To assess a putative functional activity of IgA Abs in the regulatory immune response that accompanies allergen IT, post-IT sera from five patients were fractionated into polymers and monomers of IgA1 and IgA2 as described (*Materials and Methods*), and the six final fractions were analyzed for Ab (sub)classes and protein

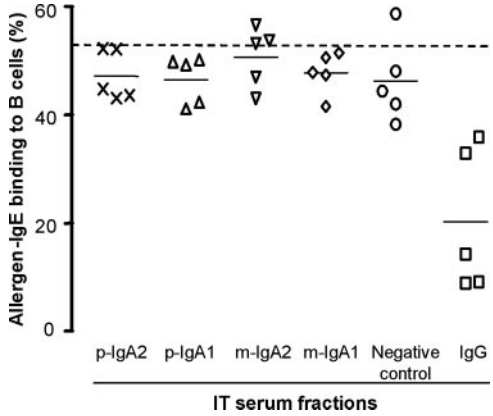

FIGURE 7. Effect of post-IT serum fractions on allergen-IgE binding to B cells. Allergen-IgE binding was 53.2% in the presence of indicator serum (dotted line). Bars represent median values ($n = 5$ IT patients).

concentrations (Fig. 4), fraction 5 being used as a control fraction containing various serum proteins but no Igs (except IgE).

Blood monocytes incubated with serum fractions of IgA produced IL-10 following stimulation with an allergen or an anti-IgA Ab (Fig. 5, *A* and *B*). High IL-10 levels were observed in supernatants of monocytes incubated with fractions enriched in p-IgA2 as well as with IgG (Fig. 5, *A* and *B*). No IL-10 was detected in monocyte supernatants incubated with IgA (or IgG) fractions alone without cross-linking (not shown).

A dose-response curve was observed with a p-IgA2-rich fraction (Fig. 5*C*), and the IL-10-inducing effect of this fraction was inhibited (by ~76%) by using the My43 FcαRI-blocking mAb (Fig. 5*C*) but not with control mouse IgM (not shown). In time course experiments, IL-10 was detected in monocyte supernatants at 4 h (39 ± 9 pg/ml, mean ± SD) and increased until 20 h (108 ± 16 pg/ml at 12 h, 171 ± 28 pg/ml at 20 h) of incubation with a p-IgA2 fraction followed by anti-IgA cross-linking.

The IL-10 response of monocytes was also induced by using purified IgA1 and IgA2 (and S-IgA) as well as purified IgG, followed by anti-Ig cross-linking (Fig. 6*A*). IL-10 expression in IgA-stimulated monocytes was confirmed by intracellular staining and at gene level by real-time PCR (Fig. 6, *B* and *C*). IgA also stimulated monocytes to secrete TGF-β, although to a much lower extent than IL-10 (~1-to 2-fold increase; data not shown).

Postimmunotherapy serum IgA does not inhibit IgE-allergen binding to B cells

No significant inhibition of binding of allergen-IgE complexes to B cells was observed with the different IgA fractions, in contrast to IgG (Fig. 7). To correct for the very different levels of Phl p Abs between IgA and IgG fractions, allergen-IgE binding was tested using fractions diluted according to their Ab content assessed by ELISA (as shown in Fig. 4). No significant inhibition could be observed with IgA while correcting for the Phl p Ab level or while using in vitro generated S-IgA (data not shown).

Discussion

Ig A is a crucial component of first-line immune mechanisms at mucosal surfaces, and peripheral and/or local IgA responses to inhaled allergens have been observed following IT in humans. This study shows for the first time that in allergic patients the serum IgA response to grass pollen s.c. IT is selective for the IgA2 subclass and parallels increases in mucosal-derived, polymeric Abs. In addition, the serum IgA2 response to IT correlates with the increased local nasal expression of TGF-β, the main cytokine inducing class

switching to IgA. We also show that whereas serum IgA fractions from IT-treated patients do not inhibit allergen-IgE binding to B cells as observed with IgG, they do trigger blood monocytes to secrete IL-10, a major immunosuppressive/regulatory cytokine.

Increases in local allergen-specific IgA Abs have been observed following IT (15–16), whereas other studies, except one (17), failed to demonstrate a serum IgA response to treatment (18, 19). Bahceciler et al. (13) recently showed that serum IgA Ab levels could be restored following IT. We report a selective IgA2 subclass response to allergen IT that may have several implications. First, it may reflect the site of the response to IT, as mucosal tissues are enriched in IgA2 (up to 50% of IgA-producing cells in the gut and salivary glands) (20, 21) while IgA1 is the predominant IgA subclass in serum (<10% IgA2-producing cells in bone marrow) and serum IgA Abs to allergens are predominantly IgA1 (14). Moreover, a significant increase in serum polymeric Abs was observed after 2 years of IT, suggesting that serum IgA Abs could be produced following treatment, at least partly, locally within nasal tissues. Second, IgA2 has unique properties as compared with IgA1, including resistance to the classical IgA(1) proteases (21) secreted by commensal bacteria of the upper aerodigestive tract. Third, selective α2 gene expression indicates that class switch recombination could potentially involve sequential switching of established high-affinity IgE-producing B cells in favor of IgA2, because within the Ig H chain locus the IgA2 (α2) H chain gene, in contrast to IgA1 (α1), is situated downstream (3′) of the IgE (ε) gene (30). No increase in specific IgA was observed during the natural (pollen) allergen exposure, indicating that the serum IgA2 response is specifically triggered following IT.

B cell switching to IgA synthesis is governed by TGF-β, known as the α-class switch factor (31), driving both α1 and α2 expression. This study shows that 2 years of allergen IT induces mucosal nasal expression of TGF-β, as previously shown in peripheral cells (17). TGF-β expression was observed in IT peak-seasonal mucosal biopsies and not in specimens obtained 2 years after the completion of treatment, outside of the pollen season. This suggests that the induction of TGF-β by IT could require both IT and natural allergen exposure, as previously observed for IL-10 (27). Also, as for IL-10, macrophages were identified as the predominant cellular source of TGF-β in the nasal mucosa of these patients in addition to T-lymphocytes, both representing probably regulatory subsets of leukocytes, although this needs further investigation. Mechanisms of selective expression of IgA2 remains under evaluation; mature IgA2 transcripts are induced in B cells by a combination of TGF-β, IL-10, and CD40 triggering (32), while dendritic cells could also provide key signals for IgA2 production (33). Besides switching to IgA, TGF-β has several immunoregulatory functions; it directly inhibits T cell proliferation and differentiation, prevents the maturation of dendritic cells, and promotes the generation of regulatory T cells (34). Although the function of TGF-β as an effector molecule of regulatory T cells remains debated (23), the suppressive effect of TGF-β on T cells appears crucial for the induction of peripheral tolerance, as suggested by TGF-β receptor type II knockout mice developing severe immunopathology (35, 36). Interestingly, in our study nasal TGF-β expression correlated with serum allergen-specific IgA2 levels, further suggesting a relationship between peripheral and mucosal responses following systemic IT. This putative relationship should also be assessed following IT using other routes, such as sublingual IT.

IgA2-expressing cells able to bind grass allergens were detected in the nasal mucosa of IT-treated patients. The low number of these cells could relate to the competition for allergen binding of the different Ab isotypes present locally, including high-affinity IgE. Interestingly, IgA Abs appear to display specificities to allergen molecules different from those of IgE (37), although this needs to be assessed in IT-treated allergic patients.

Our study provides novel insights on the functional relevance of the IgA response to IT. IgA serum fractions from IT patients induced IL-10 secretion by peripheral monocytes. IL-10 producing dendritic cells play a key role in the development of tolerance to inhaled Ags in normal immunity (38) and, accordingly, increases in IL-10 producing cells have been consistently observed following IT with pollen (27, 39, 40), mite (17), and venom (41–43) allergens and were associated with clinical improvement and increased production of IgG4 Abs (27). The source of IL-10 following IT consists of T cells and, later on, monocytic cells (27, 39, 42). The induction of IL-10 in monocytes/macrophages by IgG has previously been reported (44); Geissman et al. (45) reported on IL-10 responses to IgA in dendritic cells while Heystek et al. (46) did not observe any cytokine response of these monocyte-derived cells expressing low levels of FcαRI. In our study, freshly purified peripheral monocytes stimulated by IgA also secreted IL-10, the robust response to p-IgA2 fractions relating probably to higher IgA Ab levels, as no significant difference in IL-10 release was observed between (purified) IgA subclasses. Another possibility could be related to complexes of monomeric IgA and soluble FcαRI (CD89), which could circulate in serum as high-mass molecules (47). Our polymeric IgA fractions were however selected according to J chain (and IgA) reactivity and consisted of proteins ranging from ~200 to 330 kDa (dimeric IgA, 335 kDa), only very partly overlapping with IgA/CD89 complexes of ~300–600 kDa.

The induction of IL-10 by IgA in APCs may play a role in the development of mucosal tolerance following IT in allergic individuals. IL-10, like TGF-β, represents a major inhibitory cytokine; it prevents the differentiation of monocytes into dendritic cells while promoting the macrophage phenotype (48, 49) and inhibits MHC class II and costimulatory activity for Ag presentation to T cells (50). Although the relationships between Ab and T cell responses to IT remain complex, the induction of IL-10 in monocytes by noninflammatory isotypes (IgA and IgG4) could represent an additional mechanism to regulate immune responses to allergens in favor of tolerance. Also, in our system IL-10 induction by IgA and IgG required receptor cross-linking (via the allergen or, mimicking allergens, via anti-Ig antibody), further suggesting that IL-10 and TGF-β regulatory responses observed following IT depend on allergen exposure of mucosal tissues.

We previously developed a flow cytometry-based in vitro assay to measure the binding of allergen-IgE complexes to B cells as a surrogate for IgE-facilitated allergen presentation and activation of T lymphocytes (28). We further used the inhibition of allergen-IgE binding to B cells by post-IT serum as a surrogate functional assay for the known elevations in allergen-specific IgG that occur after IT (27, 28). In the present study it seemed important to similarly determine whether the elevations of allergen-specific IgA and its subfractions after IT could exhibit inhibitory activity in our system. In contrast to the stimulation of monocyte IL-10 production, the binding of allergen-IgE complexes to B cells was unaffected by IgA. However, in parallel experiments there was a marked inhibition by post-IT fractionated allergen-specific IgG. Previous studies also failed to demonstrate a suppressive role of serum-specific IgA in allergen-IgE binding to B cells (25) or basophil degranulation (51). Platts-Mills et al. (15) found that nasal IgA-rich fractions could inhibit basophils from releasing histamine, suggesting that mucosal IgA could display unique properties. This interesting hypothesis could not be supported in our study, although discrepancies in findings on the suppressive activity of IgA as compared with IgG Abs on in vitro allergic responses could relate to differences in Ab levels and/or in reactivity of target cells.

This study demonstrates IgA2 and TGF-β responses to allergen immunotherapy in grass pollen allergic individuals and suggests that peripheral IgA2 Abs arise, at least in part, from the exposed nasal mucosa under the regulation of TGF-β, reflecting local activation of mucosal immunity. It is also likely that this inhibitory cytokine that is up-regulated locally after treatment may be having "protective" effects such as the inhibition of local Th2 and IgE responses that might contribute to the efficacy of immunotherapy. Also, we show that IgA and IgG Abs could contribute to immunological tolerance through distinct but partly overlapping mechanisms. Monocytes activated by IgA or IgG Abs secrete IL-10, while only IgG significantly suppresses allergen-IgE binding to B cells. These data support the concept that Ag-specific IgA responses relate to the development of tolerance in mucosal immunity and are closely associated with the expression of suppressive cytokines (IL-10, TGF-β) and protective Ab subclasses (IgG4 and IgA2). Possible therapeutic implications include whether local TGF-β and/or systemic specific IgA2 responses might be surrogate or predictive of the clinical response to immunotherapy. Such large-scale studies are in progress. Secondly, in view of the functional role of IgA in scavenging inhaled Ags (achieving a form of molecular allergen avoidance) and inducing IL-10, there remains the possibility of developing human grass-specific IgA Abs as a potential form of passive immunotherapy (52) for the management of hayfever without the attendant risks of inducing anaphylactic side-effects.

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Disclosures

The authors have no financial conflict of interest.

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