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Different Bla-g T cell antigens dominate responses in asthma versus rhinitis subjects

M. B. C. Dillon[1], V. Schulten[1], C. Oseroff[1], S. Paul[1], L. M. Dullanty[1], A. Frazier[1], X. Belles[2], M.-D. Piulachs[2], C. Visness[3], L. Bacharier[4], G. R. Bloomberg[4], P. Busse[5], J. Sidney[1], B. Peters[1] and A. Sette[1]

[1]La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA, [2]Institut de Biologia Evolutiva (CSIC-Universitat Pompeu Fabra), Barcelona, Spain, [3]Federal Systems Division, Rho Inc., Chapel Hill, NC, USA, [4]Department of Pediatrics, Washington University School of Medicine, St. Louis, MO, USA and [5]Division of Clinical Immunology, Icahn School of Medicine at Mount Sinai School of Medicine, New York, NY, USA

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Correspondence:
A. Sette, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, CA 92037, USA.
E-mail: alex@lji.org

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Summary

Background and objective The allergenicity of several German cockroach (Bla-g) antigens at the level of IgE responses is well established. However, less is known about the specificity of $CD4^+$ T_H responses, and whether differences exist in associated magnitude or cytokine profiles as a function of disease severity.

Methods Proteomic and transcriptomic techniques were used to identify novel antigens recognized by allergen-specific T cells. To characterize different T_H functionalities of allergen-specific T cells, ELISPOT assays with sets of overlapping peptides covering the sequences of known allergens and novel antigens were employed to measure release of IL-5, IFNγ, IL-10, IL-17 and IL-21.

Results Using these techniques, we characterized T_H responses in a cohort of adult Bla-g-sensitized subjects, either with (n = 55) or without (n = 17) asthma, and nonsensitized controls (n = 20). T cell responses were detected for ten known Bla-g allergens and an additional ten novel Bla-g antigens, representing in total a 5-fold increase in the number of antigens demonstrated to be targeted by allergen-specific T cells. Responses of sensitized individuals regardless of asthma status were predominantly T_H2, but higher in patients with diagnosed asthma. In asthmatic subjects, Bla-g 5, 9 and 11 were immunodominant, while, in contrast, nonasthmatic-sensitized subjects responded mostly to Bla-g 5 and 4 and the novel antigen NBGA5.

Conclusions Asthmatic and nonasthmatic cockroach-sensitized individuals exhibit similar T_H2-polarized responses. Compared with nonasthmatics, however, asthmatic individuals have responses of higher magnitude and different allergen specificity.

Keywords asthma, $CD4^+$ T cell, cockroach allergy, epitope
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Introduction

The German cockroach (*Blattella germanica;* Bla-g) is a common allergen among inner-city children and a significant health problem world-wide [1, 2]. Bla-g allergies strongly correlate with asthma development, and early exposure leads to increased Bla-g sensitization and asthma severity [1, 3–6].

Several studies defined Bla-g allergens on the basis of IgE reactivity from sensitized individuals [7–16] and correlated seroreactivity prevalence with severity of Bla-g allergies [2, 17]. However, investigation of cellular responses is limited to few of the known Bla-g allergens, with relatively few T cell epitopes identified [18, 19]. Here, we report a systematic analysis of T cell responses to all known Bla-g allergens.

We previously used a transcriptomic/proteomic approach to identify novel antigens and T cell epitopes in Timothy grass (TG) allergy [20]. This indicated that T_H2 cell reactivity is not necessarily limited to proteins recognized by IgE, but extends to proteins recognized by IgG and/or generally abundant in the pollen extract. Here, we investigated whether a similar approach could reveal new Bla-g T cell antigens.

Bla-g allergies are associated with clinical presentations ranging from allergic rhinitis (AR) without

asthmatic symptoms to asthma severity ranging from intermittent (IA) to mild, moderate (MMA) and severe (SA) [2, 21]. While some studies have examined the prevalence of IgE reactivity but not relative titres [22] against different Bla-g allergens, most studies have focused primarily on IgE reactivity to whole Bla-g extract or one or two individual allergens.

Here, we planned to use the epitope information derived from the analysis of known and novel Bla-g allergens combined, to assess whether different disease severities are associated with differential magnitude, functionality or antigen/epitope specificity at the level of T cell responses. Allergen-specific T cell responses are usually dominated by T_H2 type responses, with IL-5 secreted at the highest levels, followed by IL-13, IL-4 and IL-9 [23, 24], although involvement of different T helper subsets has also been reported. Elevated T_H17 cells have been described, particularly in the context of asthmatic reactions, as IL-17F production has been reported as increased in numerous asthmatic states [25, 26]. T_R1 cells secreting IL-10 have been implicated in negative regulation of T cell allergic responses [27]. The balance between T_H2 cells and IFNγ-producing T_H1 cells (T_H1/T_H2 polarization) is considered a potential key determinant in regulating allergic disease [1, 28]. Finally, recent studies have described T_{FH} cells, associated with production of IL-21, as key regulators of isotype switching (including IgE) [29]. Here, we accordingly sought to characterize the relative balance of IFNγ, IL-5, IL-10, IL-17, and IL-21-production in response to Bla-g derived epitopes.

Materials and methods

Study subject populations

Subjects displaying symptoms of allergic rhinitis (e.g., stuffed, blocked, or runny nose, sneezing, itching or watery eyes, or itching nose or throat) were recruited from St. Louis, New York City, Boston, and Cleveland clinics, following Institutional Review Board-approved protocols and informed consent. Subjects with positive skin-prick test (wheal ≥3 mm) and IgE titres (≥0.35 kUA/L by ImmunoCAP assay) towards Bla-g extract were classified as Bla-g IgE-sensitized. Nonsensitized controls were individuals with negative skin-prick tests and IgE titres to Bla-g extract. No information about pollen and HDM allergies is included as these data were not collected during the clinical study. The asthmatic status of Bla-g-sensitized subjects was further classified as:

- Allergic rhinitis and no asthma (AR): no self-reported asthma.
- Intermittent asthma (IA): no controller treatment and no exacerbations in the last 12 months.
- Mild or moderate asthma (MMA), further subdivided as:
  - Mild asthma: current treatment (total daily dose of inhaled steroids) of fluticasone hydrofluoroalkane-134a (HFA) ≤264 μg or fluticasone dry powder inhaler (DPI) ≤300 μg or equivalent AND no prednisone bursts in last 12 months.
  - Moderate asthma: current treatment of fluticasone HFA 264–440 μg or fluticasone DPI 300–500 μg or equivalent and 0 prednisone bursts in last 12 months OR 1 prednisone burst in last 12 months and current therapy of fluticasone HFA ≤440 μg or fluticasone DPI ≤500 μg or equivalent.
- Severe asthma (SA): current treatment of fluticasone HFA >440 μg or fluticasone DPI >500 μg or equivalent OR >1 prednisone burst in last 12 months.

Bla-g-sensitized individuals who did not meet the criteria for clear diagnosis of asthma and/or allergic rhinitis were excluded. Cohort information is summarized in Table S1. Subject information is in Table S2.

Generation of the Bla-g transcriptome

Bla-g females used for the whole transcriptome analysis were reared at the Institut de Biologia Evolutiva (CSIC-UPF) at 30 °C and 70% humidity, supplied with water *ad libitum* and commercial dog food diet. Whole transcriptome was analyzed in fat body, ovary and epidermis. Fat body and ovaries were from 3- to 5-day-old adults, while the epidermis was from the thoracic dorsum of 5- and 6-day-old 6th instar nymphs. Total RNA was extracted with the GenEluteTM Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA.) RNA samples were sequenced at GATC-Biotech (Konstanz, Germany) by Roche™ pyrosequencing technology (454) [2, 17, 30, 31]. Data are accessible at the GEO database (accession code GSE63921).

Proteomic analysis of Bla-g extract

Novel Bla-g antigens were identified as described for TG [20]. Briefly, Bla-g extract (Greer, cat#XPB46D3A4) and pooled sera from 15 Bla-g-sensitized subjects were submitted to Applied Biomics for 2D immunoblot analysis. Gels (3–10 pH range, 12% (vol/vol) acrylamide) of Bla-g extract were incubated with pooled sera, stained with goat anti-human IgE and rabbit anti-human IgG (Sigma-Aldrich) and visualized using Cy2-conjugated donkey anti-goat IgE and Cy5-conjugated donkey anti-mouse IgG antibodies (Biotium). This was followed by mass spectrometry analysis of positive IgE and/or IgG protein spots. MALDI spectra were compared against the Bla-g transcriptome using Mascot. A total of 16 unique novel potential ORFs were identified (Table S3).

For a more detailed description of proteomic identification, see Supplemental Methods.

Peptide synthesis and MHC class II binding predictions

A total of 809 15-mer peptides overlapping by five residues were synthesized, covering the Bla-g allergens Bla-g 1-2, 4-9 and 11. An additional 646 15-mer peptides predicted promiscuous binders to HLA class II [32] were synthesized from the proteomic identified sequences. Finally, 233 predicted promiscuous 15-mer peptides from recently identified antigens (Vitellogenin, Hsp60, Enolase, Triosephosphate Isomerase (TPI), Trypsin, and RACK1) were synthesized (Table S4). For detailed description of binding prediction and peptide synthesis, see Supplemental Methods.

PBMC isolation, cell cultures and ELISPOT assays

PBMC (peripheral blood mononuclear cells) were isolated from 450 mL of blood by density gradient centrifugation and cryopreserved, as described [19]. Upon thaw of cryopreserved vials, according to our standard SOP, viability of cells was determined with trypan blue staining, and only vials with >85% viability were included in the study. Specifically, the average viability was 97±2%. Our goal in the use of PBMC was to maintain physiological APC to T cell interactions. Furthermore, using purified T cells would require additional manipulations and a higher number of starting cells. Accordingly, for several donors, we would not have had enough cells to complete the screen. For these reasons, we chose to utilize whole unfractionated PBMC in our assays. Next, 1×10^5 cells per well were incubated with peptide, peptide pool or Bla-g extract (10, 5, and 10 μg/mL, respectively) on anticytokine antibody-coated ELISPOT plates (Millipore #MSIPS4510) for 22 h. Subsequently, production of IFNγ, IL-5, IL-17 and IL-10, and IL-21 in response to peptide stimulation was measured by ELISPOT, as described previously [19, 20]. For IL-21, similar procedures were used and anti-IL-21 clone MT21.4/821 and anti-IL-21-biotin clone MT21.3 m (Mabtech) were used. Each peptide pool and individual peptides were run in triplicate. Criteria for peptide pool positivity were at least 100 spot-forming cells (SFC) per 10^6 PBMC, $P \leq 0.05$ by Student's t-test when compared to negative control, and stimulation index ≥2. Criteria for peptide positivity were identical except with a threshold of 20 SFC. These criteria have been used consistently in previous studies and have been maintained for consistency [20, 33–38]. We consider 20 SFC/10^6 PBMC to be the operational lower limit of detection in our ELISPOT assay and it is thus used as the 'negative' value for donor response where a lower limit value is required for graphical or statistical purposes.

Results

Differential immune reactivity against known Bla-g allergens (BLAGA) in sensitized subjects versus nonsensitized controls

A cohort of 90 adult subjects was recruited from clinical sites in St Louis, New York City, Boston, and Cleveland (summarized in Table S1). Individual subject information is listed in Table S2. The study population was 77%:23% female to male and had a median age of 33 (± 10; range 19–56), reflecting the fact that subjects were recruited at pediatric clinics as parents of allergic children. In this population, single female parents were prevalent. Adult subjects were included in the study due to the relatively large (450 mL) volume of blood required for the epitope identification studies. Each subject was classified as either Bla-g-sensitized (Bla-g IgE titre ≥0.35 kUA/L and skin-prick test wheal ≥3 mm) or control (<0.35 Bla-g IgE titre and skin-prick test wheal < 3mm). The 20 control subjects were all non-Bla-g-sensitized and either demonstrated no clinical signs of asthma ($n = 13$) or had intermittent ($n = 5$) or moderate ($n = 2$) asthma. Sensitized subjects had an average wheal sizes of 6.1 mm and average IgE titers of 10.1 kUA/L. No information about pollen and HDM allergies was available, as these data were not collected during the clinical study. Bla-g-sensitized subjects were classified as AR, IA, MMA or SA based on clinical history, questionnaires and medication scores as detailed in the Methods section. Specific treatment information is also listed in Table S2.

We synthesized sets of 15-mer peptides, overlapping by 10 amino acids covering the entire sequences of known Bla-g allergens, that is Bla-g 1-2, 4-7, 9 and 11, referred hereafter as BLAGA. A total of 809 BLAGA peptides were pooled for screening, encompassing on average 20 peptides per pool.

To assess T cell reactivity, we utilized a strategy previously applied to the definition of epitopes from various allergens [19, 20]. Here, PBMC from each subject were stimulated *in vitro* with Bla-g extract. After 14 days, pools of overlapping 15-mer peptides were tested, and positive pools deconvoluted to identify the specific epitopes. As read-out, we utilized ELISPOT assays specific for IL-5, IFNγ, IL-10, IL-17 and IL-21, chosen as representative of T_H2, T_H1, T_R1, T_H17 and T_{FH} reactivity, respectively. To validate that cryopreservation after gradient centrifugation of PMBC did not affect responses to peptide stimulation, we assayed T cell response in three Bla-g-sensitized subjects (Figure S1). PBMC were isolated, and half of the samples

were cryopreserved in FBS with 20% DMSO in liquid nitrogen for one week, while the other half of the PBMC were immediately cultured without freezing with pools of Bla-g peptides. After 14 days, the PBMC were stimulated with the same pools and IL-5, IFNγ, IL-10, IL-17 and IL-21 release was measured by ELISPOT. As shown in Figure S1, the sums of cytokine release for the cryopreserved PBMC were statistically equivalent ($P > 0.05$) to the nonfrozen PBMC. Thus, we concluded that reactivity for the tested cytokines is not affected by cryopreservation. Vigorous overall T cell responses (expressed as total SFC/10^6 PBMC for all BLAGA and for all cytokines) were detected against BLAGA in sensitized donors (Fig. 1a). As expected, the nonsensitized controls were associated with lower responses. In sensitized donors, Bla-g 4, 5, 9 and 11 were immunodominant (Fig. 1b). However, in nonsensitized controls, Bla-g 5 and 11 were hardly recognized.

As expected, the most dominant cytokine detected in sensitized individuals was IL-5 (Fig. 1c), followed by IL-10 and IFNγ. Lower or absent levels of IL-5, IFNγ and IL-10 were observed in nonsensitized controls. Lower levels of IL-17 and IL-21 were also detected, with no significant difference between sensitized and control individuals.

Definition of novel Bla-g T cell antigens

In the experiments above, 44% of sensitized subjects responded to extract stimulation but not to any of the BLAGA peptides (data not shown). This suggested that additional uncharacterized antigenic proteins might be present in the extract.

Here, we utilized a recently described approach based on proteomic analysis combined with HLA-binding predictions [20] to identify novel Bla-g antigens (NBGA). Transcripts from Bla-g mRNA were deep-sequenced, followed by mass spectrometry analysis of individual protein spots from 2D gels of Bla-g extract to derive sequences (Fig. 2). These transcripts, along with the MALDI-derived peptide sequences, were assembled in 16 unique novel potential ORFs, referred to hereafter as NBGA 1–16 (Table S3). Using similar proteomic techniques, six additional NBGA, including enolase, Hsp60, RACK1, TPI, trypsin and vitellogenin, were independently identified [15, 39].

In total, these studies defined 22 different NBGA, 15 of which were IgE reactive as defined by the 2D immunoblot analysis (Fig. 2) and previous studies [15, 18, 19, 39]. Promiscuous HLA class II binding peptides were predicted as previously described [20, 33], and a

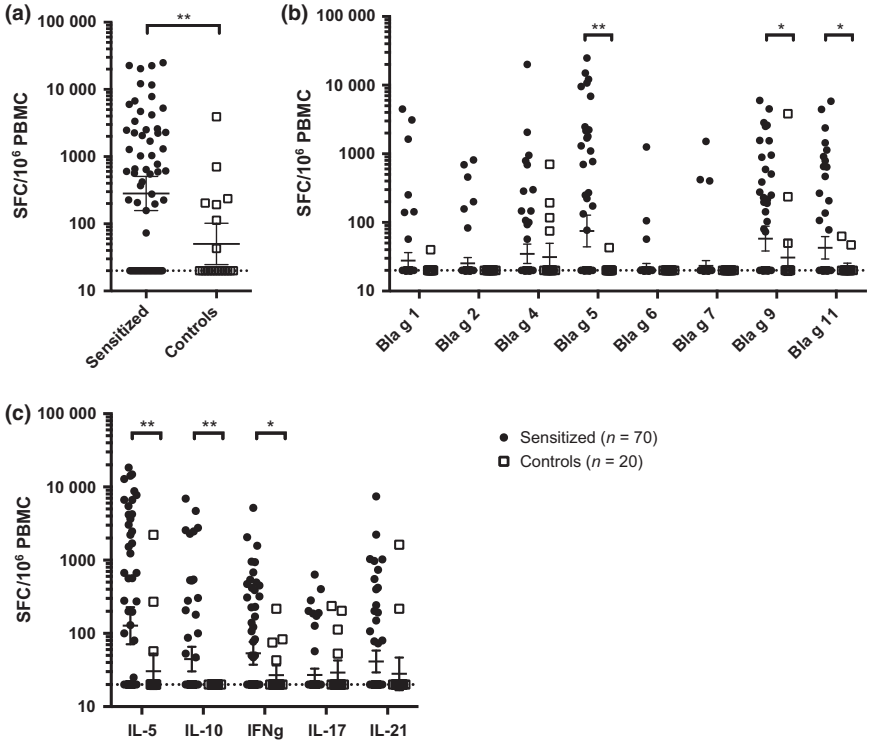

Fig. 1. CD4$^+$ T cell reactivity to known Bla-g allergens (BLAGA). (a) Overall responses (sum of peptide and cytokine responses) after stimulation with BLAGA peptides. (b) Overall responses to individual BLAGA. (c) Pattern of cytokine responses to BLAGA. Geometric means and 95% confidence intervals are shown. Black circles represent Bla-g-sensitized subjects, and grey open circles represent nonsensitized controls. *$P \leq 0.05$, **$P \leq 0.005$ by nonparametric Mann–Whitney *t*-test. Dotted lines at 20 SFC indicate the operational lower limit of detection in our ELISPOT assay used as the 'negative' value for donor response. Each symbol represents the response from an individual subject.

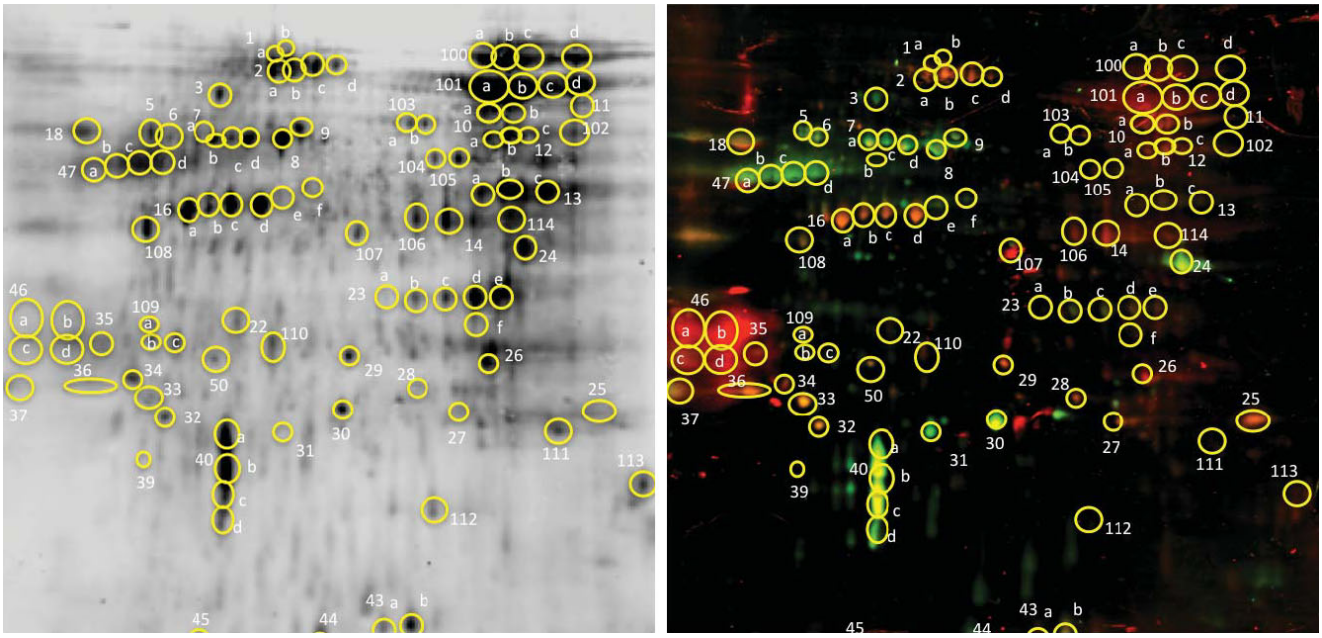

Fig. 2. Identification of novel Bla-g antigens (NBGA) by 2D gel immunoblot. Left, Coomassie stain of 2D gel of Bla-g extract. Right, Bla-g extract stained with pooled sera of Bla-g-sensitized subjects. Green spots indicate IgE binding; red spots indicate IgG binding; and yellow spots indicate dual IgE/IgG binding. Yellow circles indicate sections selected for proteomic analysis.

total of 879 peptides were synthesized. Cytokine release in response to each peptide following 2-week *in vitro* extract stimulation was measured as above. A total of 13 NBGA were associated with detectable cytokine release in more than one subject (Fig. 3a). While the novel antigen NBGA1 dominated the response (Fig. 3a), significant reactivity was also observed for several others, including NBGA2-5, NBGA7, TPI and RACK1. By contrast, ten other antigens (NBGA8-16 and vitellogenin) were negative for any cytokine responses.

Remarkably, most of the reactivity was encompassed by IgE-reactive (IgE+) NBGA (shown in the left of Fig. 3a), while, with the exception of NBGA5, IgE-unreactive (IgE-) NBGA (shown in the right of Fig. 3a) were essentially negative. IgE+ NBGA reactivity for all cytokines was stronger compared to IgE- NBGA (Fig. 3b). Non-sensitized control subjects also preferentially recognized IgE+ NBGA, but with lower magnitude responses (Fig. 3b).

Breadth and immunodominance at the epitope level

Overall, 356 nonredundant epitopes recognized in at least one subject were identified. A total of 56% of the sensitized subjects recognized at least one BLAGA epitope. Combining BLAGA and NBGA epitopes increased the percentage of subjects recognizing at least one epitope to 79% (Fig. 4a). Thus, combined use of epitopes derived from BLAGA and NBGA achieves greater coverage of Bla-g-sensitized individuals.

Overall, 50% of the subjects recognized 6 peptides or more (Fig. 4a). A total of 90% of the total response was encompassed by the top 164 epitopes, and the top 23 epitopes captured 50% of the total response (Fig. 4b). These 164 epitopes, and the corresponding average magnitudes and response frequencies, are listed in Table S5.

Polarization of T cell responses correlates with sensitization status, while magnitude of T cell responses correlates with asthma status

Next, we correlated magnitude and functionality of T_H responses with sensitization and asthma status (Fig. 5). Similar to what was observed for TG [20, 33, 40] allergy, the cytokine patterns in Bla-g-sensitized patients were polarized, with responses dominated by IL-5, regardless of the AR versus asthma status of the sensitized donors. By contrast, responses in control subjects were not polarized, with similar proportions of IL-5 (32%), IFNγ (21%) and IL-10 (25%) being detected.

A response magnitude of 251 SFC/10^6 donor cells was noted in nonsensitized controls (Fig. 5). The response/donor of all asthmatic subjects combined was significantly higher than controls ($P < 0.05$). The magnitude of responses progressively increased in subjects with AR (491 SFC), IA (605 SFC), and MMA (1786 SFC,

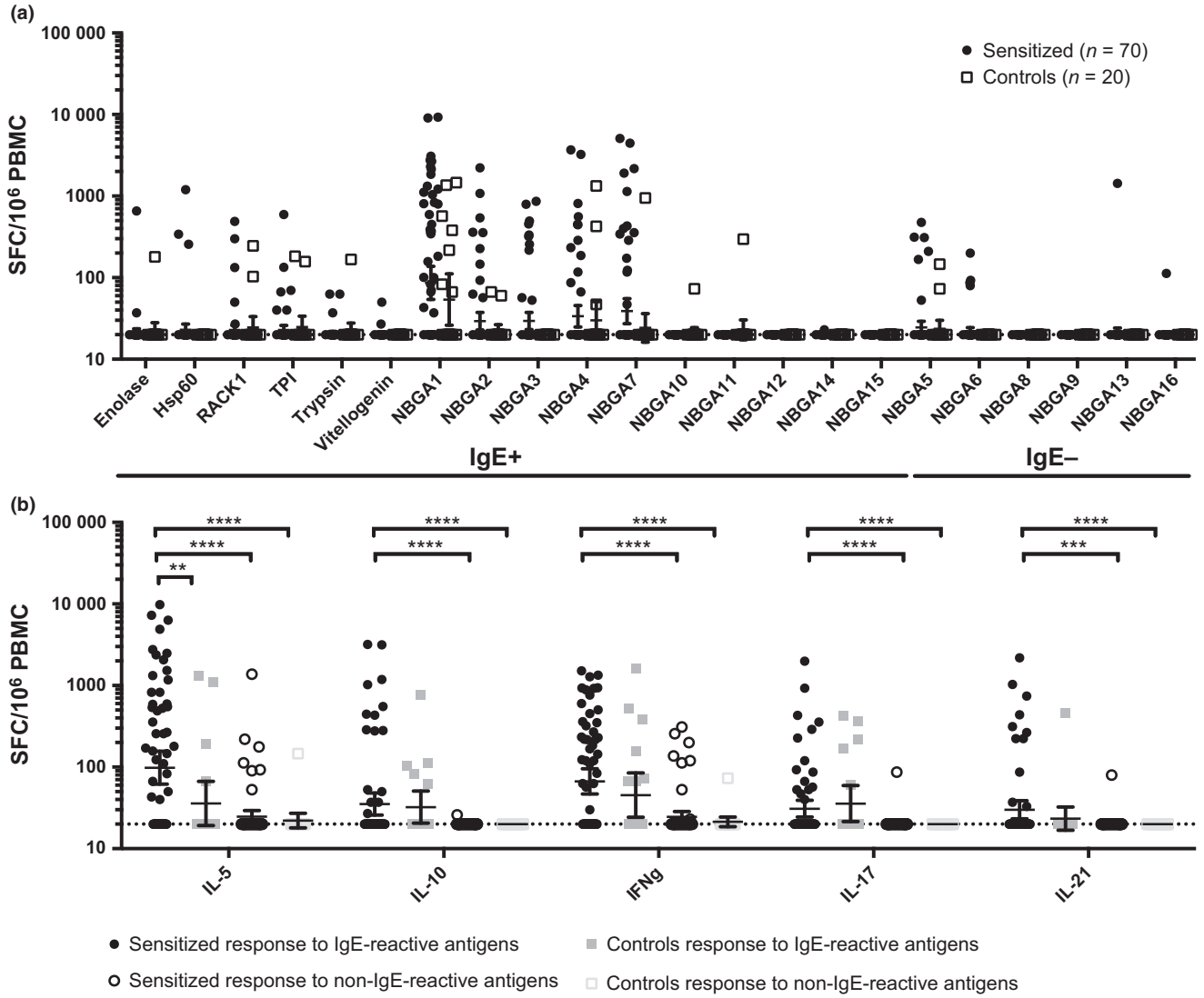

Fig. 3. CD4$^+$ T cell reactivity to novel Bla-g antigens (NBGA). (a) Individual NBGA responses (sum of all cytokines) of Bla-g-sensitized and control subjects after stimulation with NBGA peptides. (b) Pattern of cytokine responses detected against IgE-reactive and non-IgE-reactive NBGA in sensitized and control subjects. Geometric means and 95% confidence intervals are shown. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, by nonparametric Mann–Whitney *t*-test. Dotted lines at 20 SFC indicates the lower limit of detection in our ELISPOT assay used as the 'negative' value for donor response. Each symbol represents the response from an individual subject. NBGA antigens are classified as IgE+ or IgE- based on the gels shown in Fig. 2 and as described in the results.

$P < 0.005$ to controls). Responses were somewhat lower in SA (1134 SFC), although still significantly higher than the control group ($P < 0.05$), perhaps reflective of the immunosuppressive treatments administered to the SA group, who received regular higher doses of steroids (either >440 μg of fluticasone HFA or >500 μg of fluticasone DPI or equivalent OR >1 prednisone burst in last 12 months, as described in the Study Subject Population in the Materials and Methods) than the less severe asthma groups.

We examined whether different clinical groups also display differences in IgE levels. As shown in Figure S2a, no significant difference in IgE titre to whole Bla-g extract was apparent between the different allergic groups. Here, we calculated the correlation between T cell responses and IgE titres (Figure S2b,c). While T cell responses tend to increase as a function of both total response and IL-5 responses, these trends were not significant, thus confirming previous findings [19, 20].

Differential immunodominance of BLAGA and NBGA in control, rhinitis and asthmatic subjects

We next investigated the immunodominance patterns for BLAGA and NBGA antigens. Table 1 lists percent-

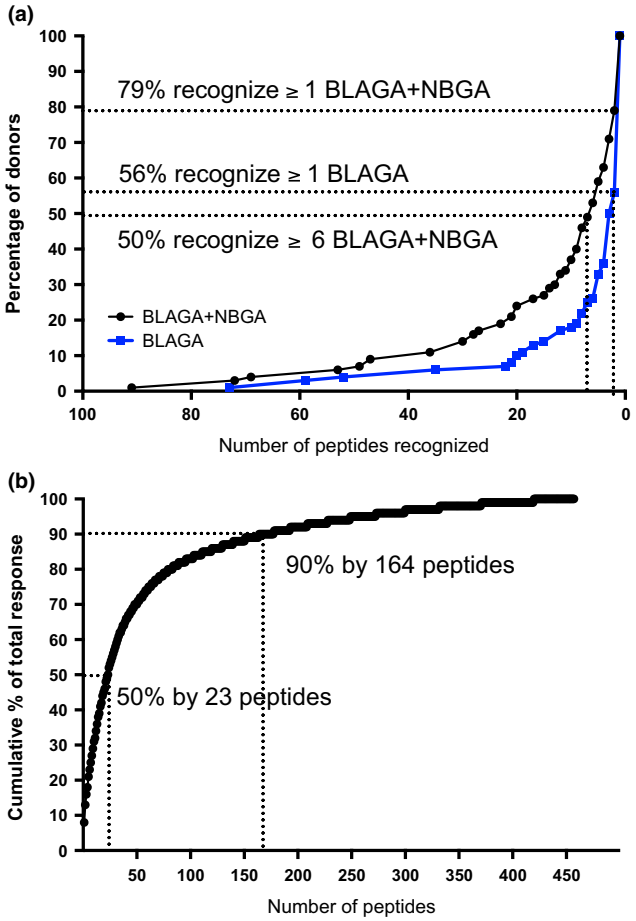

Fig. 4. Immunodominance of Bla-g epitopes. (a) Comparison of the percentage of the total cytokine response per epitope. BLAGA represented by blue circles. Combined BLAGA and NBGA represented by black circles. (b) Comparison of the number of epitopes recognized per subject among the Bla-g-sensitive subjects.

ages of subjects responding and average total response/donor associated with each particular antigen. Figure 6 shows the same data in a pie chart, with study subjects divided into nonsensitized controls, sensitized nonasthmatics, and sensitized asthmatics. We expected, based on the data shown in Figs 1 and 3, different patterns of immunodominance between sensitized and nonsensitized individuals.

Indeed, in nonsensitized controls, >70% of the total response was encompassed by the two antigens, Bla-g 9 and NBGA1 (Fig. 6a and Table 1). In AR subjects (Fig. 6b and Table 1), NBGA1 still encompassed a significant proportion of the response (21.3%); however, Bla-g 4 was the most dominant (31.8% of response), and the response to Bla-g 5 (21.6%) was equivalent to that seen in NBGA1 (21.3%). The pattern further shifted in the sensitized asthmatic donors (Fig. 6c and Table 1). Bla-g 5 and NBGA1 still accounted for large proportions of the response (32.7% and 11.9%, respectively), but responses to Bla-g 4 were nearly absent (1.6%). Responses to Bla-g 9 increased to 24.4%, and Bla-g 11, which had low responses in controls and rhinitis, increased to 8.1%.

Bla-g 2, which is a dominant target of IgE responses along with Bla-g 5, accounted for a minor fraction (<1%) of T cell reactivity among both rhinitis and asthmatic subjects, highlighting the lack of correlation between immunodominance for IgE and T cell responses, in agreement with previous reports in other allergies [19, 33, 36].

As observed in Fig. 5 in terms of the total response to all antigens, IL-5 was the dominant cytokine detected in both rhinitis and asthmatic subjects for the immunodominant antigens Bla-g 4, 5, 9, 11 and NBGA1 (Table 2), encompassing at least 50% of the response to each antigen. In control subjects, the response to the immunodominant antigens was less polarized to IL-5, representative of T_H2 cytokines. IFNγ responses were in general the second most vigorous response, accounting for 5–18% of the response to the various antigens in sensitized donors. Against the important antigen NGBA1, responses were polarized to IL-5 in sensitized AR and asthmatic donors and to IFNγ in controls. In the case of Bla-g 9, similar polarization was noted in AR and asthmatic subjects, with 65–70% of the response accounted for by IL-5, and 4–16% of the responses by other cytokines; this contrasts with what was observed in control subjects, where 45% of the response was accounted for by IL-17, and only 30% by IL-5. We additionally note a relatively high IL-10 response (31.1% of the total) to Bla-g 4 in AR, whereas IL-10 response to Bla-g 4 was negligible in asthmatics. In contrast, IL-10 Bla-g 5 responses in asthmatics accounted for 16.7% of the total, but were nonexistent in AR.

Only minor differences were observed in the pattern of responsiveness among different asthma severities. For example, NBGA1 and 7 were recognized most prominently in IA subjects (Table 1), but overall similar patterns were observed in the different categories of asthmatic patients. No significant differences linked to age were observed between subjects or groups, and thus, the differences observed were representative of a large range of ages. Additionally, the differences in response between groups were not due to heterogeneity of class II MHC between groups as no class II MHC allele was found to be significantly associated with asthma or rhinitis.

Differential recognition with disease-specific epitope pools

Based on the above results, we hypothesized that the epitopes identified could be partitioned into sets associated

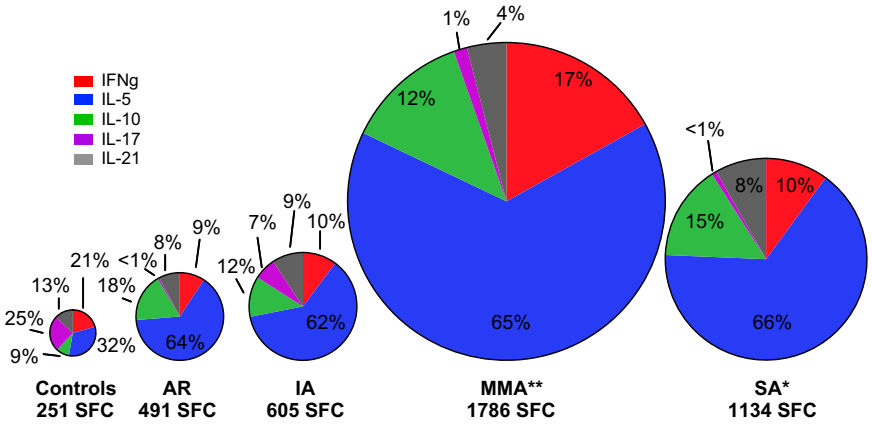

Fig. 5. Changing magnitude and polyfunctionality of responses among asthma severities. Diameter of pie chart is proportional to geometric mean of the total sum of responses for subject group. Values indicated are percentage of total response encompassed by individual cytokine. Red denotes relative proportion of IFNγ responses, blue IL-5, green IL-10, purple IL-17, and grey IL-21. $*P < 0.05$, $**P < 0.005$, calculated by non-parametric Mann–Whitney t-test.

Table 1. Differential immunodominance of Bla-g antigens as a function of allergic clinical status

	Controls		AR		Asthmatic		IA		MMA		SA	
Antigen	%	SFC	%	SFC	%	SFC	%	SFC	%	SFC	%	SFC
Bla-g 1	5	2	–	–	15	188	6	9	44	1044	15	24
Bla-g 2	–	–	12	44	9	44	3	26	33	154	8	12
Bla-g 4	4	54	18	1389	20	85	21	58	33	286	8	11
Bla-g 5	1	2	41	945	29	1704	21	1179	56	1827	31	2872
Bla-g 9	3	314	12	192	47	1275	39	715	67	2281	54	1914
Bla-g 11	2	6	18	170	36	424	30	416	44	676	46	271
NBGA1	7	290	29	932	47	621	39	700	56	406	62	579
NBGA2	2	6	12	78	13	78	12	40	11	246	15	53
NBGA3	–	–	6	21	24	123	15	71	33	85	39	273
NBGA4	3	30	24	289	16	136	18	151	22	197	8	59
NBGA5	2	11	12	33	7	19	6	17	–	–	15	37
NBGA7	1	50	29	194	16	287	18	442	22	96	8	49
NBGA11	1	15	–	–	–	–	–	–	–	–	–	–
Enolase	1	9	6	3	6	42	3	27	11	151	8	3
RACK1	2	30	–	–	9	19	12	31	11	3	–	–
TPI	2	17	6	13	11	31	6	6	11	83	23	54

'%' denotes percentage of subjects responding to given antigen. 'SFC' denotes the mean magnitude of response per donor. '–' denotes no response. The 'Asthmatic' combines the responses across IA, MMA, SA.

with preferential recognition by specific patient groups. The *in silico* analysis shown in Table S6 defines a set of 55 epitope sequences accounting for 84% of the total response in the rhinitis subjects, but only 20% of the response of asthmatic subjects. Similarly, a set of 147 epitopes (asthma pool) could be identified that is associated with preferential recognition (55% of total response) in subjects with asthma, as compared to rhinitis subjects (5%).

We next validated whether pools of peptides corresponding to these different epitope sets were indeed differentially recognized in Bla-g-sensitized subjects. PBMC from AR and asthmatic subjects were cultured *in vitro* for 14 days in the presence of Bla-g extract, followed by overnight assay with epitope pools described in Table S6, and responses were measured by ELISPOT. The fraction of total response attributed to each epitope set was calculated.

The mean percentage of responses of asthmatic subjects was highest in response to the asthma pool as compared to either the control or rhinitis pool (Fig. 7a). In fact, in 12 of 14 responses from asthmatic subjects, the highest response was directed to the asthma pool. Conversely, six of the seven responses in the AR (sensitized but nonasthmatic) subjects were dominated by the rhinitis pool (Fig. 7b). The discrimination achieved with the disease-specific epitope pools between AR and asth-

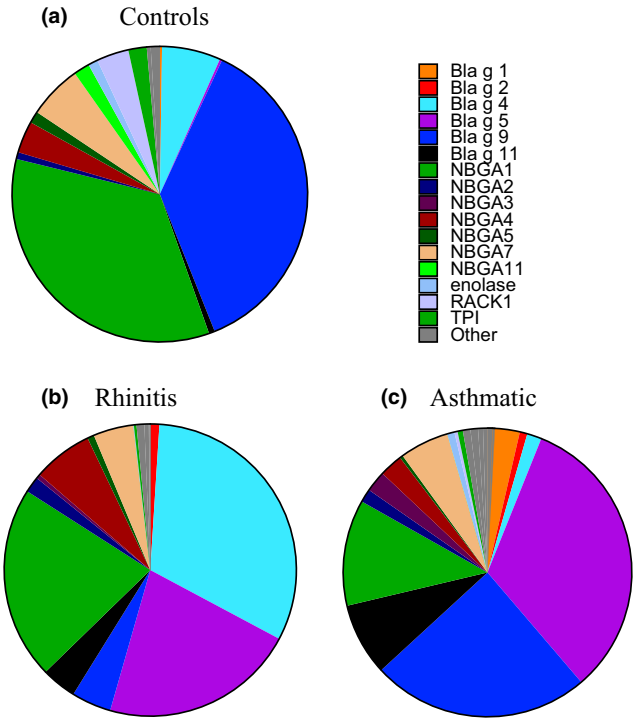

Fig. 6. Differential immunodominance of Bla-g antigens as a function of allergic clinical status. Percentage response accounted by individual antigens of total cytokine response to all Bla-g Antigens for (a) control, (b) allergic rhinitis and (c) asthmatic sensitized subjects. 'Other' category encompasses antigens accounting individually for <1% of total response for all three groups.

Table 2. Functional response to immunodominant antigens similar between AR and asthma subjects

		Percentage of total response to individual antigen				
Antigen	Group	IFNg	IL-5	IL-10	IL-17	IL-21
Bla-g 4	AR	11.1	57.5	31.1	–	0.3
Bla-g 4	Asthma	17.6	55.4	1.0	20.7	5.4
Bla-g 4	Control	34.4	30.0	–	15.6	19.9
Bla-g 5	AR	5.1	69.0	–	1.2	24.8
Bla-g 5	Asthma	9.3	67.4	16.7	0.4	6.2
Bla-g 5	Control	100.0	–	–	–	–
Bla-g 9	AR	6.6	70.4	3.9	3.9	15.3
Bla-g 9	Asthma	8.1	64.2	11.6	8.8	7.3
Bla-g 9	Control	1.2	31.0	2.5	42.5	22.7
Bla-g 11	AR	5.3	72.5	19.6	–	2.7
Bla-g 11	Asthma	13.9	51.6	6.0	6.5	22.1
Bla-g 11	Control	–	–	–	100.0	–
NBGA1	AR	13.5	67.6	13.8	1.9	3.2
NBGA1	Asthma	17.4	56.3	10.3	8.4	7.6
NBGA1	Control	45.0	25.3	18.1	3.7	7.9

Percentage of cytokine response detected to single antigen for control, AR, and asthmatic subjects. '–' denotes no response.

matic subjects was statistically significant ($P = 0.0032$ by Fisher's exact test). Results were similar when absolute response magnitude was considered (Fig. 7c,d).

Discussion

In this study, we characterized $CD4^+$ T cell responses in Bla-g allergy as a function of sensitization and asthmatic status, at the antigen and epitope level, and at the level of functionality of associated T cell responses. Surprisingly, we observed differences in antigens dominantly recognized in Bla-g-sensitized subjects with and without asthma. To the best of our knowledge, this represents the first report of differential recognition between asthmatic and nonasthmatic subjects of T cell antigens in respiratory allergens in general, and Bla-g-sensitized individuals in particular.

While further studies are necessary to address the mechanisms involved, it is possible to speculate why different antigens would be targeted in different forms of allergic disease. As the different patterns of immunodominance are seemingly not related to intrinsic immunogenicity at the antigen level, it is possible that Bla-g antigens might be differentially processed and presented in the lung/bronchial versus the nasal environment, resulting in characteristically different immunosignatures. Alternatively, differences in the local microbiome in the two different anatomical locations might influence the specificity patterns of allergic reactions.

These studies were enabled by a large-scale epitope identification screening strategy. Overall, the study investigated the reactivity of approximately 1600 different Bla-g-derived synthetic peptides. Over 164 different epitopes were recognized in multiple donors, and 23 accounted for 50% of the total response. Previous to the present study, the nature of human T cell epitopes had not been systematically investigated in this allergy system and only 30 Bla-g T cell epitopes had been described [19, 23, 24]. Accordingly, our study greatly expanded the number of epitopes available for future studies dissecting the role of T cell responses in Bla-g allergies.

Initially, we investigated the conventional allergens, Bla-g 1-2, 4-9, and 11. As these allergens only accounted for responses in 56% of Bla-g-sensitized individuals, we employed a transcriptomic/proteomic approach with Bla-g extract and sera from Bla-g-sensitized individuals to find the 'missing' allergens. We previously used a similar approach in TG allergy to discover novel antigens associated with T cell reactivity [20, 25, 26]. Here, we identified 16 novel Bla-g antigens (NBGA) that had IgE and/or IgG reactivity in Bla-g-sensitized serum, and re-identified additional antigens recently described by others [15, 27, 39]. These NGBA induced T cell responses in 43% of Bla-g-sensitized subjects, and the combined sets of antigens were recognized by more than 79% of subjects. As the

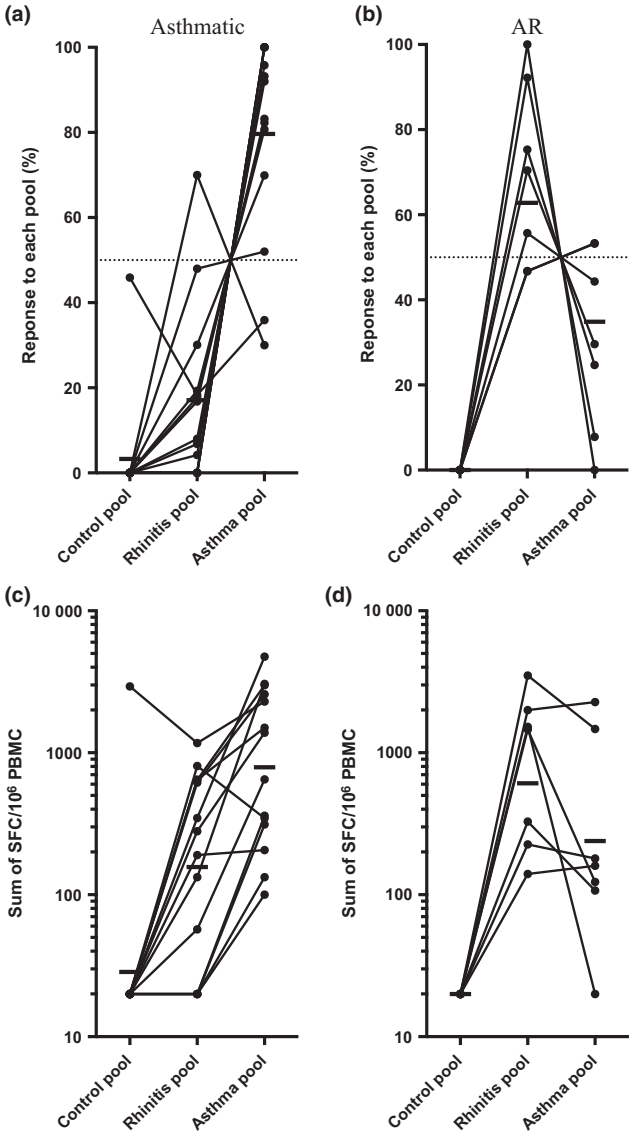

Fig. 7. Epitope set reactivity as a function of asthma status. Response to epitope set as a percentage of total response for (a) asthmatic and (b) AR subjects after stimulation with epitope pools following culture with Bla-g extract and corresponding magnitudes of response (in SFC per 10^6 PBMC) to each pool (c, d). Bars indicate median values (a, b) or geometric means (c, d). Each symbol represents the response from an individual subject.

proteomic approach was successfully applied to identification of both TG and Bla-g allergens, it is reasonable to assume that it might be applicable to other allergy systems as well.

In contrast to what we detected for TG allergy, the non-IgE-reactive antigens in Bla-g extract had negligible T cell activity in all $CD4^+$ subsets, suggesting a strong link between IgE and T cell activity in Bla-g allergy. It is not apparent why different allergic species would have different patterns in T cell:IgE linkage at the antigen level. One possibility is that the T cell responses to TG epitopes from antigens that are not targeted by IgE are actually cross-reactive T cell responses specific for antigens from other pollen species that are targeted by IgE. Indeed, we found that many TG epitopes show high cross-reactivity to other grass pollens (Westernberg et al., submitted). For Bla-g antigens, a lesser degree of exposure to homologous antigens from other species may explain the stronger T cell:IgE linkage observed.

While the data support the notion that the antigens recognized by IgE and T cell responses are largely overlapping, IgE reactivity is not predictive of which antigens will be immunodominant at the level of T cell responses. We observed that the Bla-g 2 allergen, known to be a dominant antigen in terms of IgE responses, accounted for <4% of the total $CD4^+$ T cell response to the Bla-g antigens. Conversely, allergens less dominant at the IgE level, such as Bla-g 9, Bla-g 4 and NBGA1, were immunodominant for T cell responses and accounted cumulatively for more than 75% of the response. Similar observations were previously reported [19, 34]. Along the same lines, the NBGA5 antigen was recognized at the T cell level, despite not being targeted by IgE responses. Overall, the data suggest that specific allergen immunotherapy approaches aimed at modulating T cell responses might not need to target the most dominant IgE-binding proteins. Indeed, targeting the most dominant T cell antigens that are less prominent in terms of IgE binding might offer an effective and safer immunotherapeutic approach.

In terms of functionality and magnitude of T cell responses, as expected, in the sensitized donors, the majority of the response was polarized to the T_H2 subset, but significant responses were also detected for other cytokines. Furthermore, in contrast to recent reports on the correlation between T_H17 responses and asthma [26], the relative contribution of IL-17 response to Bla-g antigens was equivalent between AR and asthmatic groups. The magnitude of responses was progressively increased in AR subjects, IA, and MMA. Responses were somewhat lower in the SA group, perhaps reflective of the immunosuppressive nature of medications they received. The functionality of the specific T helper response in asthma and rhinitis could be analyzed through the use of epitope-loaded tetramers; however, the process is not straightforward, given that the large heterogeneity of epitopes recognized would dictate the generation of a large number of tetramers and that the restriction of the epitopes has not yet been determined. The present study required screening the reactivity of 92 donors for 1688 peptides and 5 cytokines. Therefore, the ELISPOT assay was utilized because of its amenability to high-throughput analysis of large numbers of samples. The identification of the specific epitopes described herein will enable additional

experiments using intracellular staining for subset-specific transcription factors (e.g., GATA-3, T-bet, FoxP3, ROR-γt), which would be useful to further assess the presence of T_H2, T_H1, T_{reg} and T_H17 cells, respectively, and further characterize the phenotype of the responding T_H subsets.

Accordingly, more in-depth characterization will have to be addressed in future studies utilizing selected epitopes. The present study was focused on the complementary approach, namely the comprehensive definition of the epitopes and antigens recognized.

We utilized the observed preferences in antigen recognition associated with clinical status to develop epitope sets that differentiate between rhinitis and asthmatic Bla-g-sensitized subjects. These epitopes could be used to isolate the corresponding allergen-specific cells and examine whether differences exist at the level of their transcriptional or epigenetic profiles, or whether changes in the associated signatures might precede or follow the evolution of allergic disease, asthma remission or exacerbation. While further validation and optimization is necessary, it is possible to speculate that these epitope sets may have diagnostic value in a standardized laboratory test for asthmatic status.

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Conflict of interest

The authors have no conflict of interest to declare.

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Supporting Information

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

Figure S1. Cryopreservation of PBMC does not affect cytokine response to peptide stimulation.

Figure S2. No Significant correlation between IgE titres to Bla-g extract and T cell responses.

Tables S1. Summary of subject cohorts

Table S2. Demographic and clinical information.

Table S3. Novel Bla-g proteins discovered in proteomic screen.

Table S4. Bla-g Peptides included in epitope screen.

Table S5. Top 164 Bla-g T cell epitopes.

Table S6. Bla-g-sensitivity and asthma classification by epitope sets.