

ORIGINAL ARTICLE

Allergen-specific T cell responses to immunotherapy monitored by CD154 and intracellular cytokine expression

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Summary

Background A sensitive measurement of low numbers of intracellular cytokine-expressing antigen-specific T cells from peripheral blood mononuclear cells (PBMC) is possible using CD154 as a marker of recently activated T cells. This technique may have potential for monitoring peripheral blood T cell responses to immunotherapy.

Objective To evaluate the applicability of this method for measuring changes in cytokine production by allergen-specific T cells in a clinical trial setting.

Methods *Ex vivo* ragweed-specific CD154 and intracellular cytokine expression were evaluated using a subset of subjects in an environmental chamber study of allergic rhinitis immunotherapy. PBMC were collected and cryopreserved from Amb a 1-immunostimulatory oligodeoxynucleotide conjugate (AIC)-treated ($n = 17$) and placebo-treated ($n = 15$) ragweed-allergic subjects both after pre- and post-treatment ragweed exposures. *In vitro* allergen-stimulated CD3⁺CD4⁺CD154⁺ T cell intracellular IL-4, IL-5, IL-13, and IFN- γ expression were evaluated by flow cytometry.

Results Compared with the T helper type 2 (Th2) cytokine expression measured after pre-treatment ragweed exposures, placebo-treated subjects demonstrated a significantly elevated ragweed- and Amb a 1-specific T cell IL-4 and IL-13 co-expression ($P = 0.005$ and $P = 0.022$, respectively) and a significantly elevated ragweed-specific IL-5 expression ($P < 0.001$) following post-treatment ragweed exposures. In contrast, AIC-treated subjects demonstrated no increases in allergen-specific Th2 cytokine expression following post-treatment ragweed exposures. IFN- γ expression remained low and un-changed in both groups. Subject reported total nasal symptom scores demonstrated modest but significant correlations with Amb a 1- and ragweed-stimulated intracellular Th2 cytokine responses. **Conclusion** Combined CD154 and intracellular cytokine staining in PBMC can be used to sensitively monitor changes in antigen-specific T cell subset frequencies in clinical studies. Antigen-specific cytokine expression moderately correlated with the reported levels of allergic symptoms.

Trial Registration NCT00537355

Keywords: CD154, cytokines, humans, immunostimulatory DNA sequences, ragweed, seasonal allergic rhinitis

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Introduction

Sensitive and reproducible monitoring of allergen-specific T cell cytokine responses in immunotherapeutic treatment regimens that aim to alter the balance of antigen-specific CD4⁺ T cell subsets has been challenging because these cells occur at a very low frequency in the peripheral circulation in most patients. *Ex vivo* cell culture

and ELISPOT methods can be sensitive but do not detect non-cytokine-producing antigen-specific T cells, and do not allow for further phenotypic characterization of T cells [1–4]. Major histocompatibility complex (MHC)-tetramer reagents are limited by the specificity for individual MHC alleles and are not available for most antigens [5, 6]. In contrast, a technique described recently for detecting antigen-activated CD4⁺ T cells based on transiently

increased expression of the activation marker CD154 (CD40 ligand) offers the advantage of evaluating a large repertoire of antigen specificities using mixes of antigens or peptides [7–9]. Detection of antigen-specific T cells by CD154 expression is independent of cytokine production, but may be combined with staining for intracellular cytokines permitting enumeration of functional T cell subsets. Increased CD154 expression has been shown to be a very specific marker for T cells activated *in vitro* by antigen in mouse, human, and non-human primates [7–11]. Although this approach represents a significant experimental advance, its utility in clinical studies of new therapeutic interventions has not been evaluated previously.

Ragweed-allergic rhinitis immunotherapy with the major ragweed allergen Amb a 1 conjugated to immunostimulatory oligodeoxynucleotide (AIC) represents a promising alternative to conventional allergen immunotherapy. Previous clinical studies of AIC have demonstrated a reduction in symptoms of allergic rhinitis, compared with the placebo, during the first and second ragweed seasons following 6 weekly treatments with AIC [12, 13]. AIC treatment resulted in a reduced frequency of ragweed-specific T helper type 2 (Th2) cells and a corresponding increase in T helper type 1 (Th1) cells in the nasal mucosa [12]. In a separate study, ELISA analysis of *ex vivo* cell culture supernatants demonstrated a lower ragweed-stimulated IL-5 production and an elevated IFN- γ production from peripheral blood mononuclear cells (PBMC) of AIC-treated subjects compared with placebo-treated subjects [14].

A minimally invasive technique for monitoring allergen-specific T cell responses during clinical studies of immunomodulatory therapies would significantly improve the optimization of dose, regimen, and duration for such therapies. However, concerns have been raised that blood may be depleted of allergen-specific T cells due to the migration of cells to mucosal sites after an allergen exposure and may not reflect the T cell subset balance in the airways [15]. To evaluate the potential for monitoring allergen-specific T cell subsets in blood by combining staining for CD154 and intracellular cytokine expression, we examined changes in the frequency of allergen-specific Th1 and Th2 cells in AIC- or placebo-treated subjects undergoing controlled ragweed pollen exposures in an environmental ragweed-exposure chamber study.

Materials and methods

In vitro antigen-specific peripheral blood mononuclear cells responses

PBMC were harvested from blood using sodium heparin Vacutainer CPT tubes (BD, Franklin Lakes, NJ, USA) and cryopreserved. Antigen-specific human T cell responses

can be retained in PBMC that have been cryopreserved and thawed, and these responses are comparable with those of freshly isolated PBMC [16, 17]. We used the optimized protocols described in detail by Disis *et al.* [17] for freezing and thawing human PBMC samples. In our study, PBMC were frozen in liquid nitrogen for up to 8 months and the cells demonstrated consistently >95% viability upon thawing (average 97.5% and 97% for pre- and post-treatment PBMC samples, respectively). For a comparative analysis of antigen-specific T cell responses, pre- and post-treatment PBMC for each individual subject were thawed and stimulated on the same day.

Duplicate 200 μ L of PBMC cultures at 5×10^6 cells/mL were incubated in round-bottomed 96-well plates with (a) no stimulus (medium only), (b) defatted-ragweed allergen extract (*Ambrosia artemisiifolia*; Greer Laboratories, Lenoir, NC, USA) at 15.6, 62.5, or 250 μ g/mL, (c) Amb a 1 at 30 or 90 μ g/mL, or (d) phytohaemagglutinin (PHA from *Phaseolus vulgaris*; Sigma, St Louis, MO, USA) at 5 μ g/mL. Anti-CD28 (BD Biosciences, San Jose, CA, USA) was added to all cultures at 1 μ g/mL as a co-stimulus. As a control for day-to-day assay variability, cytomegalovirus (CMV) antigen-specific T cell responses were examined daily using frozen aliquots of CMV-reactive PBMC from a single donor stimulated with CMV PepMix pp65 (2 μ g/mL; JPT Peptide Technologies, Berlin, Germany). PBMC were cultured for 6 h and Brefeldin A (Epicentre, Madison, WI, USA) was added at a final concentration of 5 μ g/mL for the last 4 h to allow intracellular accumulation of CD154 and cytokines. Meier *et al.* [18], as well as our own method evaluations, have shown that the cell density (5×10^6 cells/mL) and assay duration (6 h) conditions used in this study result in the optimal detection of CD154⁺ antigen-specific T cells.

Flow cytometry

After *in vitro* re-stimulation, PBMC were left in the original culture plates and stained with anti-human CD3 (PerCP-Cy5.5-conjugated) and anti-human CD4 (APC-Cy7), and then fixed in 2% paraformaldehyde (Sigma) for 30 min. The cells were washed with FACS buffer (phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide) and stored at 4 °C overnight. The following day, cells were permeabilized in 0.5% Saponin (Sigma) to facilitate the staining of intracellular CD154 and cytokines. Cells were stained with anti-human CD154 (FITC), and either anti-human IL-4 (APC) and anti-human IL-13 (PE) antibodies, or with anti-human IL-5 (APC) and anti-human IFN- γ (PE) antibodies. Isotype control antibodies demonstrated minimal background staining (data not shown). All antibodies were obtained from BD Biosciences. Duplicate samples were pooled and analysed using a BD LSR II flow cytometer using BD FACSDiva 6.1.1. CD154⁺ Cytokine⁺ responses were

examined on lymphocyte- and CD3⁺CD4⁺-gated cells. Approximately, 6×10^5 total events were collected per sample.

Subjects and clinical study design

Evaluation of ragweed-specific Th2 responses was performed using a subset of patients recruited to a randomized, double-blind study of AIC immunotherapy for ragweed, which was carried out using an environmental allergen-exposure chamber (EEC) (Trial Registration NCT00537355). This study was reviewed and approved by Institutional Review Board Services (Aurora, Ontario, Canada). Written informed consent was obtained from all study subjects. The study was carried out at Allied Research International sites in Mississauga and Montreal, Canada from September 2007 to March 2008.

Subjects meeting the inclusion criteria [ragweed clinical history, skin prick reactivity, and $\geq 50\%$ maximal total nasal symptom scores (TNSS)] were randomized to receive AIC ($n = 136$) or a placebo ($n = 135$). Selection of subjects for blood sampling was independent of randomization and was based solely on optional voluntary consent. Blood was collected at a single centre and a total of 17 AIC-treated subjects and 15 placebo-treated subjects donated PBMC for the analysis of antigen-specific intracellular cytokine responses. PBMC were cryopreserved on the day of collection. The study design is outlined in Fig. 1. Briefly, subjects were screened (clinical history and skin prick test) at visit 1. On visits 2–5, subjects were given daily allergen challenges via a controlled dose of ragweed pollen (average concentration of 3500 ± 500 pollen grains per m^3 for all visits in the study) for 4 h in the EEC. The first EEC ragweed challenge was given 2–4 weeks after the end of the ragweed season. Subjects meeting the inclusion criteria were enrolled and randomized. Pre-treatment PBMC were collected at visit 5 after the EEC session. Subjects were then given weekly subcutaneous injections of AIC (3.0, 9.0, 30, 30, 30, and 30 μg) or placebo during visits 5–10. Two post-treatment ragweed EEC challenges were administered at visits 11 and 12 (3 weeks after concluding the treatment regimen) and four

more daily ragweed EEC challenges were given 3 weeks later during visits 13–16. Post-treatment PBMC were collected at visit 16 after the EEC session. The study was completed before the start of the spring ragweed season. Overall, there were no safety concerns identified during the study.

AIC is a covalent conjugate of the purified major allergen of short ragweed (Amb a 1) and an immunostimulatory phosphorothioate oligonucleotide (1018 ISS). AIC was manufactured by Sigma and was formulated in a solution containing 20 mM histidine, 50 mM glycine, and 210 mM sucrose (pH 7.5). Histatrol[®] was given as placebo injections and was purchased from Western Allergy Services/Quorum Pharmaceuticals (Mississauga, Ontario, Canada).

The primary end-point of the AIC ragweed EEC study was defined as the difference in mean change from pre-treatment symptom score (PSS) between treatment groups in patient-rated instantaneous TNSS for the last hour in the EEC during visits 15 and 16. PSS was defined as patient-rated scores from the last hour in the EEC during visits 4 and 5. TNSS were calculated as the sum of the individual scores for congestion, rhinorrhoea, nose itchiness, and sneezing, equally weighted, and all assessed on a 0–3 rating scale (none, mild, moderate, and severe).

Statistics

The mean change from PSS patient-rated instantaneous TNSS was assessed using an analysis of covariance (ANCOVA) where PSS, TNSS, site, and treatment group (AIC or placebo) were treated as the covariates. Interactions between site and treatment, and PSS and treatment were ruled out. Pre- to post-treatment changes in the cytokine expression within AIC or placebo groups were analysed by Wilcoxon's signed-rank test. To compare pre- to post-treatment changes in the AIC-treated group to that in the placebo-treated group, the Mann-Whitney *U*-test was used. Significance of correlations was assessed using the Spearman's rank order correlation coefficient. Statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA, USA).

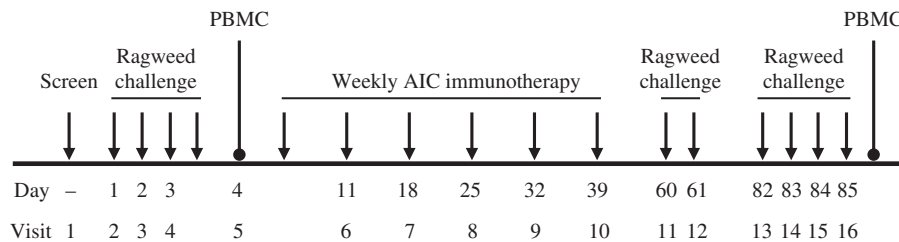


Fig. 1. Study design for ragweed Amb a 1-immunostimulatory oligodeoxynucleotide conjugate (AIC) immunotherapy. Qualifying subjects were screened for history and ragweed skin prick tested at visit 1. Subjects with total nasal symptom scores (TNSS) ≥ 6 were enrolled and randomized to AIC or placebo treatment at visit 5. Peripheral blood mononuclear cells (PBMC) were collected after a ragweed challenge but before treatment at visit 5, and again after the last ragweed challenge given post-treatment at visit 16.

Results

Detection of allergen-specific T cell responses in peripheral blood mononuclear cells from ragweed-allergic subjects

Measurement of allergen-specific T cell responses within PBMC has been challenging because of the low frequency of these cells in circulation. The recent demonstration that antigen-specific T cells can be directly identified *in vitro* by the stabilized expression of the activation marker CD154 provides a potentially powerful new tool [8]. Identification of antigen-activated CD154⁺ T cells using flow cytometry enables the direct detection of cells activated by a variety of antigenic epitopes (such as complex protein antigens or peptide pools) in a manner that permits the characterization of both surface and intracellular markers on these cells. We evaluated the applicability of this technique to monitoring allergen-specific T cell responses in a clinical trial of AIC immunotherapy for ragweed-allergic rhinitis (Fig. 1). We found that a 6 h *in vitro* re-stimulation with ragweed resulted in allergen-specific expression of CD154 and detectable intracellular IL-4, IL-5, or IL-13 expression in CD154⁺CD3⁺CD4⁺ T cells of over 90% of ragweed-challenged allergic donors (representative flow cytometry

plots shown in Fig. 2a). Allergen-specific IFN- γ was expressed at very low levels. Pilot experiments with CMV-peptide-reactive PBMC demonstrated that while antigen-specific CD154⁺ cytokine responses were evident as early as 3 h after stimulation (i.e. 1 h after the addition of Brefeldin A to the culture), a 6 h incubation was necessary to achieve an optimal intensity of CD154 expression in activated cells (data not shown). All ragweed allergen-stimulated cells positive for Th2 cytokines were part of the CD154⁺ population, and CD154⁺ Th2 cytokine responses were absent or at minimal background levels in allergic PBMC not re-stimulated *in vitro* (Fig. 2b) and in ragweed allergen-stimulated PBMC from ragweed skin prick-test-negative individuals (data not shown). These findings confirm the antigen-specific nature of the response. In preliminary experiments, we found that allergen-specific Th2 responses were comparable in fresh and frozen PBMC from the same individuals, and that responses were optimal in freshly thawed vs. thawed and overnight-rested cells. In addition, PHA-induced responses were similar in T cells from placebo- and AIC-treated groups (data not shown). Indeed, it has been demonstrated previously that antigen-specific and polyclonal T cell stimulation may result in a qualitatively different cytokine expression [19], highlighting the importance of evaluating antigen-specific responses.

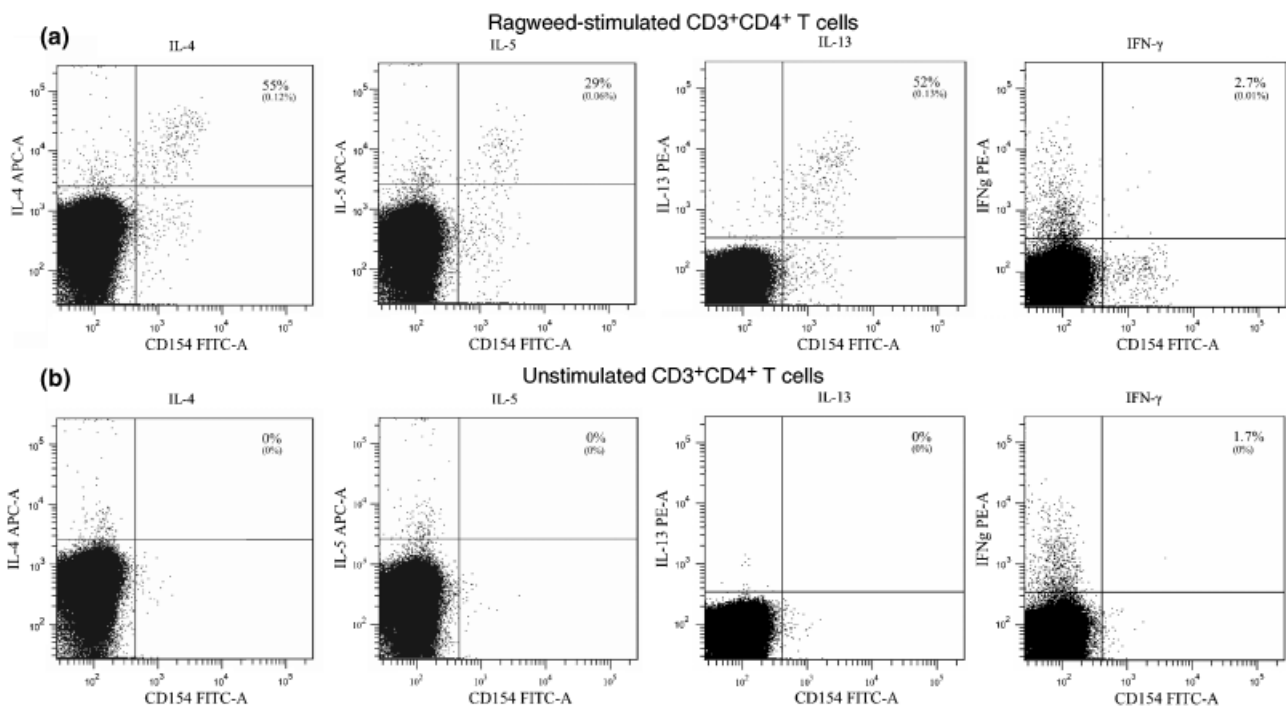


Fig. 2. Representative plots of CD3⁺CD4⁺-gated ragweed-specific CD154⁺ cytokine⁺ staining in T cells. Th2 cytokine responses were readily detectable within the CD154⁺ T cell population following ragweed (250 μ g/mL) activation of peripheral blood mononuclear cells (PBMC, row a). CD154⁺ cytokine⁺ T cell responses were absent in un-stimulated PBMC (row b). Numbers shown are cytokine⁺ cells as a percentage of the total antigen-specific (CD154⁺) population in the sample. Cytokine⁺ cells as a percentage of all CD3⁺CD4⁺ T cells in the sample are shown in brackets. Data from one representative subject are shown.

Assay sensitivity for the detection of ragweed antigen-specific T helper type 2 responses

The ability to simultaneously detect T cells reactive to all immunogenic epitopes in a complex antigen mix via the induction of CD154 expression is an important advantage when examining low-frequency responses. However, it is important to determine whether responses are of a sufficient magnitude for reproducible measurements. For all subjects in this study, the numbers of ragweed-allergen-specific T cells expressing IL-4 or IL-13 ranged from 0 up to approximately 175 events at pre-treatment (Fig. 3), representing up to 60% of the allergen-specific (i.e. CD154⁺) T cells. Compared with IL-4 and IL-13, there were fewer ragweed-specific IL-5⁺ T cells and very low numbers of ragweed-specific IFN- γ ⁺ T cells (Fig. 3). As allergen-specific T cells were limited, we examined repeated same day measures of CMV-antigen-specific IL-4 and IFN- γ T cell responses in PBMC from one individual. We found coefficients of variation (CVs) of 13% for IL-4 expression (approximately 20% of CMV-specific T cells were IL-4⁺) and 5% for IFN- γ expression (approximately 80% of CMV-specific T cells were IFN- γ ⁺) with CMV peptide stimulation. For day-to-day variability, the CVs for CMV-specific IL-4 and IFN- γ expression were 36% and 16%, respectively. Taken together, these data indicate that there is a window for identifying a treatment or an allergen challenge-related shift in Th2 frequency. To minimize variability in the low signal range, the pre- and post-treatment time-point samples of a particular subject were always analysed concurrently on the same day.

The CD154/intracellular cytokine quantification technique also proved a highly sensitive measure of antigen-

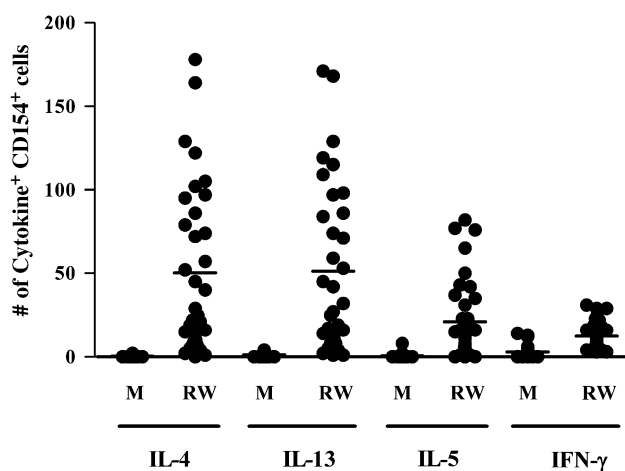


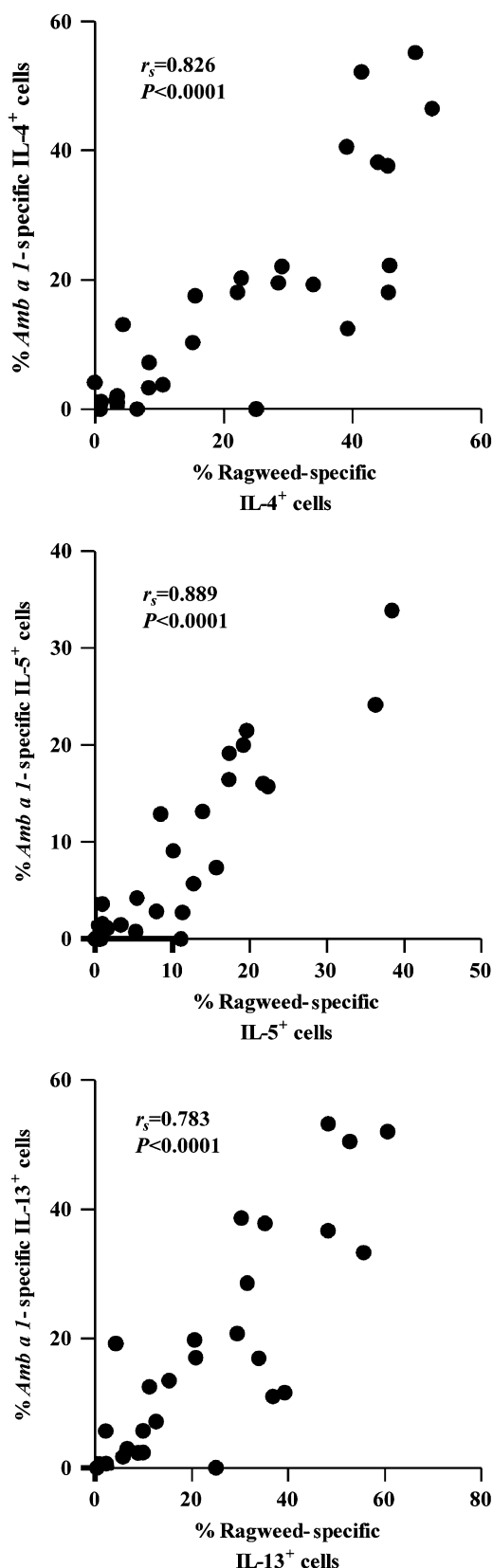
Fig. 3. Peripheral blood mononuclear cells (PBMC) from allergic rhinitis subjects respond to ragweed allergen with a quantifiable expression of Th2 cytokines. Numbers of ragweed (250 μ g/mL)-specific CD154⁺ cytokine⁺ CD3⁺ CD4⁺ T cells/ 6×10^5 total PBMC analysed/sample ($n = 32$, all subjects, pre-treatment). M, Medium (no stimulation); RW, Ragweed. Mean bars are indicated.

specific T cell responses as evidenced by the ability to detect the induction of IL-4, IL-5, and IL-13 (but not IFN- γ) in T cells of ragweed-allergic subjects in response to ragweed stimulation at a range of concentrations (supporting information Fig. S1). IL-4- and IL-13-expressing cells were consistently found at higher frequencies than IL-5⁺ cells in most subjects. Based on these observations, a dose of 250 μ g/mL of ragweed allergen extract was chosen as the optimal concentration for all subsequent stimulations.

Subjects in the EEC study were treated with AIC, which is Amb a 1 linked to the CpG-oligonucleotide 1018 ISS. Subjects were then challenged with controlled levels of ragweed pollen. Accordingly, we determined *in vitro* antigen-specific Th2 cytokine responses to both Amb a 1 and ragweed allergen extract. Pilot studies identified 90 μ g/mL as the optimal dose for Amb a 1-stimulated responses (data not shown). There was a highly significant correlation between ragweed- and Amb a 1-induced T cell IL-4, IL-5, and IL-13 expression (Fig. 4). Amb a 1 is the immunodominant ragweed allergen as defined by IgE reactivity of ragweed-sensitized individuals [20]. Our data demonstrate that Amb a 1 is also an immunodominant T cell antigen in the ragweed extract used for *in vitro* stimulations.

Pre- to post-treatment changes in allergen-induced T helper type 2 cytokine responses of Amb a 1-immunostimulatory oligodeoxynucleotide conjugate- and placebo-treated subjects

Antigen-specific Th2 cytokine expression was evaluated in PBMC from 17 AIC-treated subjects and 15 placebo-treated subjects. Following the initial series of ragweed pollen challenges and before subject randomization and treatments, most individuals demonstrated allergen-specific Th2 cytokine expression above that observed in medium-stimulated cells (Figs 5a and b). There were no significant differences in pre-treatment levels of Th2 cytokine expression between the subjects randomized to the placebo and AIC treatment groups. Placebo-treated subjects responded to the six ragweed pollen exposures given post-treatment with an increased frequency of ragweed-specific Th2 cells, as measured by CD154 and intracellular cytokine expression (Fig. 5a). Ragweed-specific IL-5 and IL-13 expression were significantly increased in CD3⁺ CD4⁺ T cells of placebo-treated subjects from pre- to post-treatment ($P < 0.001$ and $P = 0.018$, respectively) whereas IL-4 expression trended to increase ($P = 0.055$). Similar responses were observed when PBMC from placebo-treated subjects were re-stimulated *in vitro* with Amb a 1. There was a significant increase in Amb a 1-specific IL-13-expressing T cells ($P = 0.048$) from pre- to post-treatment and nearly significant increases in IL-4 ($P = 0.052$) and IL-5-expressing Amb a 1-specific T cells



($P = 0.052$). No changes were evident in the levels of IFN- γ ⁺ Amb a 1- or ragweed-specific T cells.

In contrast, the series of six post-treatment ragweed pollen exposures did not change the Th2 cell frequency significantly in AIC-treated subjects (Fig. 5b). Levels of ragweed- and Amb a 1-specific IL-4, IL-5, and IL-13 expression were similar in T cells from pre- and post-treatment samples taken from AIC subjects. IFN- γ -expressing T cells were at a low frequency and showed no difference from pre- to post-treatment in the AIC group. In comparing pre- to post-treatment changes in expression of each Th2 cytokine between AIC and placebo groups, no significant differences were observed; however, there was a strong trend towards a difference in pre- to post-treatment changes in T cell Amb a 1-specific IL-4 and IL-13 expression levels between placebo- and AIC-treated subjects ($P = 0.057$ and $P = 0.070$, respectively). CD154 expression as a percentage of CD3⁺CD4⁺ T cells did not change appreciably from pre- to post-treatment for either treatment group (supporting information Fig. S2), suggesting that the differences observed in the Th2 cell frequency were reflective of phenotypic rather than gross quantitative changes within the allergen-specific T cell population.

In summary, most ragweed-allergic subjects in this study had measurable allergen-specific Th2 responses. The multiple ragweed exposure regimen used might be expected to increase the allergen-specific Th2 response of study subjects and an increase in frequency of CD154⁺ Th2 cells was, in fact, observed in the PBMC of placebo-treated but not AIC-treated subjects. Increases in Th2 cytokine expression in placebo-treated subjects were significant or trended to significance. In this context, a larger sample size may have been expected to demonstrate more significant changes.

Relationship between ragweed allergen-specific T helper type 2 cytokine expression and total nasal symptom scores

In order to determine whether antigen-specific T cell responses in the peripheral circulation were reflective of subject-reported allergic rhinitis symptoms, we examined the correlations of Th2 cell frequency with TNSS in all PBMC donors before treatment. Amb a 1-induced IL-4, IL-5, and IL-13 expression each had a moderately positive but significant correlation with TNSS for all PBMC donors

Fig. 4. Amb a 1- and ragweed-specific Th2 cytokine responses strongly correlate. Cytokine⁺ cells are expressed as a percentage of allergen-specific CD154⁺ CD3⁺ CD4⁺ T cells. Ragweed (250 μ g/mL)-induced responses are plotted against Amb a 1 (90 μ g/mL)-induced responses. Spearman's rank order correlation coefficient was used to test correlation significances. Data are for all subjects with peripheral blood mononuclear cells (PBMC) cultured at both conditions pre-treatment ($n = 28$).

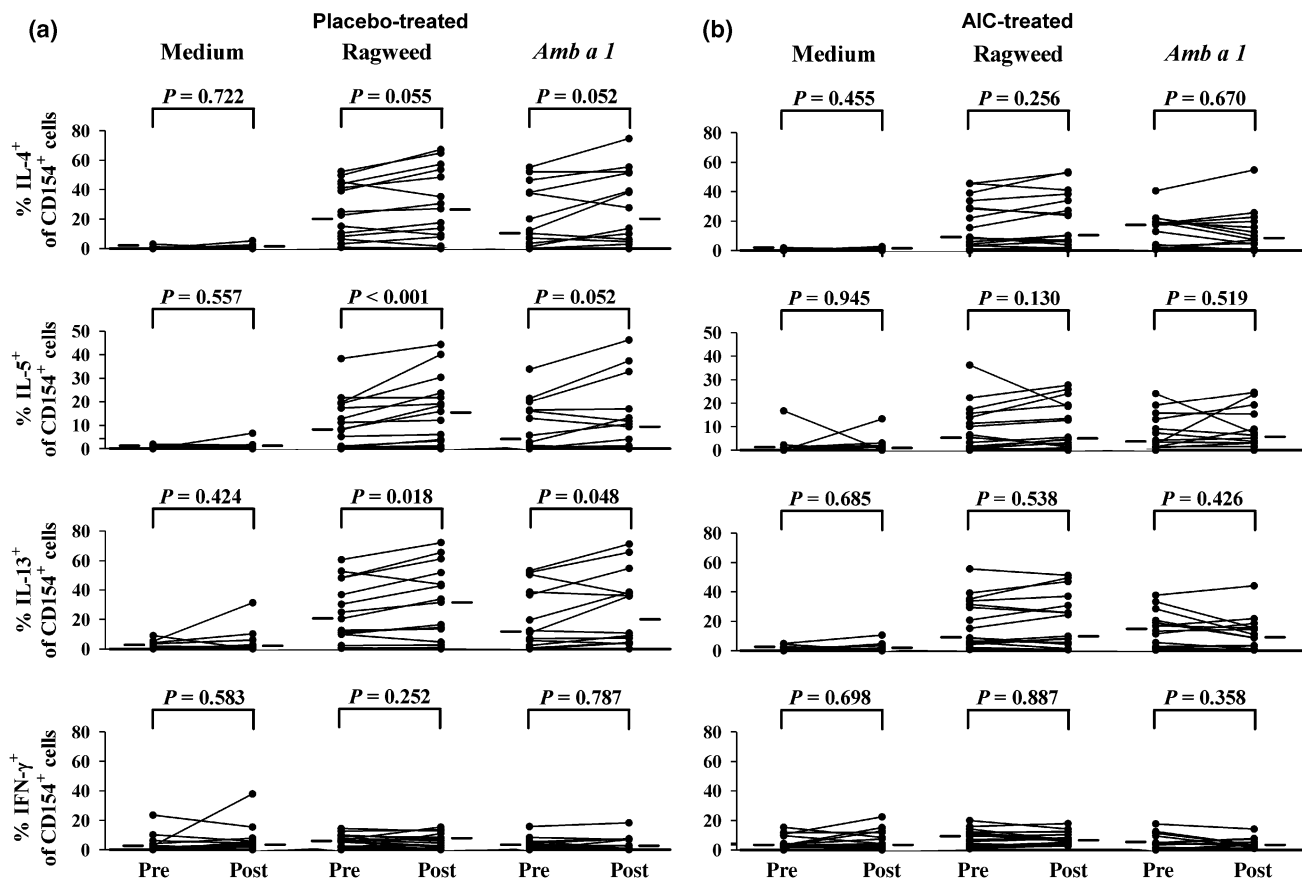


Fig. 5. Amb a 1-immunostimulatory oligodeoxynucleotide conjugate (AIC)- and placebo-treated subjects demonstrate divergent allergen-specific Th2 intracellular cytokine expression. Medium, ragweed (250 µg/mL), and Amb a 1 (90 µg/mL)-stimulated cytokine responses are shown for pre- and post-treatment peripheral blood mononuclear cells (PBMC) from placebo- (a) and AIC-treated (b) subjects. Cytokine⁺ cells are expressed as a percentage of allergen-specific CD154⁺ CD3⁺ CD4⁺ T cells. Wilcoxon's signed-rank test was used to evaluate intra-group pre- to post-treatment comparisons. Placebo ($n = 15$), AIC ($n = 17$). Median lines are indicated.

(Fig. 6). For ragweed-induced Th2 responses, a modest but significant correlation was observed only between IL-5 expression and TNSS. These data suggest an overall positive correlation between clinical symptoms and the numbers of circulating allergen-specific Th2 cells, although this frequency does not accurately predict the magnitude of patient-scored symptoms in individual subjects. This positive correlation is important as it suggests that there is not a preferential depletion of allergen-specific cells from the blood in patients experiencing symptoms in the nasal mucosa. Hence, CD154⁺ cytokine⁺ responses in peripheral blood may have potential as a tool for monitoring the effect of immunotherapeutic interventions on allergen-specific T cell responses.

Pre- to post-treatment changes in T helper type 2 cells defined by interleukin-4 and interleukin-13 co-expression

Allergen-specific T cell IL-4, IL-5, and IL-13 expression were highly correlated within all subjects in this study (supporting information Fig. S3). Accordingly, to evaluate

the effect of allergen-challenge and AIC treatment on multiple cytokine-secreting T cells, we examined IL-4/IL-13 double-positive Th2 cells. Allergen-specific T cells were co-stained with antibodies to CD3, CD4, CD154, and pairs of intracellular cytokines, either IL-4 and IL-13, or IL-5 and IFN-γ. The majority of allergen-specific T cells secreting either IL-4 or IL-13 express both cytokines (median >80% for all groups). Pre- to post-exposure changes in Th2 cytokine expression in the placebo group tended to show a greater statistical significance when measured as IL-4/IL-13 double-positive cells (Fig. 7) rather than single cytokine-positive cells (Fig. 5). These data suggest that monitoring double cytokine-positive cells may provide a more robust indicator of Th2 population changes. As with single cytokine-positive T cells, there were no significant pre- to post-treatment changes in IL-4/IL-13 double-positive T cells in the AIC-treated group. Correlations between ragweed-specific or Amb a 1-specific IL-4/IL-13 co-expressing cells and TNSS ($r_s = 0.321$, $P = 0.073$ and $r_s = 0.464$, $P = 0.01$, respectively) were comparable with those observed between single

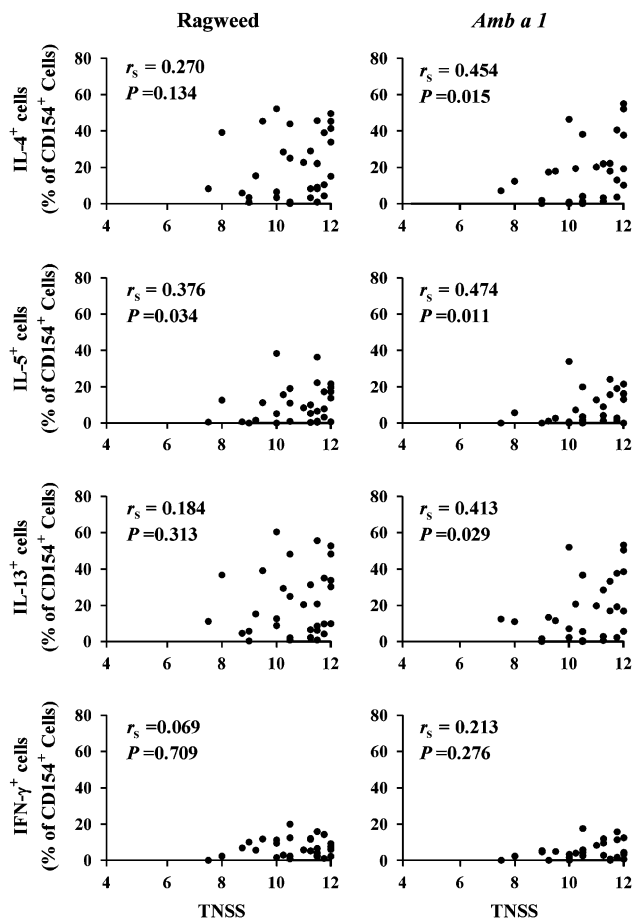


Fig. 6. Allergen-specific IL-4, IL-5, and IL-13 cytokine expression demonstrate modest correlations with subject-reported total nasal symptom scores (TNSS). Cytokine data are expressed as the percentage of allergen-specific CD154⁺ CD3⁺ CD4⁺ T cells. Subject-reported nasal symptoms are expressed as TNSS as described in Methods. The relationship between cytokine expression and TNSS was evaluated by Spearman's rank order correlation coefficient. Data are for all subjects pre-treatment ($n = 32$).

cytokine-positive cells and TNSS (Fig. 6). Interestingly, there was a significant difference between placebo- and AIC-treated groups in terms of changes in Amb a 1-induced IL-4/IL-13 co-expressing T cells from pre- to post-treatment (Fig. 7). Taken together, these data suggest that defining Th2 cells by IL-4 and IL-13 co-expression facilitates a more sensitive detection of the effects of allergen challenge and AIC treatment on allergen-specific Th2 cells within PBMCs.

Discussion

We describe here the evaluation of a technique published recently for directly assessing antigen-specific T cells in a clinical trial with well-established expectations in terms of Th2/Th1 cytokine responses. This assay is based on the transient increase in CD154 expression observed in T cells

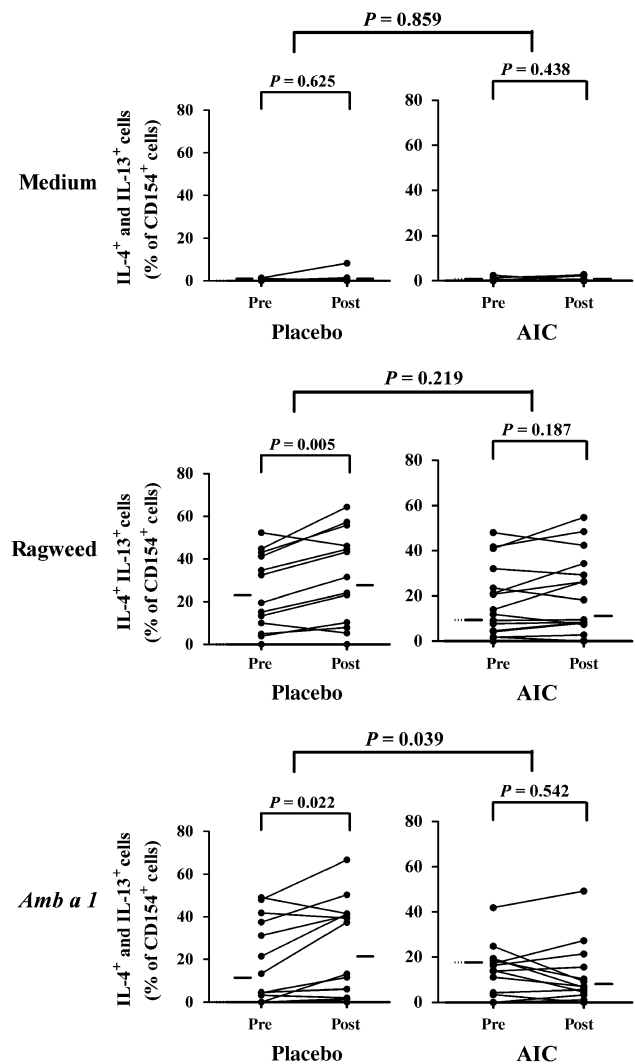


Fig. 7. Defining Th2 cells based on IL-4 and IL-13 co-expression enhances the ability to detect differences between Amb a 1-immunostimulatory oligodeoxynucleotide conjugate (AIC)- and placebo-treated subjects. Medium, ragweed (250 µg/mL), and Amb a 1 (90 µg/mL)-stimulated cytokine responses are shown for pre- and post-treatment peripheral blood mononuclear cells (PBMC) from placebo- and AIC-treated subjects. IL-4⁺IL-13⁺ double-positive cells are expressed as a percentage of allergen-specific CD154⁺ CD3⁺ CD4⁺ T cells. Wilcoxon's signed-rank test was used to evaluate intra-group pre- to post-treatment comparisons. Inter-group comparisons were analysed by Mann-Whitney *U*-test. Placebo ($n = 15$), AIC ($n = 17$). Median lines are indicated.

activated by an appropriately presented antigen. Using a subset of donors from an EEC study of AIC therapy for ragweed allergic rhinitis, we demonstrate that multiple controlled ragweed pollen exposures resulted in a significantly increased ragweed and Amb a 1-specific Th2 cell frequency in PBMC of placebo-treated subjects. This increase in Th2 cell frequency was not observed in subjects receiving AIC treatment, suggesting that the response to the experimental pollen exposures was blunted in treated subjects. Allergen-specific and

dose-dependent CD3⁺ CD4⁺ CD154⁺ IL-4⁺, IL-5⁺, and IL-13⁺ T cell responses were readily detectable in PBMC of most allergic rhinitis subjects exposed to ragweed pollen challenges, and responses to the major allergen Amb a 1 and ragweed extract were highly correlated. Collectively, these data demonstrate that this method of measuring peripheral antigen-specific T cell responses can quantify allergen-specific Th2 cells in the majority of patients showing a positive skin prick test for the allergen, and suggest that this assay may be able to measure changes in the frequency of these cells resulting from therapeutic interventions. This approach holds promise for a direct and objective way to optimize the dose and regimen of candidate immunomodulatory therapies, such as AIC.

The use of stabilized CD154 expression to identify antigen-activated T cells has been described for mouse, monkey, and human cells [8–11]. The transient nature of CD154 expression following antigen activation of T cells ensures that only *in vitro* re-activated cells are detected via intracellular CD154 expression when Brefeldin A is included in the re-stimulation culture. Although this is an advantage permitting exquisite sensitivity in detecting individual antigen-specific T cells, the assay is based on a short-term (6 h) *in vitro* re-stimulation step that does not allow for amplification of the antigen-stimulated T cell response, as would occur in cell cultures lasting several days. Thus, there is a need to determine whether the low numbers of antigen-specific T cells in circulation are sufficient for detection. For example, Stubbe et al. [21] analysed CD154⁺ CD4⁺ T cell responses to hepatitis B surface antigen in hepatitis B vaccine immunized healthy adults and found very low levels of antigen-specific T cells. Our study design precluded the possibility of obtaining PBMC samples before the pre-treatment series of ragweed pollen exposures; however, we have found a detectable *in vitro* allergen-specific CD154⁺ T cell cytokine expression in PBMC of non-allergen-challenged individuals allergic to a variety of common aeroallergens (data not shown). In contrast, T cells of non-allergic individuals (defined as having a negative skin prick test) do not display allergen-specific Th2 responses, confirming the antigen specificity of the assay. In the present study, the series of allergen challenges given pre-treatment likely resulted in augmented antigen-specific T cell frequencies; however, proportions of CD154⁺ Th2 cytokine⁺ cells clearly increased further in response to the additional allergen challenges given post-treatment in the placebo group. In terms of day-to-day variability inherent to the assay, we found that a lower coefficient of variation was associated with higher cytokine expression levels, specifically with numbers of CMV-stimulated IFN- γ ⁺ T cells. In response to allergen stimulation, it is Th2 cytokines that are highly expressed and the pre- to post-treatment increases in Th2 frequency in this study exceeded the measurement variability inherent in the

assay, thus resulting in the detection of statistically significant changes (Fig. 5).

The CD154⁺ expression-based assay appears to be a more sensitive technique for detecting allergen-specific T cell responses than approaches used in previous AIC immunotherapy studies. Tulic et al. [12] documented that AIC-treated subjects had reduced IL-4 mRNA⁺ cells and increased IFN- γ mRNA⁺ cells in the nasal mucosa compared with placebo-treated subjects after natural environmental exposure to ragweed. Their study, however, did not examine any systemic allergen-specific T cell responses. It has been proposed that systemic responses do not reflect the local effects of immunotherapy, and indeed, in a study of grass pollen immunotherapy, changes in local but not systemic Th2 responses were detectable [15]. In contrast, however, in a AIC immunotherapy trial [14] reported previously, AIC treatment was associated with decreased levels of Th2-associated chemokines CCL17 and CCL22 as well as IL-5 in supernatants of allergen-re-stimulated cultures. In the same cultures, IL-13 levels did not change and IL-4 was not detectable [14]. In the present AIC EEC study, we were able to detect allergen-specific IL-4, IL-5, and IL-13 responses in over 90% of subjects examined, demonstrating that a direct detection of allergen-specific T cell responses via CD154 expression offers the double advantage of high sensitivity combined with the ability to directly measure IL-4, IL-5, and IL-13 in allergen-specific T cells. Recent reports have demonstrated that individual human HIV antigen-specific CD8⁺ T cells or mouse *Leishmania major* – specific CD4⁺ Th1 cells simultaneously producing IFN- γ , IL-2, and TNF- α , the so-called multifunctional T cells, provide more optimal effector functions than T cells producing a limited range of cytokines in response to antigen stimulation [22, 23]. Whether high frequencies of analogous multifunctional Th2 cells occur in allergy and result in stronger allergic responses are unclear. In any case, we found that ragweed- or Amb a 1-specific Th2 cells defined as IL-4 and IL-13 co-expressing CD3⁺CD4⁺ T cells demonstrated more marked changes in response to an allergen challenge and AIC treatment compared with Th2 cells identified by single cytokine staining. These findings suggest that identifying T cell subsets as defined by the expression of two or more cytokines is a more sensitive approach for detecting T cells via antigen-activated CD154 expression. This may partly reflect the fact that the assay has been optimized for CD154 expression but not for the kinetics of the expression of each individual cytokine.

In the present study, we did not observe any changes in the proportions of ragweed allergen-specific IFN- γ ⁺ T cells in either treatment group and they remained at or near background levels at both time-points examined (Fig. 5). In contrast, we were able to easily detect CMV peptide-stimulated CD154⁺IFN- γ ⁺ responses in PBMC with our protocol and reagents (data not shown), ruling

out a technical issue preventing the detection of allergen-specific IFN- γ^+ T cells. The lack of detectable systemic allergen-specific IFN- γ^+ T cells in our study may be reflective of AIC having a partial effect in reducing TNSS that did not reach significance in the present study ($P=0.09$), or alternatively, a longer time post-treatment may be necessary for the detection of elevated peripheral allergen-specific IFN- γ^+ T cells [12]. It has been demonstrated that AIC (and free ISS) stimulate Th1-like responses locally and systemically [12, 14, 24]. However, the mechanism of action of AIC is under active investigation and may involve selective Th2/Th1 re-balancing and/or, possibly, the induction of a regulatory T cell response [25].

To assess the relevance of systemic changes in Th2 cell frequency to subjective symptom readouts, we examined the correlation between allergen-specific CD154⁺ cytokine responses and TNSS. There was a moderate, but significant, correlation between these parameters for Amb a 1-restimulated T cells (Fig. 6). These correlations are not high (ranging from $r=0.38$ to $r=0.47$), but do exceed correlations that we have observed previously between TNSS and commonly used indicators of atopy, such as weal and erythema ($r=0.2$ – 0.29) in allergic individuals [26]. This weak, but positive, correlation indicates that Th2 frequencies cannot accurately predict the TNSS of individual subjects, but do reflect a parameter that is a relevant objective measure of disease status in groups of subjects. For all subjects in this EEC study ($n=253$), the mean reduction in TNSS score after treatment was 41% greater in the AIC-treated group than in the placebo group, although this difference did not quite reach statistical significance ($P=0.09$). The safety and efficacy evaluations from this study will be reported elsewhere.

In summary, we have demonstrated for the first time that allergen-specific T cell responses, as detected by *in vitro* allergen-stimulated CD154 expression, may be used to monitor the effect of immunomodulatory therapeutic approaches for allergic disease. Monitored PBMC responses of a relatively small subset of subjects in an EEC trial of AIC immunotherapy for allergic rhinitis demonstrated divergent responses in AIC- and placebo-treated groups. Placebo-treated individuals responded to multiple ragweed pollen exposures with elevated allergen-specific IL-4, IL-5, and IL-13 responses. In contrast, AIC treatment prevented this increase, but did not decrease the allergen-specific Th2 response. Amb a 1-specific Th2 cell responses showed a moderate but significant correlation with TNSS, demonstrating an association between allergen-specific T cell responses and subject symptom reporting. Thus, this technique described recently for a direct assessment of antigen-specific T cell responses shows clear promise for monitoring changes in specific Th subset frequencies in studies of new immunomodulatory clinical interventions.

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Supporting Information

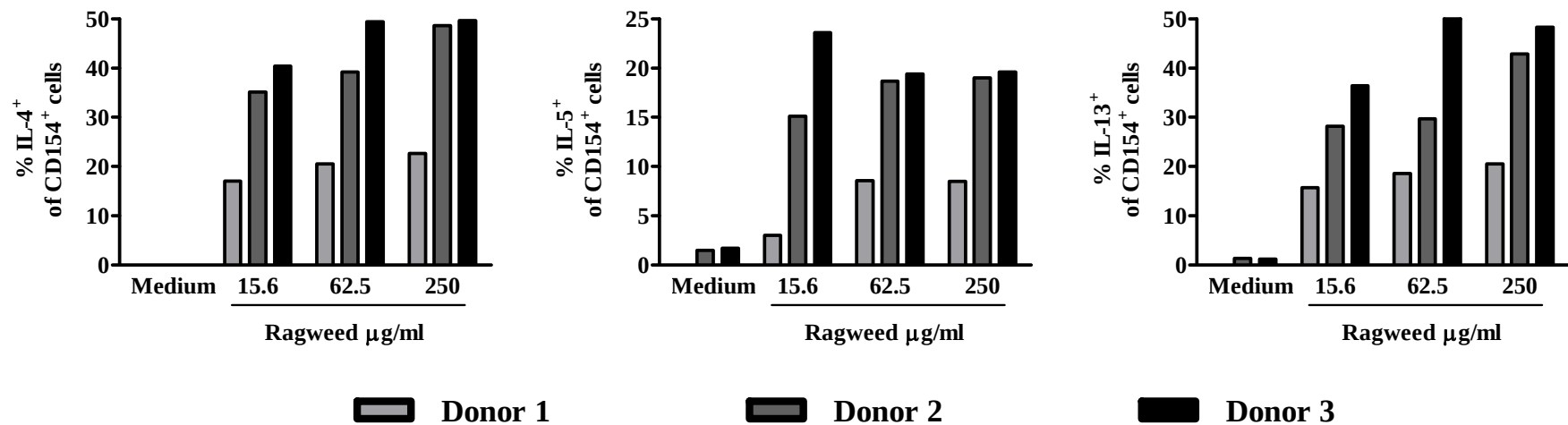
Additional Supporting Information may be found in the online version of this article:

Figure S1. Dose-dependence of allergen-specific Th2 cytokine expression. Ragweed- specific cytokine⁺ cells are expressed as a percentage of allergen-specific CD154⁺CD3⁺CD4⁺T cells after stimulation with different concentrations of ragweed. Representative data are shown for 3 PBMC donors.

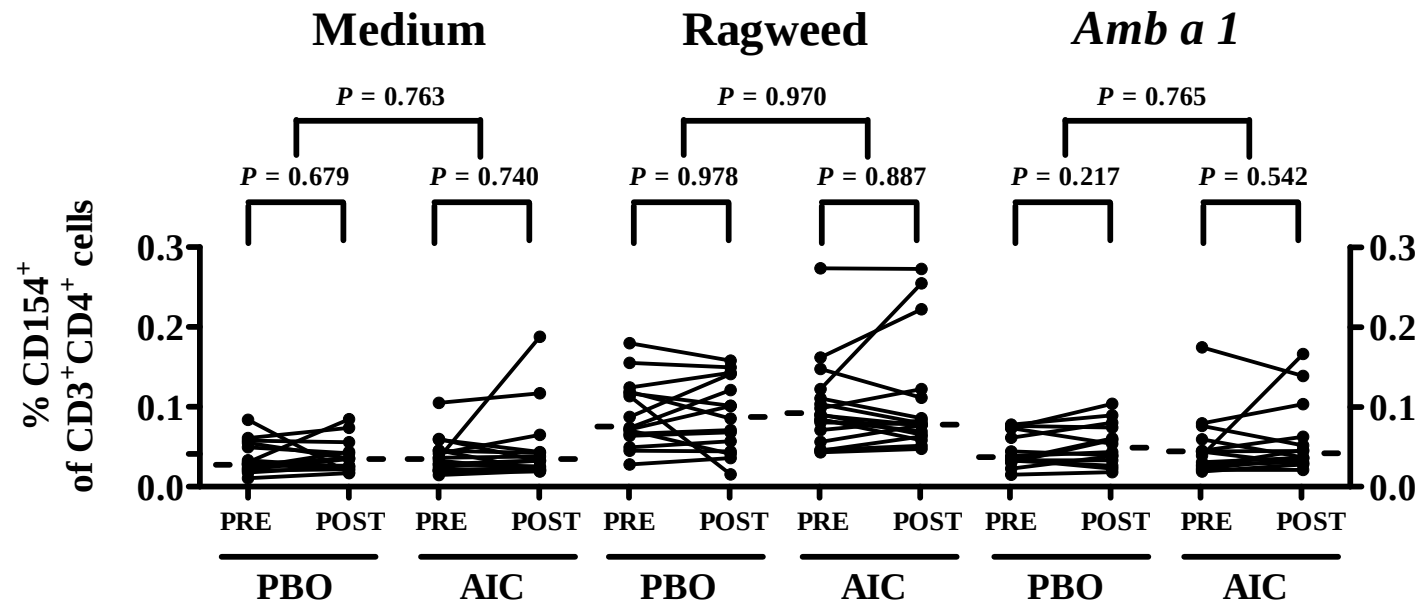
Figure S2. Dose-dependence of allergen-specific Th2 cytokine expression. Ragweed- specific cytokine⁺ cells are expressed as a percentage of allergen-specific CD154⁺CD3⁺CD4⁺T cells after stimulation with different concentrations of ragweed. Representative data are shown for 3 PBMC donors.

Figure S3. Allergen-specific IL-4, IL-5, and IL-13 expression all strongly correlate. Cytokine⁺ cells are expressed as a percentage of Amb a 1- or ragweed-specific CD154⁺ CD3⁺ CD4⁺ T cells. Spearman rank order correlation coefficient was used to evaluate relationships between expression of IL-4, IL-5, and IL-13. Data are for all subjects pre-treatment (n = 32).

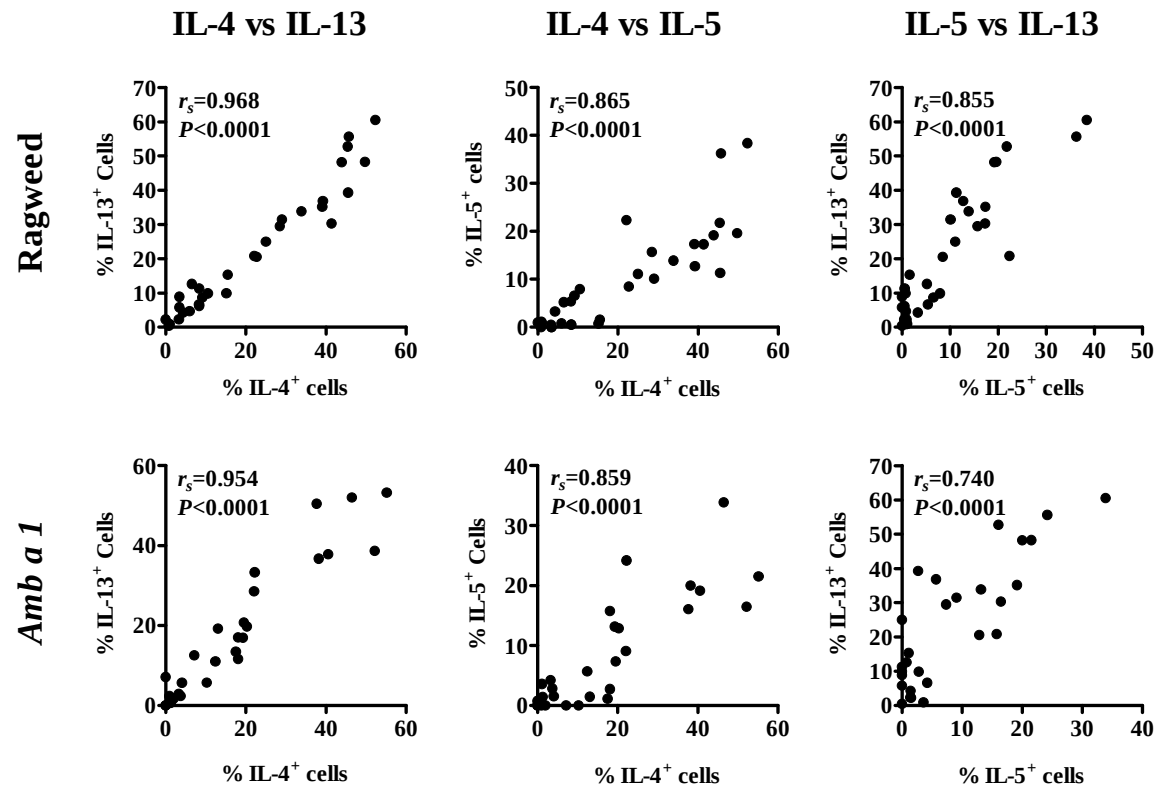
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Supplementary Fig. 1. Dose-dependence of allergen-specific Th2 cytokine expression. Ragweed-specific cytokine⁺ cells are expressed as a percentage of allergen-specific CD154⁺CD3⁺CD4⁺T cells after stimulation with different concentrations of ragweed. Representative data are shown for 3 PBMC donors.



Supplementary Fig. 2. Proportions of allergen-specific CD154⁺ T cells do not change from pre- to post-treatment and do not differ between AIC and placebo (PBO) groups. CD154⁺ cells expressed as a percentage of the CD3⁺CD4⁺ T cell population show no significant differences from pre- to post treatment (Wilcoxon signed rank test) or between treatment groups (Mann-Whitney U test). Placebo (n=15), AIC (n=17). Median lines are indicated.



Supplementary Fig. 3. Allergen-specific IL-4, IL-5, and IL-13 expression all strongly correlate. Cytokine⁺ cells are expressed as a percentage of *Amb a 1*- or ragweed-specific CD154⁺ CD3⁺ CD4⁺ T cells. Spearman rank order correlation coefficient was used to evaluate relationships between expression of IL-4, IL-5, and IL-13. Data are for all subjects pre-treatment (n=32).