Allergic Sensitization Underlies Hyperreactive Antigen-Specific CD4+ T Cell Responses in Coincident Filarial Infection


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Allergic Sensitization Underlies Hyperreactive Antigen-Specific CD4+ T Cell Responses in Coincident Filarial Infection

Pedro H. Gazzinelli-Guimarães,*,† Sandra Bonne-Année,*, Ricardo T. Fujiwara,† Helton C. Santiago,‡ and Thomas B. Nutman*

Among the various hypotheses put forward to explain the modulatory influence of helminth infection on allergic effector responses in humans, the IL-10–induced suppression of Th2–associated responses has been the leading candidate. To explore this helminth/allergy interaction more fully, parasite- and allergen-specific CD4+ T cell responses in 12 subjects with filarial infections, and coincident allergic sensitization (atopic [FIL]+allergy [A]+) were compared with the responses to three appropriate control groups (FIL−A− [n = 13], FIL−A+ [n = 12], FIL+A− [n = 11]). The most important findings revealed that FIL+A+ had marked (p < 0.0001 for all cytokines) increases in parasite Ag-driven Th2 (IL-4, IL-5, IL-13), Th9 (IL-9), and the regulatory (IL-10) cytokines when compared with FIL+A−. Moreover, using multiparameter flow cytometry, filarial parasite Ag induced a marked increase in not only the frequency of CD4+ T cells producing IL-4, IL-5, IL-2, and TNF-α in FIL+A+ when compared with FIL+A− patients, but also in the frequencies of multifunctional Th2-like (CD4+IL-4+IL-5+ and CD4+IL-2+IL-4+IL-5+TNF-α+) cells. The Th2–associated responses seen in the FIL+A+ group were correlated with serum IgE levels (p < 0.01, r = 0.5165 for IL-4; p < 0.001, r = 0.5544 for IL-5; and p < 0.001, r = 0.4901 for IL-13) and levels of circulating eosinophils (p < 0.0116, r = 0.5656) and their degranulation/activation products (major basic protein [p < 0.001, r = 0.7353] and eosinophil-derived neurotoxin [p < 0.01, r = 0.7059]). CD4+ responses to allergen were not different (to a large extent) among the groups. Taken together, our data suggest that allergic sensitization coincident with filarial infection drives parasite Ag-specific T cell hyperresponsiveness, which is characterized largely by an augmented Th2–dominated immune response. The Journal of Immunology, 2016, 197: 2772–2779.
hyperresponsiveness, characterized largely by an augmented Th2-dominated immune response. These responses are associated with a marked elevation of IgE, eosinophilia, and eosinophil activation, with the latter potentially responsible for the associated clinical signs (angioedema, urticaria) often seen in these infections.

## Materials and Methods

### Study population

From a previously described cohort of 308 subjects (16, 20) used to identify crossreactive epitopes among parasite Ags and Aeroallergens, 49 individuals were included in the current study population based solely on the availability of sufficient numbers of cryopreserved PBMCs. The study population is described in Table I and consisted of four study groups based on the presence or absence of atopy and/or filarial infection: group 1, filarial (Fil) allergy (A)* (n = 12), group 2, Fil+A* (n = 11), group 3, Fil- A* (n = 13), and group 4, Fil- A* (n = 13).

All individuals (and all samples collected) were part of registered protocols approved by the Institutional Review Boards of the National Institute of Allergy and Infectious Diseases (NCT00001230 and NCT00001345) for the filarial-infected subjects and of the Department of Transfusion Medicine, Clinical Center, National Institutes of Health (IRB 99-CC-0168) for the healthy donors. Written informed consent was obtained from all subjects.

### In vitro culture

For the analysis of cytokine production by PBMCs after in vitro stimulation by parasite Ag and environmental allergens, aliquots of cell suspensions in RPMI 1640 medium supplemented with 10% human sera, 1% i-glutamine (Sigma-Aldrich), 1% antibiotic (Invitrogen), and 1% nonessentials amino acids (Sigma-Aldrich) were cultured in media alone, with crude extract from Brugia malayi adult worms (BMA; 10 µg/ml), with dust mite (Dermatophagoides pteronyssinus [Der p]) extract (Alk-Abello, Port Washington, NY; Der p extract at 15 allergen-specific units), or with PMA/ ionomycin (P/I; Sigma-Aldrich) (0.5/0.05 pg/ml) for 72 h in 5% CO2 at 37°C for the measurement of cytokine production. To identify the source of these cytokines by flow cytometry, PBMCs of all patient groups were cultured and stimulated under the same conditions as mentioned above, but for 12 h, with the addition of CD28/CD45d costimulatory molecules (BD FastImmune; BD Biosciences) and 10 µg/ml brefeldin A/ monensin (Sigma-Aldrich).

### LumineX assay

The quantification of IL-2, IL-4, IL-5, IL-9, IL-10, IL-17A, IL-22, TNF-α, and IFN-γ levels was assessed by a LumineX multiplex assay (Milliplex; Millipore) in accordance with the manufacturer’s recommendations. The eosinophil granule proteins were measured using a previously described assay (21).

### Multiparameter flow cytometry

To identify the source of the Th1/Th2/Th9/Th17-associated and regulatory cytokines, cells from in vitro cultures were harvested and washed with FACS buffer (PBS, 2% FBS, and 0.1% sodium azide) and then incubated with Fc blocking solution for 20 min. The cells were then stained for viability (Live/Dead fixable blue, Molecular Probes), washed with FACS buffer, and then incubated with fluorescein-conjugated anti-CD3, anti-CD4, and anti-CD8 for 30 min in the dark at room temperature. The cells were next washed twice with FACS buffer, then fixed and permeabilized using a Fix/Perm buffer kit (BioLegend) for 20 min in the dark at 4°C. The cells were washed twice with Perm buffer (BioLegend) and re suspended with the fluorochrome-conjugated anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-10, anti-IL-17A, anti-TNF-α, anti-IFN-γ, and anti-CD25 pool (Supplemental Table I) for 30 min in the dark at 4°C. Finally, the cells were washed twice with Perm buffer and then acquired using the BD LSRSFortessa flow cytometer (BD Biosciences) and FACSDiva software (BD Biosciences) for acquisition. All analyses were performed using FlowJo v10.8.0. For the flow cytometry multiparameter analyses, PESTLE/SPICE v5.0 (National Institute of Allergy and Infectious Diseases) software was used. Additionally, the frequency of CD4+ T cells producing single and multiple cytokines was analyzed by the FlowJo software based on the gating strategy shown in Supplemental Fig. 1.

### Data analysis

All statistical analyses were performed using Prism 5.0 for Windows (GraphPad Software). Unless stated otherwise, geometric means (GMs) and the upper 95% confidence interval of GM (between parentheses) were used as measures of central tendency. The D’Agostino and Pearson normality test was used to determine whether data sets were normally distributed. A Kruskal–Wallis test followed by Dunn multiple comparison tests were used to evaluate the statistical differences in the cytokine production by the different groups, as well as the frequency of CD4+ T cells producing cytokines. Correlations were performed using the Spearman rank correlation. Net production of cytokines (in picograms per milliliter) and net frequency of CD4+ T cell-producing cytokines (in percentage) were calculated by subtracting the baseline level from the level following stimulation. The differences were considered statistically significant when the corrected p values were < 0.05.

### Results

#### Study population

The clinical diagnoses, IgE (total and allergen-specific) levels, and peripheral eosinophil counts for the study population can be found in Table I. Peripheral blood eosinophil counts were greater in the Fil+A* patients compared with those filarial-infected patients without allergy (Fil+A*) (GM = 2269 [4449.0] versus 590 [1312.0] cells/µl; p = 0.0151). Interestingly, the Fil+A* patients also demonstrated a significant increase in the levels of IgE (GM = 1644 [3865] kU of allergen-specific Ags [kUA/l]) when compared with Fil- A* patients (GM = 122 [323.1] kUA/l; p < 0.05). Fil- A* individuals (GM = 84.0 [168.1] kUA/l; p < 0.05), and Fil-A* healthy donors (GM = 9.0 [19.74] kUA/l; p < 0.001).

Environmental allergy induces a hyperresponsiveness in the concomitant immune response to filarial infection mediated by parasite Ags

To elucidate the influence of atopy on the parasite- and allergen-induced immune responses, we first assessed cytokine production. As shown in Fig. 1, when spontaneous production of the various Th2-associated cytokines was measured (in the absence of a stimulus), there were no differences among the four groups in any of the cytokines measured (Fig. 1A–E). In marked contrast, when stimulated with the filarial parasite Ag BMA, those filarial-infected individuals with concomitant atopy (Fil+A*) had marked and significant increases in the levels of IL-5 (GM = 232.1 [504.3] versus 4.9 [38.34] pg/ml; p < 0.05), IL-13 (GM = 826.9 [1732] versus 14.9 [175.6] pg/ml; p < 0.05), IL-9 (GM = 83.1 [178.8] versus 2.8 [24.69] pg/ml; p < 0.05), and IL-10 (GM = 36.1 [65.45] versus 9.6 [52.82] pg/ml; p < 0.05) when compared with Fil-A*. These differences were even more pronounced when the IL-4 (GM = 0.1 [0.24] pg/ml, p < 0.01 and GM = 0.1 [0.21] pg/ml, p < 0.01), IL-5 (GM = 0.7 [2.98] pg/ml, p < 0.01 and GM = 0.7 [2.89] pg/ml, p < 0.001), IL-13 (GM = 6.3 [35.66] pg/ml, p < 0.001 and GM = 4.2 [32.76] pg/ml, p < 0.001), IL-9 (GM = 0.6 [1.99] pg/ml, p < 0.01 and GM = 0.5 [1.82] pg/ml, p < 0.01), and IL-10 (GM = 0.5 [1.56] pg/ml, p < 0.01 and GM = 0.2 [0.72] pg/ml, p < 0.001) responses were compared with Fil-A*. These differences were no different when comparing Fil-A* and Fil- A* respectively Fig. 1F–J).

No relevant differences were observed among the groups in the cytokine production after Der p allergen stimulation (Fig. 1K–O). Cytokine responses following P/I stimulation showed that Fil-A* had a marked increase in the levels of IL-4 (p < 0.01), IL-5 (p < 0.001), and IL-9 (p < 0.001) when compared with Fil-A* (Fig. 1P, 1Q, 1S). When levels of IL-13 were evaluated, the Fil-A* had levels of IL-13 that were strikingly more pronounced than those seen in comparison with Fil-A* (p < 0.05) and Fil-A* (p < 0.001) (Fig. 1R). No differences were seen among the groups for P/I-driven IL-10. The analysis of spontaneous production and BMA, Der p allergen, and P/I-driven type 1– and type 17–associated cytokines showed no differences in the production of IL-2, TNF-α, IFN-γ, IL-17, and IL-22 production among the four groups (Supplemental Fig. 2).
When the frequencies of cytokine-producing CD4+ T cells were assessed ex vivo and in response to antigenic and mitogenic stimuli, a unique pattern of immune response was seen in those filarial-infected individuals with concomitant atopy (Fil+A+). In the ex vivo analyses, there were no significant differences among the four groups in the frequency of T cells producing a given cytokine in the absence of a stimulus (Supplemental Fig. 3). However, when stimulated with the filarial parasite Ag BMA, those in the Fil+A+ group had a marked increase in the per cell surface expression of CD4+IL-2+TNF-α+ cells, CD4+IL-4+IL-5+ TNF-α+ cells, and CD4+IL-2+ cells. However, in response to the filarial Ag BMA, those in the Fil+A+ group had a marked increase in the

### Table I. Description of the study population

<table>
<thead>
<tr>
<th>Group</th>
<th>ID</th>
<th>Diagnosis</th>
<th>Phadiatop (kUA/L)</th>
<th>Der p–Specific IgE (kUA/L)</th>
<th>Total IgE (kUA/L)</th>
<th>Eosinophils (cells/mm³)</th>
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<tr>
<td>Fil+A+</td>
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<td>Loa loa</td>
<td>22.5</td>
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<td>497.0</td>
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<td>755.0</td>
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<td>38.5</td>
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<td>3,267</td>
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<td>141</td>
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<td>88</td>
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<td>960</td>
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<td>2.6</td>
<td>107.0</td>
<td>—</td>
</tr>
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<td>8.7</td>
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<td>5.7</td>
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</table>

*aAllergen-specific IgE levels >0.35 kUA/L were considered allergic/atopic.*
frequency of CD4⁺IL-4⁺IL-5⁺ T cells (GM = 0.053 [0.01]%), when compared with Fil+A² (GM = 0.004 [0.01]%, p < 0.05), Fil A⁺ (GM = 0.003 [0.007]%, p < 0.001), and Fil A⁻ (GM = 0.001 [0.001]%, p < 0.001) (Fig. 3E). Interestingly, the Fil A⁺ group also had a marked increase in the frequency of the relatively infrequent polyfunctional CD4⁺ subset CD4⁺IL-2⁺IL-4⁺IL-5⁺TNF-α⁺ when
compared with Fil−A− patients (GM = 0.018 [0.043] versus 0.002 [0.004]%; p < 0.05) (Fig. 3F). There were no differences among the groups when multiparameter flow cytometric analyses were examined in response to dust mite extract.

The polarized Th2 response mediated by filarial parasite Ag may be responsible for sustaining the elevated levels of blood eosinophils and IgE in the Fil−A+ patients

To explore the relationship between the parasite-induced T cell responses and the regulation of eosinophilia, eosinophil activation, and IgE, we examined the interrelationships among these various parameters (Fig. 4). As shown in Fig. 4A, the Fil−A+ group had a distinctly increased number of circulating peripheral eosinophils compared with Fil−A− patients (p < 0.05), and those cells were strongly and positively correlated with the IL-5 levels driven by BMA Ag (p = 0.005 and r = 0.560) (Fig. 4B). Although increases in the number of peripheral blood eosinophils are often seen in helminth infections (and some allergic disorders as well), it is the activation of these eosinophils that often drives the eosinophil-mediated pathology, activation that is reflected in the serum levels of eosinophil-specific granule proteins (major basic protein, eosinophil-derived neurotoxin, eosinophil cationic protein, and eosinophil peroxidase). As shown in Fig. 4C and 4D, there was an increase in the levels of major basic protein (p < 0.01 and p < 0.01; Fig. 4C) and eosinophil-derived neurotoxin (p < 0.0001 and p < 0.001; Fig. 4D) in the Fil−A+ patients compared with Fil−A− and Fil−A− groups, respectively. There was also a clear relationship between the levels of these granule proteins and the number of circulating eosinophils (p = 0.0017 and r = 0.7353; p = 0.0030 and r = 0.7059; Fig. 4E). Not unlike what was seen for eosinophil levels, Fil−A+ individuals demonstrated significantly increased levels of IgE when compared with Fil−A− (p < 0.05), Fil−A+ (p < 0.05), and Fil−A− (p < 0.001) groups. Moreover, the IgE was shown to be strongly correlated with the levels of IL-4 (p = 0.0002 and r = 0.5165; Fig. 4G), IL-5 (p < 0.001 and r = 0.5544; Fig. 4H), and IL-13 (p < 0.0004 and r = 0.4901; Fig. 4I) driven by BMA.

Discussion

Put into the context of the hygiene hypothesis, it is thought that the lack of exposure by children early in their development to microbes/parasites (as seen in most high- and middle-income countries) may explain the increased incidence of allergic diseases (6, 22) seen in these same countries, providing a causal link between helminth infection and protection from allergy. However, a wide range of studies in humans and model systems have provided substantive but conflicting evidence of the relationship between helminths and allergy, with different studies providing evidence of a positive association (23, 24), a negative association (25), or no association at all (26). This lack of consensus likely reflects real differences among the studies if the modulatory effects of helminth infections on allergic reactivity differ either because of species differences among particular helminths or because of differences in the timing of parasite infection in relationship to immune maturation or sensitization (27).

Although initial exposures to helminths may be associated with enhanced allergic inflammatory responses to the parasite, in long-term infections and with repeated infections, the host inflammatory response becomes more tightly controlled (28, 29). Chronic helminth infections induce potent immunoregulatory pathways (30–32), such as immunosuppressive cytokines (e.g., IL-10 or TGF-β) (33, 34) or Treg populations (35, 36) that may facilitate parasite survival. This regulation, however, may not just affect responses to parasite Ags but also to bystander Ags and aeroallergens. Such helminth-associated regulatory effects may contribute to the decreased prevalence of allergic diseases (29, 37) reported from the rural tropics.

The present study was designed to understand the mechanisms underlying the parasite-driven CD4+ T cell response and the effect of coincident allergic sensitization on this process. The most important findings revealed that filarial-infected patients with coincident atopy had marked increases in parasite Ag-driven Th2
(IL-4, IL-5, IL-13) and Th9 (IL-9) cytokines and adaptive Tregs (IL-10) when compared with filarial-infected but nonallergic patients. Moreover, filarial parasite Ag induced a marked increase in the frequency of CD4+ T cells producing IL-4, IL-5, IL-2, and TNF-α in Fil’A+ when compared with Fil’A- patients, as well as a dominant Th2 cell expansion, characterized by a marked increase in the frequencies of polyfunctional (CD4+IL-4+IL-5+ and CD4+IL-2+IL-4+IL-5+TNF-α+) cells in the Fil’A+ group when

FIGURE 3. Flow cytometry multiparameter analysis highlighting the increased frequency of polyfunctional CD4+ T cell subsets. Frequency of CD4+ T lymphocytes producing multiple Th1 (A, D, G, and J), Th2 (B, E, H, and K), and mixed Th1/Th2 (C, F, I, and L) at baseline (A, B, and C) and after stimulation with the filarial Ag (BMA) (D, E, and F), Der p EXT (G, H, and I), and P/I (J, K, and L) is shown. Each dot represents a single individual, and the horizontal bars are GMs.

FIGURE 4. Eosinophilia and IgE regulation in the four groups. Eosinophils counts (A) and their association with BMA-specific IL-5 (B), eosinophil granule proteins (C and D) and their correlation with eosinophils (E), and IgE levels (F) and their association with BMA-specific Th2 cytokines (G-I) are shown. Each dot represents a single individual, and the horizontal bars are GMs. EDN, eosinophil-derived neurotoxin; MBP, major basic protein.
tricuridae. This accentuated immune responsiveness may either lead to or coexist with filarial infections (9–11). The ability of the Tregs to suppress immune-mediated pathology (38) was suggested in studies in mice infected with the intestinal nematode Heligmosomoides polygyrus, in which this parasite induced an expansion of natural Tregs (39–42) that prevented allergen-induced pathology (43, 44).

However, the present study suggests that on a background of allergic sensitization, immune responses to parasite Ags may actually be accentuated in patients with relatively acute filarial infections. This accentuated immune responsiveness may either lead to or coexist with filarial infection (59, 60). Ascaris lumbricoides is the predominant intestineuklein-10-producing cells in the circulation of filaria-infected patients. J. Infect. Dis. 197: 94–101.


Disclosures

The authors have no financial conflicts of interest.

References


