

ORIGINAL ARTICLE

T helper 2 biased *de novo* immune response to Keyhole Limpet Hemocyanin in humans

D. Spazierer*, H. Skvara*, M. Dawid*, N. Fallahi*, K. Gruber*, K. Rose†, P. Lloyd†, S. Heuerding†, G. Stingl* and T. Jung†

*Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases, Medical University of Vienna, Vienna Competence Center, 1090 Vienna, Austria and †Novartis Exploratory Development, Basel, Switzerland, Horsham, UK and East Hanover, USA

Clinical & Experimental Allergy

Summary

Background Allergen-specific T helper 2 (Th2) cells play an important role in the pathogenesis of atopic disorders. To date, no model system exists in humans that would allow the monitoring of a developing *de novo* Th2 immune response *in vivo*.

Objective The aim of the experiment was to establish an immunization protocol inducing a *de novo* Th2 response in humans using Keyhole Limpet Hemocyanin (KLH) as neo-antigen.

Methods The double-blind placebo-controlled, parallel-group study was conducted in two groups of subjects (16 healthy volunteers and 16 patients with allergic rhinitis). Subjects received three i.m. injections of 100 µg KLH adsorbed to aluminium hydroxide or matching placebo (alum alone) in intervals of 2 weeks. On day 43, KLH alone (10 µg) was given intra-dermally (i.d.) to all subjects to assess immediate and late-phase skin responses.

Results The immunization protocol was well tolerated, highly specific and efficient. Antigen-specific production of Th2-cytokines (mainly IL-5 and IL-13) by PBMCs suggested a Th2 pattern, as did the presence of KLH-specific IgG4 in sera. Intra-dermal KLH challenge induced an immediate-type of response predominantly in atopic subjects followed by a late-phase skin reaction. The latter was accompanied by the presence of IgE⁺ cells, eosinophils and a strong up-regulation of IL-4 and IL-13 along with the absence of Th1 transcripts in biopsies taken from the site of antigen challenge. IL-17 and IL-22 transcripts were detected only in healthy subjects skin following KLH challenge, while IL-1β and IL-33 expression did not differ between the healthy and the atopics.

Conclusions The immunization protocol resulted in the elicitation of a local and peripheral Th2 immune response in both healthy and atopic individuals. This may permit the investigation and monitoring of novel immunomodulatory strategies aiming to interfere with Th2 responses in man. The relevance of lack of Th17 cells in atopic skin in this model remains to be determined.

Keywords atopy, human, KLH, skin, Th1/Th2 cells, vaccination

Submitted 10 July 2008; revised 18 October 2008; accepted 21 November 2008

Correspondence:

Thomas Jung, MD, PhD, Translational Medicine Dermatology, Novartis Exploratory Development, Forum 1, WSJ-27.4.071, CH-4002 Basel, Switzerland. E-mail: thomas.jung@novartis.com

Introduction

Upon exposure to allergens, atopic individuals are prone to develop a T helper 2 (Th2) dominated immune response which mediates chronic inflammation of mucosal and skin surfaces and is associated with the induction of allergen-specific IgE [1–3]. Interfering with such pathological Th2 responses, ideally at the level of T cell priming, might be an effective way to reduce allergen-induced tissue inflammation.

To study the impact of potential immunomodulators on the process of T cell priming, a model in humans allowing the investigation of a developing Th2 response to a neo-antigen would be required. Keyhole Limpet Hemocyanin (KLH), a copper-containing protein isolated from the mollusc *Megathura crenulata*, is widely used as a carrier for peptides or other antigens in antibody production due to its immunostimulatory properties [4, 5]. KLH has been approved in a few countries for the treatment of superficial bladder carcinoma. Its safety and efficacy has been

established in randomized trials [6, 7]. Experimentally, KLH has been safely administered to humans as a neo-antigen in numerous studies. Up to 1 mg has been injected as a single dose [8–10], and 100 µg KLH has been used in multiple dose studies [11, 12]. Although the induction of KLH-specific IgM and IgG has been demonstrated, Th2 responses have not been investigated in these studies. One component to influence the type of a developing immune response is the adjuvant. Aluminium-based adjuvants, such as aluminium hydroxide or phosphate, are widely used in vaccines such as diphtheria, tetanus and pertussis, and their safety in humans has been established [13]. In mice, these adjuvants are known to stimulate Th2-type responses [14, 15]. Aluminium hydroxide alone leads to the priming of B-cells and the accumulation of IL-4 – producing GR1⁺ cells in spleens of mice [16]. Immunization of mice with KLH adsorbed to aluminium hydroxide (KLH/alum) has been shown to induce a robust Th2 response, as assessed by antigen-specific IL-4 production of T cells *in vitro*, low IFN-γ production and by the presence of KLH-specific IgG1 (the homologue of human IgG4) and IgE in serum [17–24].

To address the question whether KLH adsorbed to alum would induce a Th2-type of *de novo* immune response in humans, we conducted a vaccination study in healthy volunteers and atopic subjects and monitored the developing immune response in sera, peripheral blood cells and skin biopsies following intra-dermal (i.d.) injection of KLH.

Methods

Study participants

The study population of this double-blind placebo-controlled parallel-group trial consisted of 32 patients, that is 16 healthy volunteers and 16 patients with allergic rhinitis, all male or female Caucasians between 18 and 45 years of age. Basic demographics were not different between atopic and healthy subjects. Patients were defined as atopics by having a personal history of allergic rhinitis, demonstrating an enhanced skin prick reaction (> 6 mm erythema) to at least one allergen in a standard prick test and an elevated total IgE (> 100 kU/L) in serum at the time of the study, but having no active disease requiring medical intervention. All subjects gave informed consent and the study was approved by the ethics committee of the Medical University of Vienna (no. 380/2005). For the analysis of the cytokine response to tetanus toxoid (TT), blood from 10 healthy volunteers previously immunized with a TT vaccine was used.

Study design and late-phase skin reaction

Subjects received a single 0.5 mL isotonic i.m. injection of 100 µg KLH (IMMUCOTHEL[®]; Biosyn Arzneimittel GmbH,

Fellbach, Germany) adsorbed to aluminium hydroxide (aqueous aluminium hydroxide suspension) or matching placebo (alum alone) at day 1 (baseline), day 15 and day 29. On day 43, KLH alone (10 µg) and a saline control was given i.d. to all subjects to assess immediate and late-phase skin responses. A punch skin biopsy from the injection site was taken on day 45 (48 h post i.d. injection) from all subjects.

Anti Keyhole Limpet Hemocyanin antibody enzyme-linked immunosorbent assay

Assays were established and conducted by Charles River Laboratories, Preclinical Services, Montreal, Canada. KLH was diluted to 2.5 µg/mL in 0.05 M carbonate buffer, pH 9.6. Plates were coated with 100 µL KLH solution overnight at 4 °C, and blocked with Blocker[™] Casein (Pierce, Rockford, IL, USA). After addition of 100 µL serum samples from study subjects, detection was performed with the following antibodies: goat anti-human IgM-HRP (Bethyl Laboratories Inc., Montgomery, TX, USA), mouse anti-human IgG1-HRP (Alpha Diagnostic International, San Antonio, TX, USA) or mouse anti-human IgG4-HRP (Alpha Diagnostic International). A rat anti-KLH IgG2a antibody (Biolegend, San Diego, CA, USA) or a rat IgM antibody cross-reacting with KLH (#14-4341; eBioscience, San Diego, CA, USA) were used to generate a standard curve for the quantification of human KLH-specific IgG or IgM antibodies.

In the absence of an anti-KLH-specific IgE antibody, a multistep sandwich ELISA was established by using rat anti-KLH IgG2a and rabbit anti-KLH IgG (Bethyl) antibodies. To generate a standard curve, goat anti-rabbit IgG antibodies were coated followed by increasing concentrations of rabbit anti-KLH IgG. KLH (5 µg/mL) was added, and wells were incubated with rat anti-KLH IgG2a. Finally, a goat anti-rat IgG-HRP antibody served as the detection antibody. Human serum samples were analysed for IgE-specific anti-KLH antibodies using the same principle with the exception that instead of coating plates with a goat anti-rabbit IgG antibody, a goat anti-human IgE (KPL, Gaithersburg, MD, USA) was used. Wells were then incubated with the serum from study subjects followed by the addition of KLH, and detection was performed as above. The detection limit of this assay was in the range of 4–10 ng/mL.

Isolation of peripheral blood mononuclear cells

Blood was drawn at each visit before the subsequent KLH injections on days 1, 15, 29 and 43, and at a final visit on day 57. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by Ficoll Paque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation.

In vitro stimulation of cells, proliferation assay and cytokine measurements

1×10^5 cells were cultured in 200 μ L RPMI 1640/10% FCS at 37 °C in 96-well round bottom plates and stimulated with either 1 μ g/mL KLH, 100 ng/mL anti-CD3 antibody (SPV-T3b), 0.2 μ g TT (Calbiochem, Merck KGaA, Darmstadt, Germany) or left unstimulated. Cells were cultured for 5 days and pulsed overnight with 1 μ Ci/well ^{3} H-thymidine. For cytokine measurements, supernatants were taken after culturing cells for 40 h, and 50 μ L were subjected to a multiplex luminex method (Upstate Beadlyte Human Multi-Cytokine kit; Millipore, Billerica, MA, USA) using beadmates coated with antibodies to human IL-4, IL-5, IL-10, IL-13 and IFN- γ .

Reverse transcription polymerase chain reaction in skin biopsy specimens

Skin biopsies were homogenized in 1.2 mL Trizol reagent (Invitrogen, Carlsbad, CA, USA) using a rotor-stator homogenizer. RNA was purified with the Qiagen RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and was reverse-transcribed using the High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed with a cDNA concentration of 20 ng/well on either the ABI PRISM[®] 7700 or 7900HT Sequence Detection System (Applied Biosystems) using Assay on Demand reagents (Hs00187842_m1 for Beta-2 microglobulin, Hs00929862_m1 for IL-4, Hs00174143_m1 for IFN- γ , Hs01073447_m1 for IL-12p35, Hs01011518_m1 for IL-12p40, Hs99999038_m1 for IL-13, Hs00174383_m1 for IL-17, Hs01574154_m1 for IL-22, Hs00413259_m1 for IL-23p19, Hs01555410_m1 for IL-1 beta, Hs00369211_m1 for IL-33; Applied Biosystems) and the TaqMan[®] Universal PCR Master Mix (Applied Biosystems). PCR conditions were 50 °C for 2 min followed by 95 °C for 10 min, and 40 cycles with 15 s at 95 °C and 1 min at 60 °C. Fold difference of transcripts from KLH-immunized vs. alum-immunized patients was calculated using the formula $2^{-\Delta\Delta C_T}$, where C_T is the threshold cycle, ΔC_T is the difference in threshold cycles for the target and reference gene (Beta-2 microglobulin) and $\Delta\Delta C_T$ is the difference in ΔC_T between samples from KLH and alum-immunized individuals.

Detection of immunoglobulin E positive cells and eosinophils in skin biopsies

Frozen sections (5 μ m) were acetone-fixed and blocked with 2% BSA/PBS. A mouse anti-human IgE (clone B3102E8; Southern Biotech, Birmingham, AL, USA) and a mouse anti-human eosinophil major basic protein antibody (clone BMK13; Millipore) were used for the detection of IgE⁺ cells and eosinophils, respectively. Positive cells

were visualized with the Elite Vecta Stain mouse ABC Kit (Vector Laboratories, Burlingame, CA, USA).

Statistical analysis

The Cpm values of proliferation assays are shown as mean $\pm$ SEM (standard error of mean), one- and two-way ANOVA were used to assess differences between KLH-stimulated and unstimulated cells. For comparison of individual time-points with one another, a paired *t*-test was applied. For statistical analysis of cytokine and gene expression levels, the Mann-Whitney *U*-test was used to compare different groups. GraphPad Prism Software, Version 4.0 (GraphPad Software Inc., San Diego, CA, USA) was used for all applications. *P*-values of <0.05 were considered as statistically significant, *P*-values of higher significance (<0.01) were indicated separately.

Results

In order to establish an immunization protocol to generate a *de novo* Th2-immune response in humans, 100 μ g KLH adsorbed to aluminium hydroxide (KLH/alum) was injected i.m. three times at 14-day intervals into eight healthy volunteers and eight atopic subjects. Aluminium hydroxide (alum) alone served as a placebo control in cohorts of additional eight healthy and eight atopic subjects. The immunization regimen was well tolerated and did not cause any disease flare in atopic individuals. No adverse events were recorded during the study.

Keyhole Limpet Hemocyanin specific T cell response

KLH-mediated proliferation of PBMCs was measured *in vitro*. Blood was drawn before immunization (baseline), and at days 15, 29, 43 and 57 before subsequent injections. PBMCs were stimulated with either KLH, soluble anti-CD3 antibody (positive control), or left unstimulated (negative control). In both healthy and atopic subjects' PBMCs, a significant KLH-specific proliferative response was already detectable 2 weeks after the first immunization and further increased after subsequent immunizations with KLH (Fig. 1). In contrast, PBMCs from subjects immunized with aluminium hydroxide failed to proliferate to KLH. As expected, anti-CD3-mediated proliferation as well as proliferation of non-stimulated cells did not change significantly between the different time-points, and no differences between KLH-immunized and alum-immunized subjects were observed.

Keyhole Limpet Hemocyanin specific B-cell response

To investigate the development of KLH-specific immunoglobulins, sera were isolated before immunization on day 1 and before subsequent KLH injections on days 15, 29, 43

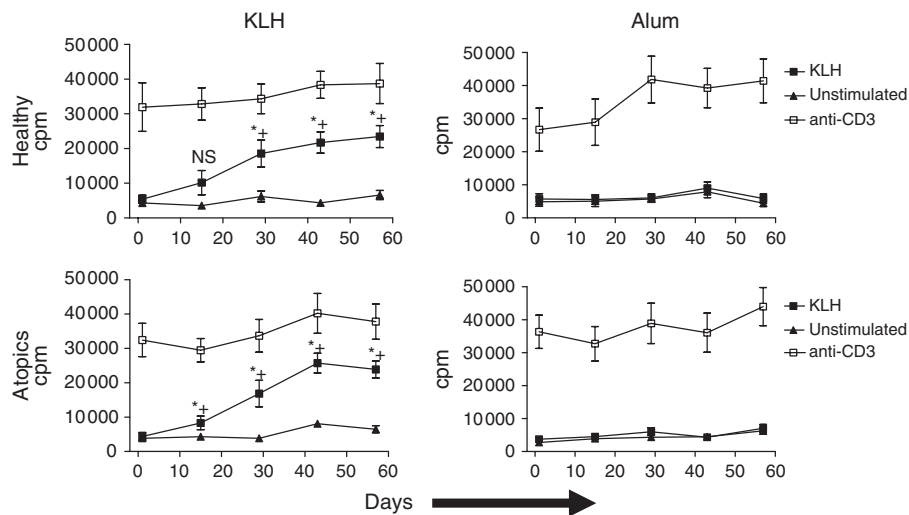


Fig. 1. Keyhole Limpet Hemocyanin (KLH) induced proliferation of peripheral blood mononuclear cells (PBMCs) is highly specific. Proliferative responses of PBMCs isolated at the indicated time-points from KLH-immunized healthy volunteers and atopics vs. alum-immunized (placebo) respective controls. PBMCs were stimulated *in vitro* with either KLH or anti-CD3 antibody, or left unstimulated. Mean values $\pm$ SEM are shown of $n = 8$ per cohort. Two-way ANOVA revealed a significant difference between KLH-stimulated and unstimulated cells from KLH-immunized patients ($P = 0.0006$ for healthy and 0.0001 for atopic individuals), while no significant difference was detected for cells from alum-immunized control patients. One-way ANOVA showed a significant ($P < 0.0001$) variation over time for KLH-stimulated cells of KLH-immunized patients. *: Significant difference from baseline value (day 0), +: significant difference from unstimulated negative control samples (paired *t*-test, $P < 0.05$), n.s.: not significant.

and 57. Small amounts of KLH-specific IgM were detected already after one immunization (Fig. 2). These levels increased after two injections (day 29) and persisted until study completion. No differences in KLH-specific IgM levels could be found between healthy and atopic subjects. Similarly, KLH-specific IgG1 and, to a lesser extent, also IgG4 levels were detectable already after one immunization and increased following subsequent KLH injections. There were no significant differences in serum levels of these IgG sub-classes between the healthy and the atopic group, although the mean KLH-specific IgG4 on day 57 was about eightfold higher in the serum of atopic subjects compared with healthy ones. This difference, however, was not significant, as just two of the eight atopic subjects accounted for this difference. Further, no KLH-specific IgE was detectable and individual levels of total IgE did not change over the course of the study in any of the subjects tested (data not shown). KLH-specific immunoglobulins were absent in the sera of alum-immunized patients.

Cytokine secretion profile of in vitro stimulated Peripheral blood mononuclear cells is indicative of a T helper 2 skewed reaction

The specific Th1/Th2 pattern of the developing KLH response was determined upon *in vitro* stimulation of PBMCs with KLH. As shown in Fig. 3a, KLH-specific cytokines were detectable after the second immunization on day 29 and showed a trend for further increase after the

third and fourth KLH injections. Cytokine production was highly specific as subjects immunized with alum alone did not show any detectable KLH-specific cytokine response (data not shown). KLH-specific IL-5 was detectable in 3/8 healthy and 4/8 atopic subjects immunized with KLH. PBMCs from all but one KLH-immunized subject produced KLH-specific IL-13 and IL-10 was detectable in 6/8 healthy and 4/8 atopic subjects. IFN- γ production was induced by KLH in cells from most KLH-immunized subjects (6/8 healthy and 5/8 atopic subjects). These levels were just above the detection limit. KLH-specific IL-4 was detectable in three KLH-immunized subjects (one healthy volunteer, two atopic patients; data not shown). In contrast, polyclonal stimulation of cells with anti-CD3 antibody as a positive control resulted in detectable IL-4 production in all KLH- as well as alum-immunized subjects, while non-stimulated cells serving as a negative control failed to produce any of the cytokines tested for (data not shown). This data suggests that the KLH immunization protocol induced a cytokine response with detectable or even high levels of the Th2 cytokines IL-13, IL-5 and IL-10 but very little IFN- γ . While KLH stimulation almost failed to induce a strong IFN- γ response, the same cells produced IFN- γ , but little IL-13 when stimulated with an anti-CD3 antibody (Fig. 3b). Furthermore, when PBMC from a different cohort of healthy subjects with a history of TT vaccination were stimulated with TT they produced IFN- γ , but little IL-13 resulting in an IFN- γ /IL-13 production ratio of 33 in contrast to 0.18 following KLH stimulation. This result is consistent with previous

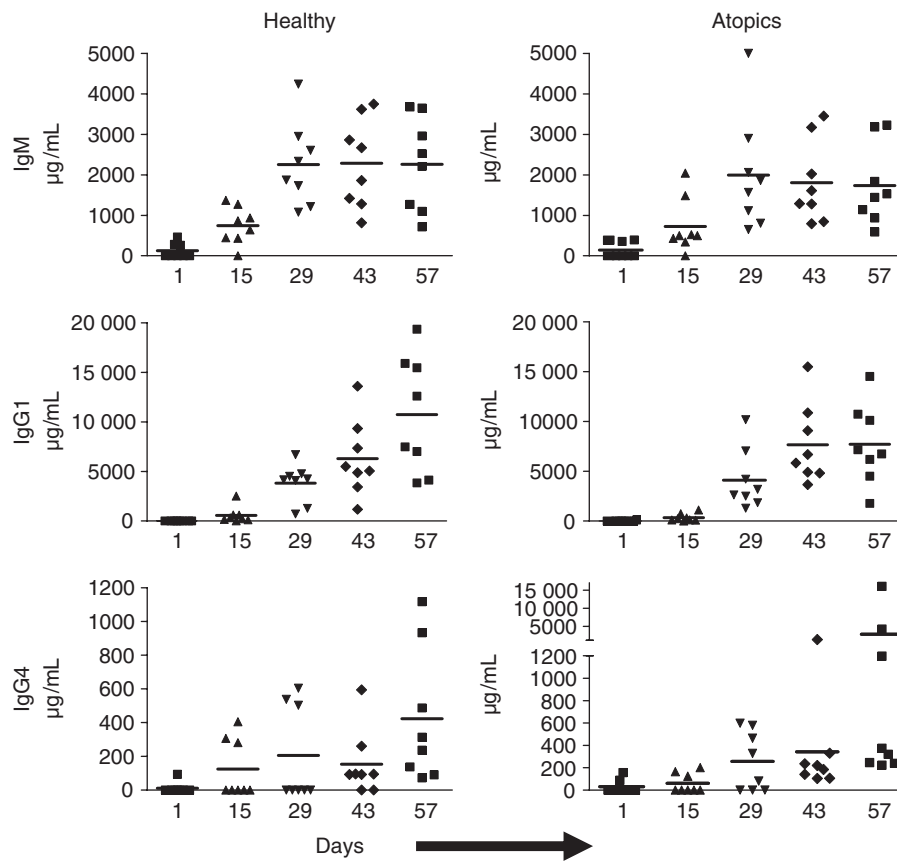


Fig. 2. Immunization with Keyhole Limpet Hemocyanin (KLH) induces strong production of antigen-specific antibodies. Serum isolated from KLH-immunized healthy volunteers and atopics at indicated time-points was analysed for KLH-specific IgM, IgG1 and IgG4 by ELISA. Horizontal bars indicate the mean of the cohort. Note: no KLH-specific antibodies were detected in the serum of alum-immunized controls (data not shown).

reports demonstrating a strong Th1 component upon TT activation [25, 26]. Thus, KLH-induced cytokine responses show a Th2 pattern, in contrast to anti-CD3 or TT stimulation.

Keyhole Limpet Hemocyanin induces a late-phase skin reaction peaking 24 h past challenge

Next, we tested immediate and delayed skin reactions to application of KLH (i.d.). Therefore, 10 µg of KLH alone were injected into the skin of all subjects on day 43. Seven out of eight KLH-immunized atopic patients and 5/8 healthy volunteers that had been vaccinated with KLH showed a flare reaction after 15–30 min, with the mean diameter being significantly larger in atopic (~2.5 cm) compared with healthy subjects (~1 cm; Fig. 4a). No immediate skin reaction to KLH was visible in any of the alum-immunized volunteers (data not shown). Induration was strongest 24 h after KLH application in most (6/8 healthy and 6/8 atopic) subjects and had disappeared in 25% of all subjects after 48 h (Fig. 4b). This pattern suggests a Th2-type late-phase skin response rather than a classical Delayed Type Hypersensitivity (DTH), in which

the peak induration occurs at 48–72 h [27–29]. Eosinophils were detected in biopsies taken 48 h after KLH injection in KLH-immunized but not in alum-immunized atopic as well as healthy individuals (Fig. 4c). Similarly, IgE⁺ cells were detected in biopsies only from KLH-immunized healthy individuals (Fig. 4d) indicating a *de novo* IgE response. Atopic patients immunized with KLH also showed IgE⁺ cells but, in contrast to healthy volunteers, there were already some positive cells in alum-treated patients, indicative of a pre-sensitization in these individuals (Fig. 4d).

To further characterize the type of the immune reaction *in situ*, transcript levels of various cytokines were determined in the skin (Figs 4e and f). Cytokine transcript changes were calculated as fold-increase between KLH-immunized/KLH-challenged and alum-immunized/KLH-challenged subjects. IL-4 levels were 9- or 14-fold increased and IL-13 levels even 18- and 14-fold elevated in the skin of healthy and atopic subjects, respectively. Levels of IL-33, a cytokine, which promotes a Th2 response at least in mice [30] were very low and not different between healthy and atopic subjects skin. IFN-γ mRNA showed only a 5- and 3.5-fold up-regulation in healthy and atopic subjects, respectively. IL-12p35,

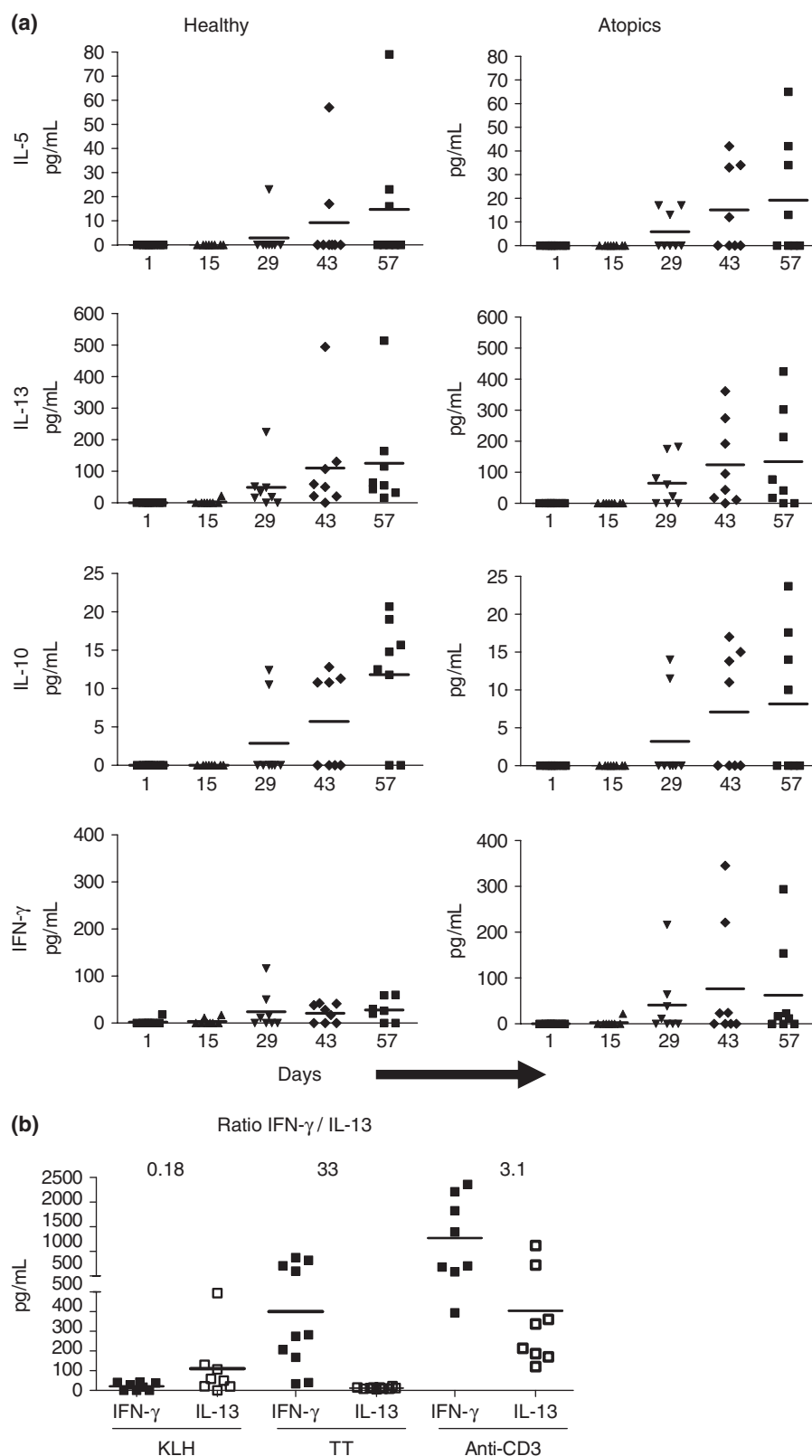


Fig. 3. Cytokine production by peripheral blood mononuclear cells (PBMCs) upon stimulation with Keyhole Limpet Hemocyanin (KLH). (a) Plots show levels of *in vitro* produced IL-5, IL-13, IL-10 and IFN- γ following KLH stimulation of PBMCs from healthy and atopic individuals as a function of time. Each dot represents one patient, horizontal lines indicate the means of the cohorts. Note: no cytokine production has been detected in cells isolated from the alum-immunized control groups (data not shown). (b) IFN- γ and IL-13 production following KLH and anti-CD3 stimulation of PBMCs from eight healthy subjects and following tetanus toxoid (TT) stimulation of PBMCs from 10 different healthy subjects. Numbers in the plot are ratios of the mean IFN- γ and IL-13 production for each stimulation condition.

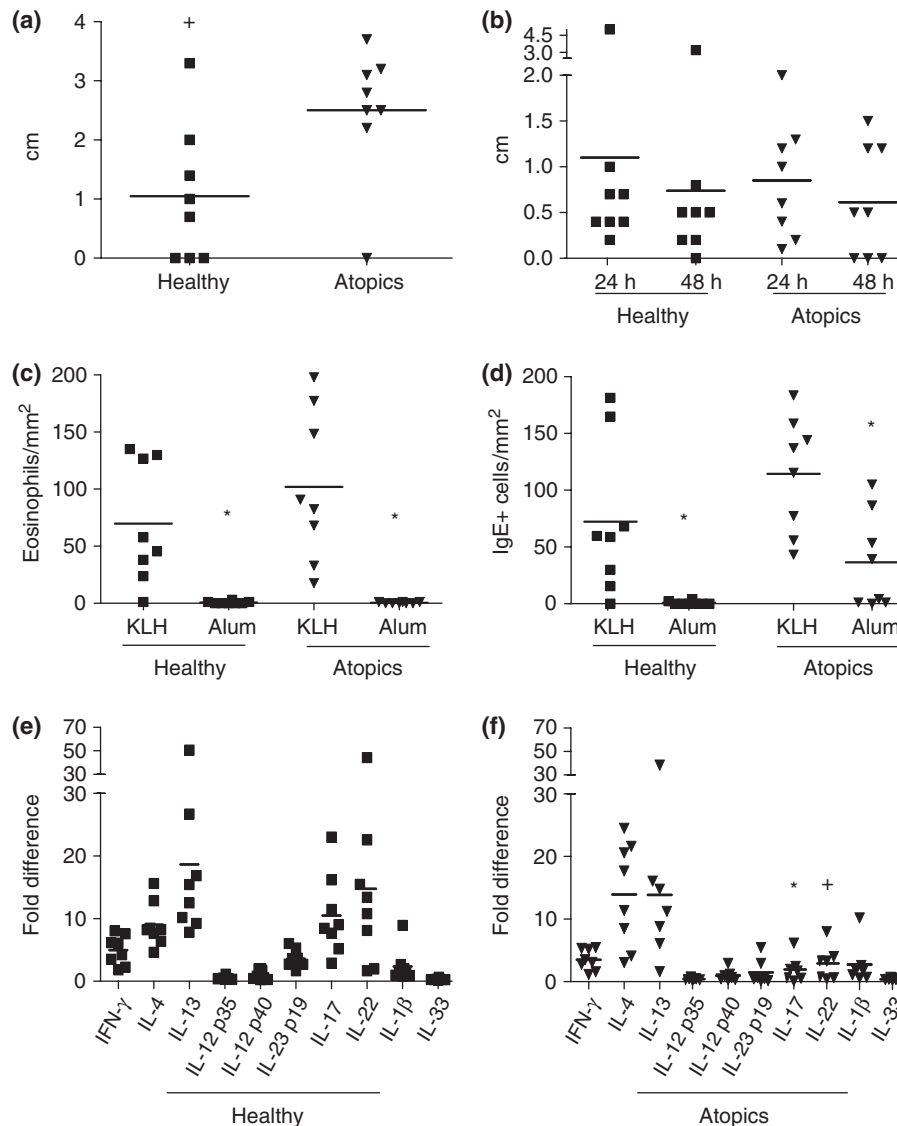


Fig. 4. Assessment of skin responses to Keyhole Limpet Hemocyanin (KLH). (a) Diameter of immediate flare assessed 15–30 min after i.d. KLH injection. Each dot represents one patient, mean is indicated with line. +: $P < 0.05$ (Mann–Whitney U -test). (b) Late-phase skin response (diameter of the induration) assessed 24 and 48 h after i.d. KLH injection. (c) Eosinophils and (d) IgE⁺ cells in cryosections from biopsies taken 48 h after challenge. *: $P < 0.01$ for KLH vs. alum. (e) Fold difference of indicated cytokine transcripts in biopsies from KLH-immunized vs. alum-immunized patients is shown for healthy (e) and atopic (f) subjects. Each dot represents one subject, mean values are indicated with horizontal lines. *: $P < 0.01$ and +: $P < 0.05$ comparing atopic and healthy individuals. Note: stronger up-regulation of IL-4 in atopic vs. healthy subjects is not significant ($P = 0.23$).

IL-12p40 and IL-23p19 levels were almost not or only minimally up-regulated. As Th17 cells have been described to be involved in skin inflammation, in psoriasis [31] as well as in acute lesions of atopic dermatitis [32], expressions of IL-17 and IL-22 transcripts were determined. Both IL-17 and IL-22 showed a significant and strong up-regulation in healthy individuals, but almost no response in atopic subjects. These data suggest that KLH immunization induced a Th2-skewed skin response associated with a Th17 response in healthy volunteers. IL-1 β , a growth factor for Th17 cells in humans [33] was expressed at low levels and not found to be different between atopics and healthy skin.

Discussion

This study describes for the first time a safe and highly specific immunization protocol to generate a *de novo* Th2-skewed immune response in man. Multiple i.m. injections of KLH adsorbed to alum (KLH/alum) induced KLH-specific serum IgG4, high IL-13 production by KLH-activated PBMCs, and a strong IL-4 and IL-13 response in KLH-induced late-phase skin responses. No such KLH-specific immune responses were detected in subjects immunized with alum alone.

In previous trials where KLH was used to immunize human subjects, antigen-specific IgG and IgM production

as well as KLH-specific T cell proliferation was reported, but most groups did not further discriminate between IgG isotypes [8, 9, 11, 12, 34]. Boelens et al. [8] detected KLH-specific IgG1–3, but no IgG4 after a 500 µg single dose. We detected this isotype already in some patients after one KLH immunization and in all subjects after three KLH injections. In contrast to Boelens et al., we used KLH adsorbed to alum that might explain the induction of IgG4. As IgG4 production, like IgE, is thought to be induced by the Th2 cytokines, IL-4 and IL-13 [35–37], the presence of KLH-specific IgG4 (besides antigen-specific IgG1) indicates that our protocol induced a Th2 component. While KLH-specific IgE in sera from KLH/alum-immunized subjects was undetectable with our assay, large numbers of IgE⁺ cells were found in skin biopsies after antigen challenge. This makes it likely that KLH-specific IgE levels in sera were too low to be detectable, but high enough at the site of challenge to induce an immediate-type skin response. The immunoglobulin pattern (high IgG4, low IgE) is therefore reminiscent of the ones reported among animal handlers and beekeepers responding to high-dose exposure to the respective allergens, rat urine and bee venom, and also to the immune response in children exposed to cat allergen [38–40], where high levels of allergen-specific IgG4, but no IgE, have been detected. Concordantly, intra-pulmonary administration of KLH (single 500 µg administration) to allergic asthma patients did not induce any KLH-specific IgE, but KLH-specific IgG4 [41]. In contrast to humans, mouse KLH-specific IgE, besides antigen-specific IgG1 (the mouse homologue of human IgG4), was detectable when KLH adsorbed to aluminium hydroxide was used as a neo-antigen to study Th1/Th2 immune responses [17, 18, 21, 23, 42]. Kolbe et al. [18] have shown that the amount of KLH used for immunization has a strong influence on IgE levels, as high amounts of antigen lead to low production of IgE and vice versa. Such a dose-dependent KLH-specific IgE response was also found in the nasal lavage of atopics after nasal exposure to KLH in conjunction with adjuvant diesel exhaust particles. While low (10 µg) doses lead to KLH-specific IgE production in all subjects, only 57% of individuals were positive for KLH-specific IgE after medium dosage (1000 µg), and only 11% after receiving a very high (100 000 µg) KLH dose [43]. Thus, the possibility remains that the amount of KLH used in our study was too high to induce a strong IgE production detectable in serum. Lower concentrations of i.m. injected KLH/alum in follow-up studies may therefore establish an even stronger Th2 response in man.

The fact that KLH-specific IgE could not be detected in sera might mirror the finding that IL-4 production was found in PBMCs from only 3/16 KLH/alum-immunized subjects. However, all donors showed an IL-4 response following a polyclonal, anti-CD3 stimulus, demonstrating that cells were in general capable of producing IL-4, but

the protocol was sub-optimal in inducing high levels of peripheral antigen-specific IL-4, likely for the reasons discussed above. KLH-specific IL-13 production was high in PBMC in all but one subject, and IL-4 and IL-13 transcripts were highly up-regulated at the site of antigen challenge. This reflects a strong local and moderate peripheral Th2 response. The high local levels of IL-4 and IL-13 might be sufficient to induce a local IgE response. As Th1 cytokine transcripts were not up-regulated in the skin following antigen challenge, these data suggest that the protocol induced a robust local Th2 response. This conclusion is further supported by the observation that the local clinical response follows the pattern of a Th2-driven late-phase skin reaction rather than a typical DTH reaction [27, 29, 44, 45]. While this reaction exhibits its peak between 6 and 24 h, a classical, Th1-mediated DTH shows the strongest reaction at 48–72 h post induction [28, 46]. Furthermore, the demonstration of a large eosinophilic infiltrate in skin biopsies supports the conclusion of a local Th2 response.

Currently, it is unclear what promoted the Th2 response in our protocol. KLH's immunostimulatory property has been mainly linked to the carbohydrate moieties [4], but not much is known about the mode of action of KLH as an inducer of a Th2 response. IL-33, a cytokine that may promote Th2 responses, was expressed at very low levels in the skin and seems therefore unlikely to be a major inducer of a strong local Th2 response. It remains to be clarified whether KLH acts directly on DCs/APCs which then in turn induce the differentiation of Th2 cells, or whether it acts indirectly, for example via Thymic Stromal Lymphopoietin (TSLP), which then activates DCs to induce a Th2 response [47].

Interestingly, transcripts of IL-17 and IL-22 were highly elevated in the skin following antigen challenge in healthy, but not atopic subjects. The significance of this finding is currently unknown. To the best of our knowledge, such a pattern has not been described yet. While IL-22 as a Th17 cytokine is involved in skin inflammation in mice [48] and is, along with IL-17, highly up-regulated in psoriasis skin [31], there is little known about its contribution to antigen-induced late-phase responses in atopics. IL-17 was identified in acute lesions of atopic dermatitis [32], but was found to be severely impaired in patients with Hyper-IgE-Syndrome [49]. IL-1β, required for the differentiation of human Th17 cells, was expressed at low levels in the skin and was not different between healthy and atopic subjects. Thus, the reason for the lack of Th17 cells in atopic subject's skin remains currently unknown.

In summary, a protocol to induce a strong local and moderate peripheral, antigen-specific *de novo* Th2 response to KLH/alum in humans has been presented. This approach might be of particular interest to study a developing Th2 response in humans and to investigate novel immunomodulatory regimens aiming to interfere with the development of a Th2 response.

Acknowledgements

This study was sponsored by Novartis Exploratory Development, all authors marked with the symbol (†) are Novartis employees. We thank Arabelle Pfrunder for her excellent logistical work to conduct the study. Dr Stingl has provided consultancy to Novartis in the last years with an income of less than 10 000 USD per year.

References

- Loza MJ, Peters SP, Penn RB. Atopy, asthma, and experimental approaches based on the linear model of T cell maturation. *Clin Exp Allergy* 2005; 35:8–17.
- Holgate ST. The epithelium takes centre stage in asthma and atopic dermatitis. *Trends Immunol* 2007; 28:248–51.
- Bullens DM, Van Den Keybus C, Dilissen E, Kasran A, Ceuppens JL. Allergen-specific T cells from birch-pollen-allergic patients and healthy controls differ in T helper 2 cytokine and interleukin-10 production. *Clin Exp Allergy* 2004; 34:879–87.
- Harris JR, Markl J. Keyhole limpet hemocyanin (KLH): a biomedical review. *Micron* 1999; 30:597–623.
- Brown MJ, Coltart J, Gunewardena K, Ritter JM, Auton TR, Glover JF. Randomized double-blind placebo-controlled study of an angiotensin immunotherapeutic vaccine (PMD3117) in hypertensive subjects. *Clin Sci (Lond)* 2004; 107:167–73.
- Lamm DL, Dehaven JI, Riggs DR. Keyhole limpet hemocyanin immunotherapy of bladder cancer: laboratory and clinical studies. *Eur Urol* 2000; 37 (Suppl. 3):41–4.
- Schenkman E, Lamm DL. Superficial bladder cancer therapy. *Sci World J* 2004; 4 (Suppl. 1):387–99.
- Boelens PG, Fonk JC, Houdijk AP *et al.* Primary immune response to keyhole limpet haemocyanin following trauma in relation to low plasma glutamine. *Clin Exp Immunol* 2004; 136:356–64.
- Miller JS, Curtsinger J, Berthold M *et al.* Diminished neo-antigen response to keyhole limpet hemocyanin (KLH) vaccines in patients after treatment with chemotherapy or hematopoietic cell transplantation. *Clin Immunol* 2005; 117:144–51.
- Rentenaar RJ, van Diepen FN, Meijer RT *et al.* Immune responsiveness in renal transplant recipients: mycophenolic acid severely depresses humoral immunity in vivo. *Kidney Int* 2002; 62:319–28.
- Kraus TA, Toy L, Chan L, Childs J, Mayer L. Failure to induce oral tolerance to a soluble protein in patients with inflammatory bowel disease. *Gastroenterology* 2004; 126:1771–8.
- Moldoveanu Z, Oliver F, Mestecky J, Elson CO. Oral tolerance in humans: failure to suppress an existing immune response by oral antigen administration. *Ann N Y Acad Sci* 2004; 1029:299–309.
- Jefferson T, Rudin M, Di Pietrantonj C. Adverse events after immunisation with aluminium-containing DTP vaccines: systematic review of the evidence. *Lancet Infect Dis* 2004; 4:84–90.
- HogenEsch H. Mechanisms of stimulation of the immune response by aluminium adjuvants. *Vaccine* 2002; 20 (Suppl. 3):S34–9.
- Lindblad EB. Aluminium adjuvants – in retrospect and prospect. *Vaccine* 2004; 22:3658–68.
- Jordan MB, Mills DM, Kappler J, Marrack P, Cambier JC. Promotion of B cell immune responses via an alum-induced myeloid cell population. *Science* 2004; 304:1808–10.
- Germann T, Guckes S, Bongartz M *et al.* Administration of IL-12 during ongoing immune responses fails to permanently suppress and can even enhance the synthesis of antigen-specific IgE. *Int Immunol* 1995; 7:1649–57.
- Kolbe L, Heusser CH, Kolsch E. Isotype-associated recognition of allergen epitopes and its modulation by antigen dose. *Immunology* 1995; 84:285–9.
- Kelso A, Groves P, Ramm L, Doyle AG. Single-cell analysis by RT-PCR reveals differential expression of multiple type 1 and 2 cytokine genes among cells within polarized CD4+ T cell populations. *Int Immunol* 1999; 11:617–21.
- Kelso A, Groves P, Troutt AB, Francis K. Evidence for the stochastic acquisition of cytokine profile by CD4+ T cells activated in a T helper type 2-like response in vivo. *Eur J Immunol* 1995; 25:1168–75.
- Kelso A, Groves P, Troutt AB, Pech MH. Rapid establishment of a stable IL-4/IFN- γ production profile in the antigen-specific CD4+ T cell response to protein immunization. *Int Immunol* 1994; 6:1515–23.
- Falcone M, Bloom BR. A T helper cell 2 (Th2) immune response against non-self antigens modifies the cytokine profile of auto-immune T cells and protects against experimental allergic encephalomyelitis. *J Exp Med* 1997; 185:901–7.
- Yeung VP, Gieni RS, Umetsu DT, DeKruyff RH. Heat-killed *Listeria monocytogenes* as an adjuvant converts established murine Th2-dominated immune responses into Th1-dominated responses. *J Immunol* 1998; 161:4146–52.
- Maecker HT, Do MS, Levy S. CD81 on B cells promotes interleukin 4 secretion and antibody production during T helper type 2 immune responses. *Proc Natl Acad Sci USA* 1998; 95:2458–62.
- Berger S, Ballo H, Stutte HJ. Distinct antigen-induced cytokine pattern upon stimulation with antibody-complexed antigen consistent with a Th1 $\rightarrow$ Th2-shift. *Res Virol* 1996; 147:103–8.
- Moverare R, Elfman L, Stalenheim G, Bjornsson E. Study of the Th1/Th2 balance, including IL-10 production, in cultures of peripheral blood mononuclear cells from birch-pollen-allergic patients. *Allergy* 2000; 55:171–5.
- Benson M, Carlsson L, Guillot G *et al.* A network-based analysis of allergen-challenged CD4+ T cells from patients with allergic rhinitis. *Genes Immun* 2006; 7:514–21.
- Dannenberg AM. Jr. Delayed-type hypersensitivity and cell-mediated immunity in the pathogenesis of tuberculosis. *Immunol Today* 1991; 12:228–33.
- Phipps S, Ying S, Wangoo A, Ong YE, Levi-Schaffer F, Kay AB. The relationship between allergen-induced tissue eosinophilia and markers of repair and remodeling in human atopic skin. *J Immunol* 2002; 169:4604–12.
- Schmitz J, Owyang A, Oldham E *et al.* IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005; 23:479–90.
- Haider AS, Lowes MA, Suarez-Farinas M *et al.* Identification of cellular pathways of “type 1,” Th17 T cells, and TNF- and inducible nitric oxide synthase-producing dendritic cells in autoimmune inflammation through pharmacogenomic study of cyclosporine A in psoriasis. *J Immunol* 2008; 180:1913–20.
- Toda M, Leung DY, Molet S *et al.* Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. *J Allergy Clin Immunol* 2003; 111:875–81.

- 33 Laurence A, O'Shea JJ. T(H)-17 differentiation: of mice and men. *Nat Immunol* 2007; **8**:903–5.
- 34 Husby S, Mestecky J, Moldoveanu Z, Holland S, Elson CO. Oral tolerance in humans. T cell but not B cell tolerance after antigen feeding. *J Immunol* 1994; **152**:4663–70.
- 35 Platts-Mills TA, Vaughan JW, Blumenthal K, Woodfolk JA, Sporik RB. Decreased prevalence of asthma among children with high exposure to cat allergen: relevance of the modified Th2 response. *Mediators Inflamm* 2001; **10**:288–91.
- 36 Poulsen LK, Hummelshoj L. Triggers of IgE class switching and allergy development. *Ann Med* 2007; **39**:440–56.
- 37 Vercelli D. One cytokine, two isotypes: a trojan horse, pandora's box, and an evolving paradigm. *Am J Respir Crit Care Med* 2000; **162**:S86–90.
- 38 Aalberse RC, Platts-Mills TA. How do we avoid developing allergy: modifications of the TH2 response from a B-cell perspective. *J Allergy Clin Immunol* 2004; **113**:983–6.
- 39 Platts-Mills T, Vaughan J, Squillace S, Woodfolk J, Sporik R. Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. *Lancet* 2001; **357**:752–6.
- 40 Aalberse RC, van der Gaag R, van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *J Immunol* 1983; **130**:722–6.
- 41 Schuyler M, Lyons CR, Masten B, Bice D. Immunoglobulin response to intrapulmonary immunization of asthmatics. *Immunology* 1997; **91**:167–75.
- 42 Doyle AG, Ramm L, Kelso A. The CD4+ T-cell response to protein immunization is independent of accompanying IFN-gamma-producing CD8+ T cells. *Immunology* 1998; **93**:341–9.
- 43 Riedl MA, Landaw EM, Saxon A, Diaz-Sanchez D. Initial high-dose nasal allergen exposure prevents allergic sensitization to a neoantigen. *J Immunol* 2005; **174**:7440–5.
- 44 Gaga M, Frew AJ, Varney VA, Kay AB. Eosinophil activation and T lymphocyte infiltration in allergen-induced late phase skin reactions and classical delayed-type hypersensitivity. *J Immunol* 1991; **147**:816–22.
- 45 Phipps S, Flood-Page P, Menzies-Gow A, Ong YE, Kay AB. Intravenous anti-IL-5 monoclonal antibody reduces eosinophils and tenascin deposition in allergen-challenged human atopic skin. *J Invest Dermatol* 2004; **122**:1406–12.
- 46 Vukmanovic-Stejić M, Reed JR, Lacy KE, Rustin MH, Akbar AN. Mantoux test as a model for a secondary immune response in humans. *Immunol Lett* 2006; **107**:93–101.
- 47 Liu YJ. Thymic stromal lymphopoietin and OX40 ligand pathway in the initiation of dendritic cell-mediated allergic inflammation. *J Allergy Clin Immunol* 2007; **120**:238–44.
- 48 Zheng Y, Danilenko DM, Valdez P *et al*. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* 2007; **445**:648–51.
- 49 Milner JD, Brenchley JM, Laurence A *et al*. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 2008; **452**:773–6.