Synchronous immune alterations mirror clinical response during allergen immunotherapy

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Background: Three years of treatment with either sublingual or subcutaneous allergen immunotherapy has been shown to be effective and to induce long-term tolerance. The Gauging Response in Allergic Rhinitis to Sublingual and Subcutaneous Immunotherapy (GRASS) trial demonstrated that 2 years of treatment through either route was effective in suppressing the response to nasal allergen challenge, although it was insufficient for inhibition 1 year after discontinuation.

Objective: We sought to examine in the GRASS trial the time course of immunologic changes during 2 years of sublingual and subcutaneous immunotherapy and for 1 year after treatment discontinuation.

Methods: We performed multimodal immunomonitoring to assess allergen-specific CD4 T-cell properties in parallel with analysis of local mucosal cytokine responses induced by nasal allergen exposure and humoral immune responses that included IgE-dependent basophil activation and measurement of serum inhibitory activity for allergen-IgE binding to B cells (IgE-facilitated allergen binding).

Results: All 3 of these distinct arms of the immune response displayed significant and coordinate alterations during 2 years of allergen desensitization, followed by reversal at 3 years, reflecting a lack of a durable immunologic effect. Although frequencies of antigen-specific T_H2 cells in peripheral blood determined by using HLA class II tetramer analysis most closely paralleled clinical outcomes, IgE antibody–dependent functional assays remained inhibited in part 1 year after discontinuation.

Conclusion: Two years of allergen immunotherapy were effective but insufficient for long-term tolerance. Allergen-specific T_H2 cells most closely paralleled the transient clinical outcome, and it is likely that recurrence of the T-cell drivers of allergic immunity abrogated the potential for durable tolerance. On the other hand, the persistence of IgE blocking antibody 1 year after discontinuation might be an early indicator of a protolerogenic mechanism. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *Allergy, immunotherapy, immune tolerance, allergen desensitization, T_H2 cells*

Allergen immunotherapy is an effective treatment option for patients with allergic rhinitis who do not respond adequately to the usual antihistamine and topical corticosteroid medications.[1] Subcutaneous immunotherapy involves weekly administration of incremental doses of allergenic material by means of injection, followed by monthly maintenance injections for several years.[2-4] Immunotherapy has been associated with overall changes in T-cell function with cytokine changes that suggest a shift from T_H2 cells toward T_H1 phenotypes or induction of regulatory T cells.[5,6] These alterations are accompanied by decreases in recruitment, activation, or both of allergic effector cells, including mast cells, eosinophils, and basophils, in target organs.[7,8] Measurement of serum immunoglobulins directed against the allergen in such immunotherapy studies indicates that specific IgG, particularly of the IgG_4 subclass, can be induced by therapy and is presumed to be mechanistically linked to clinical benefit by virtue of competitive inhibition of allergic responses triggered by specific IgE directed to the same allergens.[9-12] Alternative routes of allergen administration for immunotherapy are now under active investigation, including the sublingual[13-15] and epicutaneous[16,17] routes. For food allergens, the oral route has also shown promising results.[18,19] Because immunologic properties at each of these sites differ, the mechanisms through which these forms of allergen immunotherapy exert their therapeutic effects can differ as well.

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Research reported in this publication was sponsored by the Immune Tolerance Network and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (NIH) under award no. UM1AI109565 and NIH grant R01 AI095074. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Disclosure of potential conflict of interest: A. Renand receives grant support from the National Institutes of Health (NIH). M. H. Shamji serves as a consultant for Imperial College London and receives lecture fees from ALK-Abelló, ASIT Biotech, Allergopharma, and UCB. K. M. Harris receives grant support from the National Institute of Allergy and Infectious Diseases (NIAID)/NIH. E. Wambre receives grant support from the Immune Tolerance Network and NIH and is an employee of Benaroya Research Institute. P. A. Wurtzen is an employee of ALK-Abelló and hold stocks with ALK-Abelló. S. J. Till serves as a consultant for ALK-Abelló and Aimmune and receives grant support from Astra Zeneca and ASIT Biotech. G. T. Nepom receives grant support from NIH. W. W. Kwok receives grant support from the NIH. S. R. Durham receives grant support from the Immune Tolerance Network, NIAID, ALK-Abelló, Hørsholm Regeneron, and Biotech Tools and serves as a consultant from Anergis, Circassia, Biomay, Merck, Allergy Therapeutics, Med Update GmbH, and Food Standards. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication June 8, 2017; revised August 25, 2017; accepted for publication September 18, 2017.

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0091-6749/$36.00

https://doi.org/10.1016/j.jaci.2017.09.041

Abbreviations used
CRTH2: Chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes
FAB: Facilitated allergen binding
GRASS: Gauging Response in Allergic Rhinitis to Sublingual and Subcutaneous Immunotherapy
PE: Phycoerythrin
PP: Per-protocol

The Gauging Response in Allergic Rhinitis to Sublingual and Subcutaneous Immunotherapy (GRASS) clinical trial was a randomized, placebo-controlled, double-blind study of 106 adults with a clinical history of moderate-to-severe seasonal allergic rhinitis caused by grass pollen. Study participants received 2 years of subcutaneous immunotherapy, sublingual immunotherapy, or placebo and were extensively studied over 3 years for clinical and immunologic parameters of response.[20] Clinical assessments in this trial were reported recently, demonstrating successful suppression of the nasal response to allergen challenge after 2 years of therapy through both the subcutaneous and sublingual routes, with lack of sustained benefit in the subsequent untreated third year.[20] We now report immunologic findings from this trial, including peripheral blood cellular and humoral assessments, as well as local tissue responses to allergen: evaluation of antigen-specific $CD4^+$ T cells in peripheral blood, functional outcomes from changes in the humoral response detected in serum and peripheral IgE-dependent basophil assays, and cytokine responses to allergen challenge in the nasal mucosa.

METHODS

Sample collection

Clinical characteristics of the subjects in the GRASS trial and details of the protocol have been reported previously.[20] Subcutaneous alum-adsorbed grass pollen immunotherapy (Alutard SQ Grass Pollen; ALK-Abelló, Hørsholm, Denmark) or matched placebo subcutaneous injections were given weekly for 15 weeks, followed by monthly maintenance injections until 2 years. Freeze-dried grass pollen *(Phleum pratense)* sublingual tablets (Grazax; ALK-Abelló) or matched placebo sublingual tablets were self-administered daily for 2 years. Timothy grass–specific IgE and specific IgG_4 levels were quantified by using the CAP FEIA system (Phadia, Uppsala, Sweden). Peripheral blood lymphocytes were collected and prepared for cryopreservation, as previously described.[20] Coded samples were provided to the operator.

Tetramer assays and flow cytometric analysis

Timothy grass–specific $CD4^+$ T-cell epitopes were identified by using tetramer-guided epitope mapping.[21,22] Epitope-specific pMHC tetramer reagents were generated by loading specific peptides onto biotinylated soluble DR monomers and subsequently conjugated with phycoerythrin (PE)–streptavidin.[23] These included HLA-DR04:01, HLA-DR03:01, HLA-DR04:01, HLA-DR07:01, HLA-DR10:01, and HLA-DR11:01 tetramer reagents. For *ex vivo* tetramer staining, 20 to 40 million frozen PBMCs from subjects with HLA genotypes corresponding to these tetramers were thawed and resuspended in 200 μL of T-cell culture medium and, to enhance tetramer staining, were treated with dasatinib (Sigma-Aldrich, St Louis, Mo) for 10 minutes at 37°C before tetramer staining.[24] PE-labeled pooled tetramers were then added to a final concentration of 20 μg/mL, and staining was carried out for 100 minutes at room temperature. A 1/100 fraction of the cells was saved, and the rest of the PE tetramer–positive cells were then enriched by using the anti-PE bead enrichment protocol through a magnetic column, according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany).[22,25] Cells in both the enriched fraction and the precolumn fraction were stained with a panel of antibodies of interest, including CD14 (HCD14; BioLegend, San Diego, Calif), CD19 (HIB19; BioLegend), CD45RA (HI100; BD Biosciences, San Jose, Calif), CD4 (RPA-T4; BD Biosciences), chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes (CRTH2; BM16; BD Biosciences), CD161 (PK136; BioLegend), and CD27 (O323; BioLegend) and were treated further with BD Via-Probes (BD Biosciences) before flow cytometry. Frequencies of tetramer-positive cells were calculated by using the formula n/N, where *n* is the number of tetramer-positive cells in the enriched fraction, and *N* is the total number of cells in the sample, which can be calculated by counting the number of cells in the precolumn fraction and multiplying by 100. The efficiency of recovery was optimized by using less than 30 million cells as starting material on samples with less than 300 tetramer-positive cells per million, capturing greater than 95% of the PE tetramer–stained populations.

Isolation of grass pollen allergen–reactive T cells with the CD154 upregulation assay

Global grass pollen–reactive $CD4^+$ T cells were tracked by using the CD154 assay.[26,27] Briefly, frozen/thawed PBMCs were cultured at a density of 10^6/mL with 1 μL/mL Timothy grass pollen crude extract and 1 μg/mL anti-CD40 blocking mAb (HB14; Miltenyi Biotec). After 18 hours of stimulation at 37°C, cells were harvested and labeled with PE-conjugated anti-CD154 mAb for 10 minutes at 4°C. Cells were then washed, labeled with anti-PE magnetic beads, and enriched by using a magnetic column, according to the manufacturer's instructions (Miltenyi Biotec). Magnetically enriched cells were next stained with antibodies against markers of interest and analyzed on a FACSAria II flow cytometer (BD Biosciences). Live memory $CD45RO^+CD154^+CD4^+$ T cells were sort purified for subsequent transcript analysis.

Real-time PCR expression analysis

The Fluidigm BioMark 96.96 Dynamic Array (Fluidigm, South San Francisco, Calif)[28] was used to measure gene expression in small cell populations. Ten cells per well were sorted by using FACS in quadruplicate in 96-well plates containing a reaction mix for reverse transcription (CellsDirect One-Step qRT-PCR kit; Invitrogen, Carlsbad, Calif) and preamplification with 96 selected gene primer pairs (DELTAgene assays; Fluidigm). After sorting, samples were reverse transcribed and preamplified for 18 cycles. Primers and dNTPs were removed by means of incubation with Exonuclease I (New England Biolabs, Ipswich, Mass), and samples were diluted (5×) with TE buffer and stored at −20°C. Samples and assays (primer pairs) were prepared for loading onto 96.96 Fluidigm Dynamic arrays, according to the manufacturer's recommendations. The 96.96 Fluidigm Dynamic Arrays were primed and loaded on an IFC Controller HX (Fluidigm), and real-time PCR was run on a BiomarkHD (Fluidigm). Data were collected and analyzed by using Fluidigm Real-Time PCR Analysis software (version 4.1.2).

Measurement of nasal cytokines

Nasal challenge was performed with Aquagen (ALK-Abelló) *Phleum Pratense* (Timothy grass) extract, as described previously.[20] The challenge dose was determined according to a dose-titration challenge at screening. The same dose was then used at the baseline (pretreatment) nasal challenge visit and at each subsequent challenge visit. The dose range was 1500 BU/mL (equivalent to 1.0 μg/mL major allergen) to 30,000 BU/mL (equivalent to 20.2 μg/mL major allergen).

Nasal secretions were collected by using synthetic polyurethane sponges precut to 20 × 15 × 15 mm (RG 27 grau; Gummi-Welz GmbH, Neu-Ulm, Germany) and sterilized by means of autoclaving. A single sponge was inserted into each of the participant's nostrils posterior to the mucocutaneous junction by a study physician under direct vision by using croc forceps and a nasal speculum (Phoenix Surgical Instruments, Hertfordshire, United Kingdom). Sponges were left in place for 2 minutes before removal and then added to 2-mL centrifuge tubes with indwelling 0.22-μm cellulose acetate filters (Costar Spin-X; Corning, Corning, NY). Tubes were kept briefly on ice before being centrifuged. At baseline, sponges were centrifuged "neat" without adding an elution buffer. At years 2 and 3, 75 μL of elution buffer