

Human subjects without peanut allergy demonstrate T cell-dependent, T_H2-biased, peanut-specific cytokine and chemokine responses independent of T_H1 expression

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Background: Peanut allergy is a major cause of anaphylaxis. Regulation of immune responses to peanut allergen, particularly why sensitization does not usually progress to allergic reactions, is not well investigated. Most studies focus exclusively on serologic responses and individuals with peanut allergy.

Objective: We sought to determine the existence, prevalence, and nature of peanut-specific, T cell-dependent cytokine and chemokine responses of adults who eat peanut without having symptoms.

Methods: We developed systems to examine specific immunity in peanut-tolerant individuals who had (1) negative histories and negative peanut skin test responses, (2) negative histories and positive peanut skin test responses, and (3) clinically apparent peanut allergy. After primary culture of PBMCs restimulated with whole peanut extract, we quantified responses characteristic of T_H1 (IFN- γ and CXCL10) and T_H2-like immunity (IL-5, IL-13, CCL17, and CCL22) using ultrasensitive ELISAs. Antigen-presenting cell costimulatory requirements (CD4, HLA-DR, CD80/86, and cytotoxic T lymphocyte-associated antigen 4 [CTLA4] Ig) were determined.

Results: T cell-dependent, peanut-specific IL-5, IL-13, and CCL22 were common in peanut-tolerant individuals, regardless of whether they had positive or negative skin test

responses. These were blocked by anti-CD4 and were dependent on CD28/CD86 costimulation. None of the 70 individuals studied had demonstrable IFN- γ or CXCL10 responses to peanut. All demonstrated T_H1 and T_H2 responses to the ubiquitous recall antigen streptokinase.

Conclusions: Qualitatively similar and quantitatively increasing peanut-specific T_H2 responses in the consistent absence of putatively protective T_H1 immunity were found in both peanut-tolerant individuals and those with peanut allergy. **Clinical implications:** The continuum of responses between individuals with negative and individuals with positive skin test results, rather than T_H1 versus T_H2 bias, might be important in peanut allergy. (J Allergy Clin Immunol 2006;■■■:■■■-■■■.)

Key words: Food allergy, peanut allergy, anaphylaxis, T cell, cytokine, chemokine, costimulation

Food allergies, in particular peanut allergy, are major triggers of anaphylaxis in many countries.¹⁻⁴ Exposure to peanut is ubiquitous. Sensitization, with or without subsequent development of clinically apparent peanut allergy, has increased in prevalence.⁵⁻⁷ Current estimates indicate that some 1% to 2% of populations in the developed world exhibit signs and symptoms of peanut allergy after ingestion.⁵⁻⁹

Understanding of the immunologic mechanisms that allow peanut sensitization in a substantial segment of the population and those that determine its progression to clinical manifestations in a minority of individuals remains limited. Extensive studies provide valuable insight on biochemical characterization of constituent peanut allergens and the diversity of epitope/antibody interactions.¹⁰⁻¹⁹ In contrast, the nature of the T-cell response is much less well understood.^{3,20,21} Several studies demonstrate proliferation of PBMCs in response to peanut allergen stimulation.²²⁻²⁵ This approach reveals that T-cell activation is readily observed in allergic individuals, but proliferative responses reveal little about the nature of the response evoked. In efforts to better understand immune regulation in such individuals, several groups examined IL-4 and IFN- γ mRNA transcripts^{23,26-28} or, because

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Abbreviations used

CTLA4: Cytotoxic T lymphocyte-associated antigen 4
 PMA: Phorbol 12-myristate 13-acetate
 SPT: Skin prick test
 WPE: Whole peanut extract

of the low frequency of any antigen-specific T cell *in vivo*, derived cell lines or clones of peanut-specific T cells.^{22,24,29} Such studies are informative, but their interpretation is constrained by (1) the small number of individuals and T-cell clones studied because of the effort involved in deriving T-cell clones and (2) the effect of exogenous and endogenous mediators encountered during the cycles of clonal derivation and expansion that might skew the function of T-cell clones obtained.

Using an innovative strategy combining peanut antigen stimulation during initial culture and then polyclonal activation with phorbol 12-myristate 13-acetate (PMA) plus ionomycin during analysis of cytokine responses, Turcanu et al³⁰ identified peanut-reactive T cells. In the 9 individuals with peanut allergy studied, the frequency of peanut-specific, cytokine-producing cells that underwent clonal expansion was seen as T_H2 biased, with IFN- γ /IL-4 or IFN- γ /IL-13 ratios of approximately 1:1 to 30:1. Nine individuals without peanut allergy, also stimulated with peanut antigen followed by PMA plus ionomycin, exhibited more intense IFN- γ /IL-4 or IFN- γ /IL-13 ratios, with medians ranging from 40:1 to 80:1. These data were taken in support of the notion that T_H1-biased responses underlie oral tolerance.

Understanding the immune mechanisms underlying sensitization and subsequent failure to progress to clinical sensitivity would provide important insight into future avenues of prevention, therapy, or both. Given the intense interest in developing more effective approaches to food allergy management,^{4,31-35} better understanding of the immunologic basis of naturally acquired functional tolerance that is exhibited by environmentally exposed but clinically tolerant human subjects is a logical prerequisite. However, virtually nothing is known of peanut-specific responses in human subjects who can eat peanut without having an allergic reaction because most studies focus largely or exclusively on individuals with peanut allergy.^{8,36-38}

We hypothesized that individuals who can ingest peanut without exhibiting symptoms of allergic or anaphylactic reactions (peanut-tolerant individuals) might nevertheless respond to this ubiquitous antigen immunologically and that using peanut allergen to stimulate PBMCs from these individuals would provide information about the nature of their immunologic responsiveness. Extrapolating from studies carried out with T-cell clones, intense polyclonal activators, or both^{30,36-40} for food antigens or with antigen-specific activation in primary culture for inhalant allergens,⁴¹⁻⁴⁴ we speculated that nonallergic adults would exhibit well-defined, T_H1-biased immune response to peanut concomitant with minimal or

undetectable T_H2-like activation. We tested these hypotheses by measuring antigen-driven cytokine and chemokine responses to peanut and to an unrelated control antigen in short-term primary culture of PBMCs directly *ex vivo* paired with ultrasensitive ELISAs that we developed. Surprisingly, although classical, costimulation-dependent, T_H2-associated peanut responses were readily found in many nonallergic individuals, as were T_H1 immune responses to the bacterial recall antigen streptokinase, peanut-specific T_H1-associated responses were not evident in the populations studied.

METHODS

Participants

The University of Manitoba Research Ethics Board approved the study, and written informed consent was obtained from each individual before enrollment. In total, detailed data are provided on 60 adults without peanut allergy and 10 adults with peanut allergy (convincing history and positive skin test response) aged 18 to 35 years who were recruited in response to advertisements. Among nonallergic individuals, a detailed allergy history consistent with a lack of clinical reactivity ever to peanut was obtained. These individuals took no precautions to avoid and were regularly exposed to dietary peanut without symptoms. In subsequent experiments individuals without peanut allergy were divided into 2 groups based on their response to epicutaneous testing with 1:20 wt/vol peanut/glycerin extract (Bencard Allergy Laboratories, Mississauga, Ontario). We carried out detailed analyses on a cohort of 50 individuals able to ingest peanut without exhibiting any signs or symptoms and who had negative epicutaneous test responses (<3 mm in diameter prick-through drop technique) to peanut allergen and a cohort of 10 individuals who were able to eat peanut without exhibiting any signs or symptoms of an allergic reaction but had a clearly positive (4-23 mm in diameter) wheal response to peanut allergen. These individuals were sensitized but asymptomatic to peanut. Most of these individuals with positive peanut skin prick test (SPT) responses also exhibited positive skin test responses or clinical symptoms to additional unrelated allergens. A third cohort of 10 peanut-sensitized individuals with convincing histories of anaphylaxis within the previous 5 years and who made stringent efforts to avoid environmental peanut were subsequently recruited, and their peanut-specific cytokine responses were characterized in response to the same stimuli.

PBMC isolation

Fifty milliliters of whole blood was collected into 2 mL of 2.7% EDTA. PBMCs were obtained by means of centrifugation from whole blood diluted by 50% with 0.85% NaCl to improve cell yields by using Histopaque-1077 Ficoll (Sigma Aldrich Canada, Ltd, Oakville, Ontario, Canada). Cells collected from the interface were washed twice with 0.85% NaCl and resuspended in 4 mL of RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, and 2×10^{-5} 2-mercaptoethanol. Cell viability was greater than 95%, as determined by using trypan blue exclusion.⁴¹⁻⁴⁴

Preparation of peanut antigen

Roasted peanut antigen was prepared as previously described.¹¹ Briefly, peanuts were defatted with hexane, powdered, and extracted for 24 hours in an aqueous phosphate buffer and then centrifuged, and total protein was determined. Ara h 2 represents 10% to 12% of total protein in whole peanut extract (WPE).

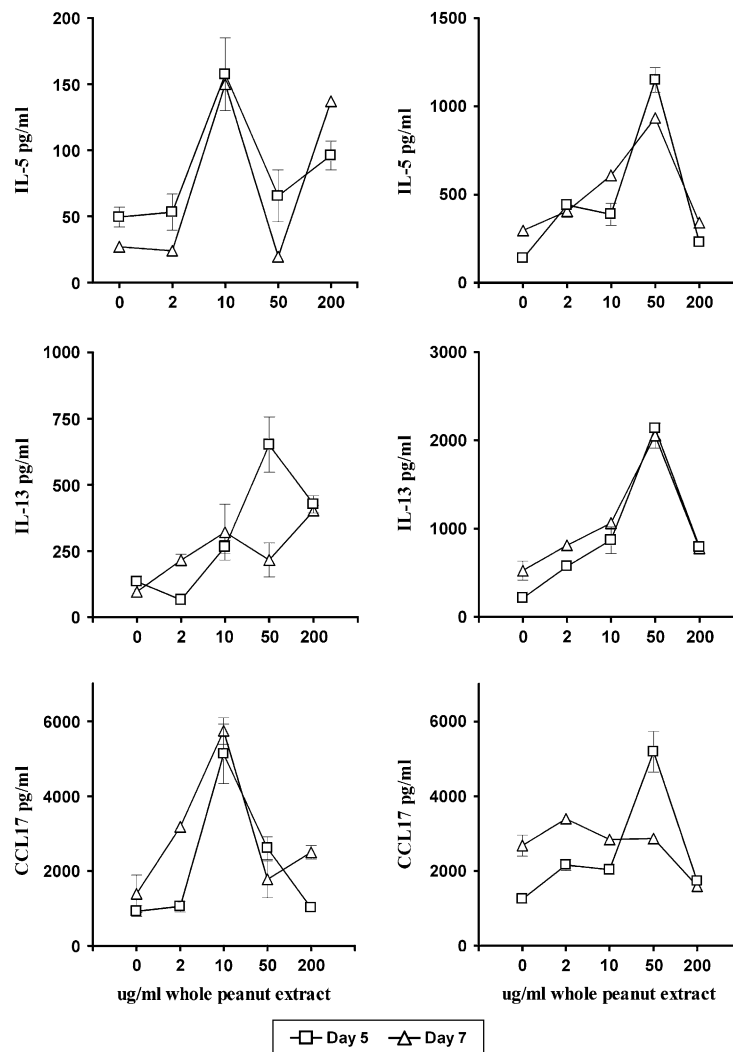


FIG 1. Optimization of time and dose-response curves for peanut extract-stimulated T_H2 responses. PBMCs from subjects without peanut allergy were cultured directly *ex vivo* with 2, 10, 50 or 200 µg/mL WPE. Supernatants were collected at 5 or 7 days (and days 3 and 9, as shown in Figs E1 and E2 in the Online Repository at www.jacionline.org) and then analyzed with cytokine- or chemokine-specific ELISAs.

Short-term primary culture of PBMCs

The culture system was established and optimized by using a range of concentrations of WPE from 2 to 200 µg/mL. Cells were cultured (5×10^5 cells per well) at 37°C for 3, 5, 7, or 9 days in 96-well round-bottom microwell plates by using a minimum of 2 wells per condition. Simultaneously, parallel cultures were set up using 5000 U/mL of the recall antigen streptokinase as a positive control. Based on the data obtained, most experiments were subsequently performed with 10 and 100 µg/mL WPE (the optimal concentrations for inducing cytokine and chemokine production) in culture for 5 days, and the time of peak responses for the indicators were examined. Analysis of costimulatory requirements for allergen-specific cytokine production was conducted with media alone versus 10 µg/mL WPE, each in the presence and absence of (1) 2 µg/mL α CD4 (BD PharMingen, Mississauga, Canada), (2) 2 µg/mL anti-HLA-DR (BD PharMingen), (3) 5 µg/mL α CD80 (BD PharMingen), (4) 5 µg/mL α CD86 (BD PharMingen), or (5) 5 µg/mL cytotoxic T lymphocyte-associated antigen 4 [CTLA4] Ig (provided by Dr Peter Nickerson, University of Manitoba).

Supernatants were collected at day 5 and stored at -20°C until analyzed by means of ELISA.

Cytokine and chemokine ELISAs

Sandwich ELISAs were used to determine IL-5, IL-13, CCL17, CCL22, IFN- γ , and CXCL10 production in cell supernatants after *in vitro* stimulation of PBMCs with WPE alone or after addition of neutralizing antibodies to coreceptors and costimulatory molecules. Recombinant cytokine and matched pair anti-cytokine and biotinylated anti-cytokine antibodies for IL-5, IL-13, and IFN- γ were purchased from BD PharMingen, Peprotech (Rocky Hill, NJ) or the National Institutes of Health (Bethesda, Md). Similar reagents for chemokine analysis were obtained from R&D Systems (Minneapolis, Minn) and Peprotech. Detection was carried out with streptavidin alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa) and *p*-nitrophenol phosphate substrate tablets (Sigma Aldrich Canada, Ltd). Plates were read at 405 to 690 nm (SOFTmax Pro 3.2.1 program, SpectraMax 190; Molecular Devices Corp, Sunnyvale, Calif). Detection limits were as follows: IFN- γ ,

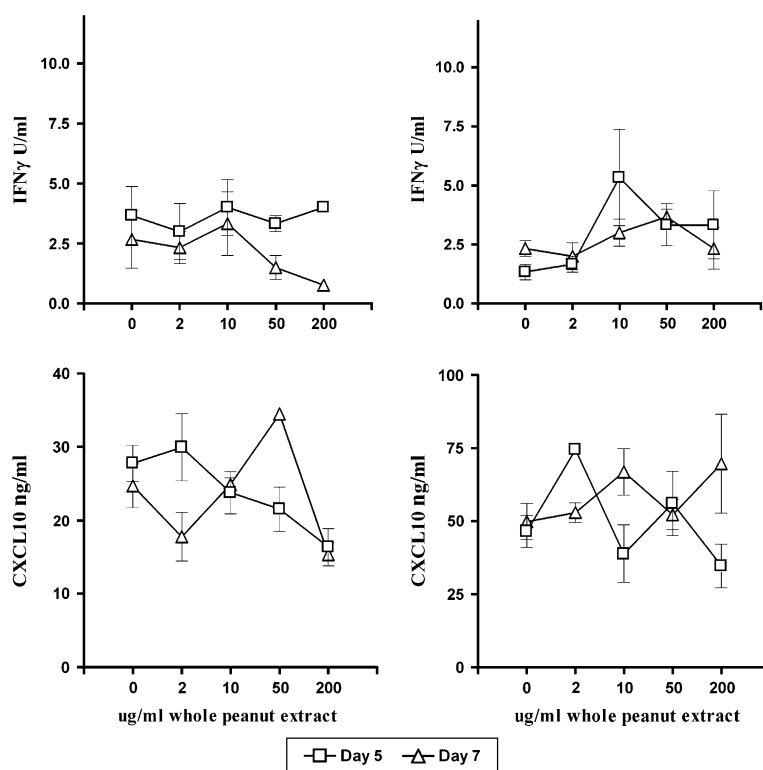


FIG 2. Optimal time and dose-response curves for peanut extract-stimulated production of T_H1 immunity-associated cytokines. PBMCs were cultured and analyzed as described for Fig 1. Mean cytokine production ($\pm$ SEM) is shown from 2 individuals representative of 7 examined in detail.

0.2 U/mL (approximately 23 pg/mL of World Health Organization standard Gxg01-902-535); CXCL10, 7.8 pg/mL; IL-5, 5.9 pg/mL; IL-13, 4.7 pg/mL; CCL17, 3.9 pg/mL; and CCL22, 7.8 pg/mL.

Statistical analysis

Data were obtained from at least 4 dilutions against a standard curve for each ELISA plate, with each condition tested in duplicate. All statistical analyses were carried out with GraphPad Prism 3 software (GraphPad, San Diego, Calif). Where sufficient data were obtained for Gaussian distributions, the Student *t* test (matched pairs) was used. Nonparametric analyses were conducted with validated Wilcoxon matched pairs. The Fisher exact test was used to compare the frequency of cytokine and chemokine responses between groups with negative skin test responses and those with positive skin test responses (both with negative histories). Differences were considered significant at a *P* value of less than .05.

RESULTS

Peanut allergen-specific stimulation of fresh PBMCs in primary culture

Given the well-established caveats of extrapolating from T-cell clones to *in vivo* conditions and the concerns about using polyclonal or pharmacologic activators as surrogates for specific allergen, we sought to establish systems that would allow characterization of responses by fresh unselected PBMCs on stimulation with peanut allergen in primary culture. Because our primary focus was on

individuals without peanut allergy and these were likely to be the most challenging patients in whom to detect responses, optimization studies for identification and measurement of peanut-specific cytokine and chemokine production were carried out in 7 individuals with negative histories (peanut-tolerant individuals) and positive skin test responses to determine (1) whether such responses were detectable in short-term primary culture *ex vivo* by using ultrasensitive ELISAs that we developed, (2) the optimal concentration of WPE eliciting peanut-specific cytokine and chemokine responses, and (3) the kinetics of cytokine production.

PBMCs were incubated for 3, 5, 7, or 9 days at concentrations of WPE ranging from 2 to 200 μ g/mL. The resulting culture supernatants were examined for indicators of peanut-driven T-cell activation. Production of representative T_H2 cytokines (IL-5 and IL-13) and chemokines (CCL17 and CCL22, data not shown) was found, with detailed results for 2 individuals shown in Fig 1. Maximal responses were similarly evident at 10 to 50 μ g/mL WPE on day 5 of culture, with kinetics much like those seen for inhalant allergen-driven cytokine production.⁴¹ However, in marked contrast to responses seen after primary culture with inhalant antigens,⁴¹⁻⁴⁴ production of type 1 cytokine (IFN- γ) and chemokine (CXCL10) responses to WPE were consistently undetectable, being no different from the low background levels seen in medium alone (Fig 2). Peanut-specific responses at days 3

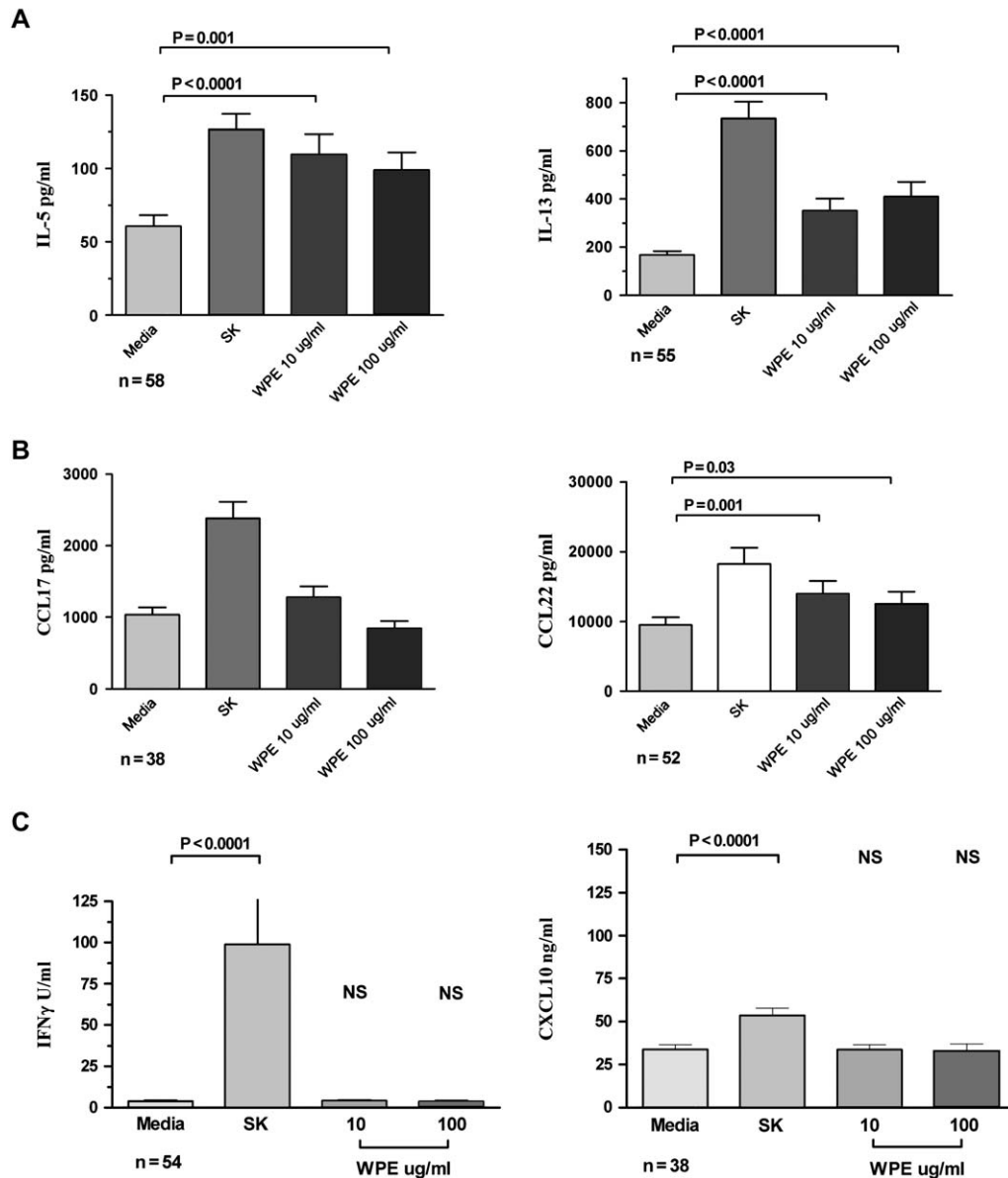


FIG 3. Peanut nonallergic populations exhibit readily detected peanut-specific T_H2 -like cytokine and chemokine production. PBMCs were cultured in the presence of medium alone, 5000 U/mL streptokinase (SK), or 10 or 100 μ g/mL peanut (WPE) for 5 days. T_H2 cytokine (A), chemokine (B), and T_H1 (C) responses are shown. Each bar represents the mean (+SEM) response seen for the population, with significance determined by using the Student *t* test. NS, Not significant.

and 9 were markedly lower (see Figs E1 and E2 in the Online Repository at www.jacionline.org), demonstrating that the apparent absence of type 1 immunity did not merely reflect differences in the kinetics of the response for different cytokines. Based on these results, concentrations of 10 and 100 μ g/mL WPE and 5-day cultures were used for stimulation in most subsequent experiments.

Peanut-specific cytokine and chemokine responses in adults with negative histories

To determine the prevalence of responses in individuals with negative histories who could eat peanut without

symptoms, we examined a panel of immunoregulatory cytokines and chemokines in a large group of individuals without peanut allergy. The population of environmentally peanut-exposed but clinically nonallergic adults exhibited increased IL-5 and IL-13 responses on WPE stimulation (Fig 3, A). Several of these individuals exhibited undetectable (<0.35 KU) peanut RAST responses, indicating the occurrence of cellular immunity in the absence of (detectable) peanut-specific IgE. When characterizing T_H2 -like chemokine responses over this clinically tolerant population, a difference in production of CCR4-binding family members CCL17 and CCL22

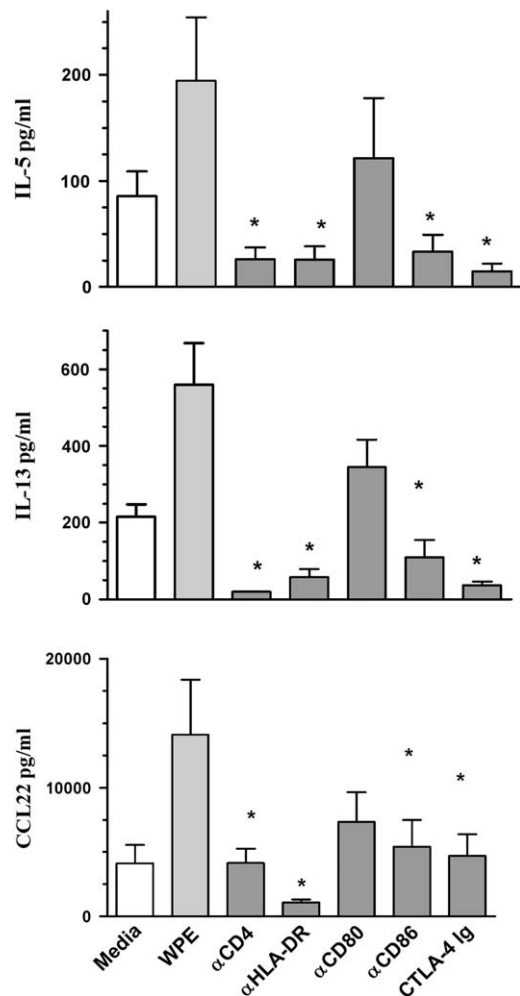


FIG 4. Peanut-driven cytokine production by adults without peanut allergy is dependent on recognition of antigen in the context of MHC class II stimulation, CD4 stimulation, and costimulation. PBMCs from 7 individuals were stimulated *in vitro* with medium or 10 μ g/mL peanut extract with or without indicated blocking antibodies or normal mouse IgG (not shown) for 5 days. Means (SEM) were compared by using the Student *t* test (* $P < .03$ -.001).

was evident. Substantive increases in peanut-driven CCL22 were evident, but CCL17 responses were sporadic and not significant in the group as a whole (Fig 3).

As seen for the initial 7 individuals recruited, neither IFN- γ nor CXCL10, characteristic T_H1 -associated immune responses, were detectable in response to WPE stimulation in any of the individuals subsequently examined (Fig 3, C; $P > .05$). The capacity and sensitivity of the assay systems used to quantify IFN- γ production are indicated by the significant responses ($P < .0001$) seen on streptokinase stimulation, ranging from 1.1 to 911 U/mL IFN- γ and 48 pg to 147,000 pg/mL CXCL10. Similarly, T_H2 -like (IL-5, IL-13, CCL17, and CCL22) cytokine and chemokine production was readily seen after primary culture of cells from these individuals with streptokinase (Fig 3).

TABLE I. Populations without peanut allergy but with positive SPT responses demonstrate greater frequencies of type 2 cytokine-producing individuals than do populations without peanut allergy but with negative SPT responses

Cytokine	SPT ⁻ /total	SPT ⁺ /total	P value (Fisher test)
IL-5	13/49	8/10	.002
IL-13	13/45	8/10	.004
CCL22	12/44	6/8	.02
IFN- γ	0/44	0/9	NS
CXCL10	0/44	0/9	NS

NS, Not significant.

T-cell and antigen-presenting cell costimulatory molecule requirements for peanut-specific responses

Peanut-stimulated PBMCs were cultured with antibodies that block key coreceptors and costimulatory molecules commonly required for antigen-mediated T-cell activation to determine the T-cell dependence of these responses and investigate immunologic mechanisms that might control peanut-specific recall cytokine and chemokine responses in individuals with negative histories. Anti-CD4 or anti-MHC class II (HLA-DR), but not normal mouse IgG (not shown), consistently reduced peanut-stimulated IL-5, IL-13, and CCL22 responses to background levels (Fig 4). These data indicate that the T_H2 -like cytokine production described above is dependent on peanut-specific stimulation rather than nonspecific triggers, such as mitogens, that could potentially be present in the peanut antigen extract.

To more precisely detail costimulatory requirements of T-cell activation by peanut in these individuals, we examined the effect of interfering with the CD28/CD80/CD86 T-cell/antigen-presenting cell pathway. Abrogation of antigen-driven responses in the presence of CTLA4Ig, blocking the active binding site for both CD80 and CD86, demonstrated that costimulation through this pathway is required for these recall responses. The differential intensity of the decreases seen on blocking CD80 versus CD86 demonstrated that costimulation is predominately CD86, rather than CD80, dependent (Fig 4).

Comparison of peanut-specific cytokine and chemokine responses in individuals with negative histories: Negative skin test responses versus positive skin test responses

Volunteers with no history of clinical peanut sensitivity were skin tested with peanut extract, and the response of their cultured PBMCs to peanut antigen stimulation was determined as described above to evaluate possible differences in cytokine responses of individuals with positive skin test responses versus those with negative skin test responses who could ingest peanut without exhibiting symptoms. We established a cohort of 10 individuals with

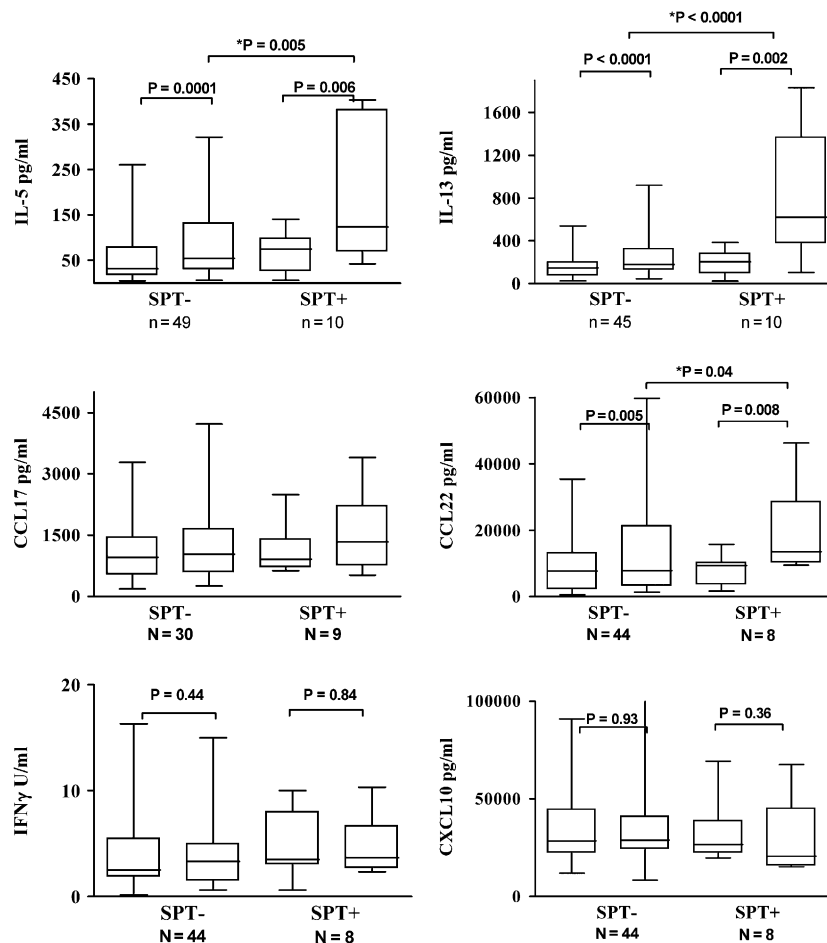


FIG 5. Peanut-specific type 2 responses are evident in both unsensitized and peanut-sensitized populations with negative histories in the absence of peanut-stimulated T_H1 immunity. PBMCs from individuals without peanut allergy with negative or positive SPT responses were cultured with medium alone or WPE for 5 days. Medians were compared by using the Wilcoxon matched-pairs test and the Mann-Whitney U test (*).

negative histories and positive SPT responses and 50 individuals with negative histories and negative SPT responses to assess WPE-driven responses. The 2 groups exhibited similar patterns of cytokine and chemokine production, but the frequency at which responses were detected (Table I) and the intensity with which they were expressed (Fig 5) were clearly higher in individuals with positive peanut skin test responses. The group with positive SPT responses had IL-5, IL-13, and CCL22 responses significantly more frequently than did the group with negative SPT responses ($P = .04$ to $.002$, Table I). When examined on an individual cytokine basis, 48% (23/49) of individuals with negative SPT responses exhibited peanut-specific IL-5, IL-13, or CCL22 responses, whereas 90% (9/10) of individuals with negative histories and positive SPT responses had quantifiable responses expressed as the production of one or more of these T_H2 -associated cytokines or chemokines on stimulation with peanut extract (Table I). However, when examining T_H1 cytokine or chemokine responses from either population, none of these individuals with negative peanut histories exhibited

peanut-dependent IFN- γ or CXCL10 responses above background values.

To enable comparison of these data obtained in clinically peanut-tolerant individuals with those obtained in individuals who exhibit convincing clinical histories of peanut allergy, we recruited 10 adults with peanut allergy who make great efforts to avoid environmental peanut exposure but who have experienced a peanut-driven anaphylactic episode in the preceding 5 years. The same pattern of cytokine production was evident, with clearly defined responses in IL-5, IL-13, and both T_H2 -immunity chemokines examined but no evidence of peanut-driven T_H1 -like recall responses to peanut (Fig 6). Thus examining the data as a whole, although the intensity of the clinical sensitivity to peanut (plus vs minus), peanut skin test intensity (ranging from 3 to 28 mm among peanut-sensitized individuals with negative histories), and peanut-stimulated T_H2 cytokine/chemokine production were clearly distinct, peanut-stimulated expression of the T_H1 cytokine IFN- γ or the T_H1 chemokine CXCL10 was consistently indistinguishable in all 3 groups.

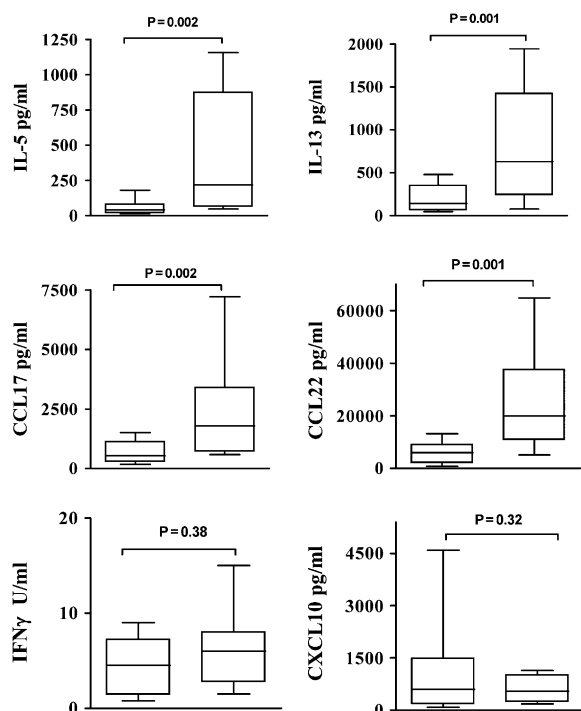


FIG 6. Peanut-driven cytokine and chemokine responses in adults with peanut allergy are dominated by T_H2 immunity. PBMCs from 10 individuals with convincing histories of peanut allergy were cultured in the presence of medium alone or WPE for 5 days. Medians, complete ranges, and 25% to 75% quartiles were compared by using the Wilcoxon matched-pairs test.

DISCUSSION

Allergic individuals have maladaptive immune responses and display clinically apparent symptoms to specific food allergens or other allergens to which they have been previously exposed. Current theories argue that in contrast healthy individuals mount protective immune responses against these allergens, become immunologically tolerant to them, or both. Although there is extensive literature examining serologic responses, published information about cytokine or chemokine responses and mechanisms of T-cell activation to food allergens is scant. Here we developed sensitive systems to detect and quantify T-cell responses in short-term primary culture from individuals who are not clinically allergic to peanut in comparison with those with convincing clinical histories of peanut allergy. Our focus on this population was driven by the ubiquitous nature of peanut exposure in many countries and by the fact that sensitization, generally defined as skin test positivity, is much more prevalent than are clinically apparent signs and symptoms of disease.

Peanut-driven skin test and cytokine/chemokine responses are frequent in individuals with negative histories who tolerate peanut ingestion. They are characterized by substantial levels of production of peanut-specific T_H2 cytokines, including IL-5 and IL-13. Production of

peanut-specific CCL22 responses in the absence of peanut-specific CCL17 responses indicates differential production of CCR4-binding chemokine family members in peanut-sensitized populations with negative histories versus those with positive histories. Moreover, subanalysis of T_H2 cytokine and chemokine production between the 2 groups with negative histories, as stratified by skin test positivity, revealed differences in cytokine production. When comparing IL-5, IL-13, and CCL22 responses, those by individuals with positive SPT responses were substantially more intense than those of individuals with negative SPT responses ($P < .05$ to .0008, Fig 5). As an alternative approach, comparing the frequency of T_H2 between individuals with negative and individuals with positive SPT responses, the group with positive SPT responses exhibited IL-5, IL-13, and CCL22 responses more frequently than the group with negative SPT responses ($P = .04$ to .002, Table I). Moreover, up to 48% (23/49) of individuals with negative SPT responses versus 90% (9/10) of individuals with positive SPT responses displayed either IL-5, IL-13, or CCL22. Thus a substantial proportion of peanut-tolerant individuals demonstrate commitment to readily quantified T_H2 responses.

Although a diverse pattern of type 2 responses was evident in individuals in each population, IL-13 and CCL22 proved to be the most consistent biomarkers to parallel skin test positivity. Extensive careful work has been done to determine a quantitative relationship between peanut RAST values or skin test intensities and clinical outcomes.⁴⁵⁻⁴⁷ Although valuable at the extremes of the spectrum (very high or undetectable RAST values having a strong positive predictive value), there is a soft relationship between specific quantities of circulating IgE, quantitative intensity of skin test responses, and predictive value of the frequency or intensity of clinical reactivity across the broad midrange of RAST values. Our data extend this analysis by demonstrating that even among a proportion of individuals with negative skin test responses and RAST results, peanut-specific cytokine responses can be readily evident. Thus any single indicator in isolation (skin test response, RAST result, or cytokine production) is not predictive of clinical reactivity.

Interestingly, we found that peanut-specific IL-5 and IL-13 production by individuals with positive skin test responses and negative histories was seen here at concentrations equal to or greater than grass pollen-driven responses from individuals without grass pollen allergy⁴¹ by using highly similar experimental systems. Although this reflects responses from small numbers of individuals (10 for peanut and 20 for grass pollen), it suggests that peanut allergens might be more potent stimulators of cytokine production than aeroallergens such as grass pollen. Studies with a larger cohort of sensitized individuals who are clinically tolerant to peanut are currently underway.

Notwithstanding the hypothesis that oral tolerance to peanut^{26,30} or cow's milk antigens³⁹ is associated with dominant "protective T_H1 -biased" cytokine production, we were consistently unable to detect differences in peanut-specific (T_H1 , IFN- γ , or chemokine CXCL10)

responses when comparing peanut-stimulated and media control cultures for 70 individuals seen on multiple occasions. This differs from the findings of Dorion et al,²⁶ working with 6 subjects without peanut allergy and purified Ara h 2, or Turcanu et al,³⁰ who found that cloned peanut-specific lymphocytes from 9 nonallergic children, as well as several children who had outgrown their peanut allergy, displayed a clearly dominant T_H1 phenotype after peanut extract combined with PMA-ionomycin stimulation. This might be attributable to differences in the populations studied (adults vs children), the means used to assess recall responses (secreted protein vs intracellular staining), stimulation conditions used to evoke responses (primary culture of peanut-stimulated PBMCs vs PMA-ionomycin stimulation of peanut-derived, short-term T-cell clones), the antigen used (WPE vs purified Ara h 2), or the small numbers of individuals examined. In this regard the recent report by Castro et al,³⁷ using conditions similar to those used by Turcanu et al,³⁰ that found that nonproliferating peanut-specific cells, rather than CFSE-low proliferating cells,³⁷ are responsible for most cytokine production, is important to consider. Clearly, more attention to defining the immunoregulatory response in both sensitized/allergic and sensitized/nonallergic human subjects will be important.

In light of the lack of evidence in our study for an association of clinical tolerance with dominant T_H1-biased immunity, an observation evident in groups with or without positive peanut skin test responses, the mechanism or mechanisms responsible for prevention of sensitization or progression to clinically apparent disease remain unclear. Although our data from a cohort of 60 adults without peanut allergy argues strongly against putatively protective T_H1 biases in such individuals, several alternative mechanisms are possible. Among these, there is much interest in the role of differential expression of TGF- β , IL-10, and/or CTLA4 expression by various cell types that might include T regulatory cells.^{48,49} Because the primary objective of our study was to determine whether evidence of peanut-driven, T cell-dependent cytokine production was seen in peanut-tolerant adults and because of analysis of the cytokines and chemokines listed above and other readouts in which detectable responses were not evident (ie, eotaxin, data not shown), there was an insufficient sample to examine IL-10 (or TGF- β) levels. Similarly, IL-10 was not examined in other peanut-specific T-cell activation studies.³⁰

Preferential involvement of specific HLA-DR haplotypes has been suggested in the presentation of peanut allergen-immunodominant epitopes and thus in the absence or development of clinically evident peanut allergy.^{50,51} When peanut-stimulated PBMCs were cultured with blocking antibodies against key coreceptors and costimulatory molecules required in the classical CD28:CD80/CD86 T-cell/antigen-presenting cell activation pathway, we were able to show for the first time that TcR/CD4 recognition of peanut allergen peptides in the context of MHC class II molecules is essential for peanut-specific IL-5, IL-13, and CCL22 production in

peanut-tolerant individuals. Moreover, although virtually all peanut-specific cytokine and chemokine production was CD86 dependent, blocking of CD80 generally had no effect on expression of these responses, demonstrating that costimulation is CD86 mediated and not CD80 mediated.

In summary, these data show that a substantial proportion of adults who eat peanut without allergic symptoms or signs mount immune responses to this ubiquitous antigen, regardless of the presence or absence of skin test positivity to peanut. We speculate that increased T_H2 cytokine production in anaphylactic individuals with positive peanut skin test responses and positive histories, rather than differences in putatively protective T_H1-biased cytokine and chemokine production, differentiates individuals who are clinically tolerant to peanut from those who exhibit clinical signs and symptoms caused by peanut. Taken together with differences in the intensity of peanut-specific cytokine production and the frequency of responses between individuals without peanut allergy with negative and those with positive SPT responses, this suggests that a spectrum of intensity of T_H2 responses, as opposed to control of their induction and consequent presence or absence, serves as a key factor in determining the presence versus absence of clinical allergy symptoms to peanut. Studies are currently underway to test this hypothesis.

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REFERENCES

1. Sampson H. Food allergy: When mucosal immunity goes wrong. *J Allergy Clin Immunol* 2005;115:139-41.
2. Roberts G, Lack G, and the Avon Longitudinal Study of Parents and Children Study Team. Diagnosing peanut allergy with skin prick and specific IgE testing. *J Allergy Clin Immunol* 2005;115:1291-6.
3. Sicherer SH, Leung DY. Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs, and insects. *J Allergy Clin Immunol* 2005;116:153-63.
4. Li XM. Beyond allergen avoidance: update on developing therapies for peanut allergy. *Curr Opin Allergy Clin Immunol* 2005;5:287-92.
5. Kagan RS, Joseph L, Dufresne C, Gray-Donald K, Turnbull E, Pierre YS, et al. Prevalence of peanut allergy in primary-school children in Montreal, Canada. *J Allergy Clin Immunol* 2003;112:1223-8.
6. Sicherer SH, Munoz-Furlong A, Sampson HA. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J Allergy Clin Immunol* 2003;112:1203-7.
7. Grundy J, Matthews S, Bateman B, Dean T, Arshad SH. Rising prevalence of allergy to peanut in children: data from 2 sequential cohorts. *J Allergy Clin Immunol* 2002;110:784-9.
8. Lewis SA, Grimshaw KE, Warner JO, Hourihane JO. The promiscuity of immunoglobulin E binding to peanut allergens, as determined by Western blotting, correlates with the severity of clinical symptoms. *Clin Exp Allergy* 2005;35:767-73.
9. Vander Leek TK, Liu AH, Stefanski K, Blacker B, Bock SA. The natural history of peanut allergy in young children and its association with serum peanut-specific IgE. *J Pediatr* 2000;137:749-55.
10. Burks AW, Williams LW, Helm RM, Connaughton C, Cockrell G, O'Brien T. Identification of a major peanut allergen, Ara h I, in patients with atopic dermatitis and positive peanut challenges. *J Allergy Clin Immunol* 1991;88:172-9.
11. Burks AW, Williams LW, Thresher W, Connaughton C, Cockrell G, Helm RM. Allergenicity of peanut and soybean extracts altered by

- chemical or thermal denaturation in patients with atopic dermatitis and positive food challenges. *J Allergy Clin Immunol* 1992;90:889-97.
12. Clarke MC, Kilburn SA, Hourihane JO, Dean KR, Warner JO, Dean TP. Serological characteristics of peanut allergy. *Clin Exp Allergy* 1998;28:1251-7.
 13. Burks AW, Shin D, Cockrell G, Stanley JS, Helm RM, Bannon GA. Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. *Eur J Biochem* 1997;245:334-9.
 14. Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brien TJ, Helm RM. Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J Allergy Clin Immunol* 1992;90:962-9.
 15. Maleki SJ, Kopper RA, Shin DS, Park CW, Compadre CM, Sampson H, et al. Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *J Immunol* 2000;164:5844-9.
 16. de Leon MP, Drew AC, Glaspole IN, Suphioglu C, O'Hehir RE, Roland JM. IgE cross-reactivity between the major peanut allergen Ara h 2 and tree nut allergens. *Mol Immunol* 2006 Mar 29 [Epub ahead of print].
 17. Barre A, Borges JP, Rouge P. Molecular modeling of the major peanut allergen Ara h 1 and other homotrimeric allergens of the cupin superfamily: a structural basis for their IgE-binding cross-reactivity. *Biochimie* 2005;87:499-506.
 18. Shreffler WG, Lencer DA, Bardina L, Sampson HA. IgE and IgG4 epitope mapping by microarray immunoassay reveals the diversity of immune response to the peanut allergen, Ara h 2. *J Allergy Clin Immunol* 2005;116:893-9.
 19. Piersma SR, Gaspari M, Hefle SL, Koppelman SJ. Proteolytic processing of the peanut allergen Ara h 3. *Mol Nutr Food Res* 2005;49:744-55.
 20. Eigenmann PA, Frossard CP. The T lymphocyte in food-allergy disorders. *Curr Opin Allergy Clin Immunol* 2003;3:199-203.
 21. Braunstahl G-J. The unified immune system: Respiratory tract-nasobronchial interaction mechanisms in allergic airway disease. *J Allergy Clin Immunol* 2005;115:42-8.
 22. de Jong EC, Spanhaak S, Martens BP, Kapsenberg ML, Penninks AH, Wierenga EA. Food allergen (peanut)-specific TH2 clones generated from the peripheral blood of a patient with peanut allergy. *J Allergy Clin Immunol* 1996;98:73-81.
 23. Laan MP, Tibbe GJ, Oranje AP, Bosmans EP, Neijens HJ, Savelkoul HF. CD4+ cells proliferate after peanut-extract-specific and CD8+ cells proliferate after polyclonal stimulation of PBMC of children with atopic dermatitis. *Clin Exp Allergy* 1998;28:35-44.
 24. Higgins JA, Lamb JR, Lake RA, O'Hehir RE. Polyclonal and clonal analysis of human CD4+ T-lymphocyte responses to nut extracts. *Immunology* 1995;84:91-7.
 25. Hourihane JO, Dean TP, Warner JO. Peanut allergic subjects' peripheral blood mononuclear cell proliferative responses to crude peanut protein. *Clin Exp Allergy* 1998;28:163-8.
 26. Dorion BJ, Burks AW, Harbeck R, Williams LW, Trumble A, Helm RM, et al. The production of interferon-gamma in response to a major peanut allergen, Ara h II correlates with serum levels of IgE anti-Ara h II. *J Allergy Clin Immunol* 1994;93:93-9.
 27. Hong SJ, Michael JG, Fehring A, Leung DY. Pepsin-digested peanut contains T-cell epitopes but no IgE epitopes. *J Allergy Clin Immunol* 1999;104:473-8.
 28. van Reijssen FC, Feliuss A, Wauters EA, Bruijnzeel-Koomen CA, Koppelman SJ. T-cell reactivity for a peanut-derived epitope in the skin of a young infant with atopic dermatitis. *J Allergy Clin Immunol* 1998;101:207-9.
 29. Glaspole IN, de Leon MP, Rolland JM, O'Hehir RE. Characterization of the T-cell epitopes of a major peanut allergen, Ara h 2. *Allergy* 2005;60:35-40.
 30. Turcanu V, Maleki SJ, Lack G. Characterization of lymphocyte responses to peanuts in normal children, peanut-allergic children, and allergic children who acquired tolerance to peanuts. *J Clin Invest* 2003;111:1065-72.
 31. Morafo V, Srivastava K, Huang CK, Kleiner G, Lee SY, Sampson HA, et al. Genetic susceptibility to food allergy is linked to differential TH2-TH1 responses in C3H/HeJ and BALB/c mice. *J Allergy Clin Immunol* 2003;111:1122-8.
 32. Appelt GK, Chan-Yeung M, Watson WTA, Dimich-Ward H, Ferguson A, Manfreda J, et al. Breastfeeding and food avoidance are ineffective in preventing sensitization in high risk children. *J Allergy Clin Immunol* 2004;113(suppl):S99.
 33. Sampson HA. Food allergy—accurately identifying clinical reactivity. *Allergy* 2005;60(suppl 79):19-24.
 34. Chehade M, Mayer L. Oral tolerance and its relation to food hypersensitivities. *J Allergy Clin Immunol* 2005;115:3-12.
 35. Pons L, Palmer K, Burks W. Towards immunotherapy for peanut allergy. *Curr Opin Allergy Clin Immunol* 2005;5:558-62.
 36. Kolopp-Sarda MN, Moneret-Vautrin DA, Gobert B, Kanny G, Guerin L, Faure GC, et al. Polyisotypic antipeanut-specific humoral responses in peanut-allergic individuals. *Clin Exp Allergy* 2001;31:47-53.
 37. Castro RR, Shreffler WG, Pei-Chi LS, Sampson HA. Characterization of T cell responses in peanut-allergic patients by polychromatic flow cytometry. *J Allergy Clin Immunol* 2004;113(suppl):S251.
 38. Scott-Taylor TH, Harper J, Strobel S. Patterns of food allergen-specific cytokine production by T lymphocytes of children with multiple allergies. *Clin Exp Allergy* 2005;35:1473-80.
 39. Pohjavuori E, Viljanen M, Korpela R, Kuitunen M, Tiittanen M, Vaarala O, et al. Lactobacillus GG effect in increasing IFN- γ production in infants with cow's milk allergy. *J Allergy Clin Immunol* 2004;114:131-6.
 40. Turcanu VH, Winterbotham M, Kelleher P, Lack G. Peanut-specific B and T cell responses are correlated in peanut allergic individuals whereas tetanus-specific B and T cell responses are not. *J Allergy Clin Immunol* 2004;113(suppl):S48.
 41. Li Y, Simons FER, HayGlass KT. Environmental antigen-induced IL-13 responses are elevated among subjects with allergic rhinitis, are independent of IL-4, and are inhibited by endogenous IFN-gamma synthesis. *J Immunol* 1998;161:7007-14.
 42. Simons FE, Shikishima Y, Van Nes G, Eiden JJ, HayGlass KT. Selective immune redirection in humans with ragweed allergy by injecting Amb a 1 linked to immunostimulatory DNA. *J Allergy Clin Immunol* 2005;113:1144-51.
 43. Campbell JD, Gangur V, Simons FE, HayGlass KT. Allergic humans are hyporesponsive to a CXCR3 ligand-mediated Th1 immunity-promoting loop. *FASEB J* 2004;18:329-31.
 44. Imada M, Simons FE, Jay FT, HayGlass KT. Antigen mediated and polyclonal stimulation of human cytokine production elicit qualitatively different patterns of cytokine gene expression. *Int Immunol* 1995;7:229-37.
 45. Sampson HA. Improving in vitro tests for the diagnosis of food hypersensitivity. *Curr Opin Allergy Clin Immunol* 2002;3:257-61.
 46. Hill DJ, Heine RG, Hosking CS. The diagnostic value of skin prick testing in children with food allergy. *Pediatr Allergy Immunol* 2004;15:435-41.
 47. Verstege A, Mehl A, Rolinck-Werninghaus C, Staden U, Nocon M, Beyer K, et al. The predictive value of the skin prick test weal size for the outcome of oral food challenges. *Clin Exp Allergy* 2005;35:1220-6.
 48. Wilson MS, Maizels RM. Regulatory T cells induced by parasites and the modulation of allergic responses. *Chem Immunol Allergy* 2006;90:176-95.
 49. Van Wijk F, Hoeks S, Nierkens S, Koppelman SJ, van Kooten P, Boon L, et al. CTLA-4 signaling regulates the intensity of hypersensitivity responses to food antigens, but is not decisive in the induction of sensitization. *J Immunol* 2005;174:174-9.
 50. Boehncke WH, Loeliger C, Kuehn P, Kalbacher H, Bohm BO, Gall H. Identification of HLA-DR and -DQ alleles conferring susceptibility to pollen allergy and pollen associated food allergy. *Clin Exp Allergy* 1998;28:434-41.
 51. Howell WM, Turner SJ, Hourihane JO, Dean TP, Warner JO. HLA class II DRB1, DQB1 and DPB1 genotypic associations with peanut allergy: evidence from a family-based and case-control study. *Clin Exp Allergy* 1998;28:156-62.