

Original article

Analysis of the CD4⁺ T cell responses to house dust mite allergoid

Background: Modified allergen extracts (allergoids) with reduced IgE-binding capacity are successfully used in immunotherapy of atopic allergy. Their reduced T-cell stimulatory capacity is less well studied and is a subject of the present study.

Methods: We compared the ability of native house dust mite extract (*Dermatophagoides pteronyssinus*; HDM) and the glutaraldehyde-modified allergoid (HDM-GA) to induce the proliferation and cytokine production by fresh PBMC and by DC-stimulated polyclonal Th cells and HDM-specific Th cell clones.

Results: Freshly isolated T cells showed a partially reduced responsiveness to HDM-GA, differentially pronounced in different donors. HDM-specific Th cell clones prepared from three donors showed either a complete loss of reactivity to HDM-GA, or completely preserved responsiveness. The frequency of nonreactive clones was donor-dependent (2/3, 3/10 and 1/10). GA modification of HDM did not interfere with the cytokine production profile of HDM-specific T cell clones.

Conclusions: The reduced stimulatory potential of HDM-GA results mainly from a loss of certain Th cell epitopes, rather than impaired allergen uptake and presentation, or induction of suppressive factors. Varying frequencies of allergoid-nonreactive HDM-specific Th cells may result in differential responses of individual patients to immunotherapy.

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Atopic allergic diseases (e.g. atopic dermatitis, allergic rhinitis and allergic asthma) are immune disorders caused by aberrant Th2 cell-dominated responses to otherwise innocuous substances, such as proteins from house dust mite (HDM) or grass or tree pollen (1). Allergen-specific immunotherapy is frequently used for the treatment of HDM and pollen allergies. The mechanism of this therapy, although still not entirely clear, is based on the counteraction of the undesirable Th2 bias in allergen-specific Th cells and downregulation of the Th2-type cytokines IL-4, IL-5 and IL-13, upregulation of Th1 cytokine IFN- γ and the induction of IL-10 (1–10), a cytokine with a general immunosuppressive function (11). While in human cells IL-10 production can be induced in both Th1 and Th2 cells (12), the recently defined

T regulatory (Tr1 or Treg) cells that selectively produce IL-10 (13) are good candidates as regulatory cells induced by immunotherapy (14, 15).

The safety of allergen immunotherapy is improved by the glutaraldehyde or paraformaldehyde modification of allergen extracts (resulting in the so-called allergoids), which destroys or masks their IgE-binding sites and reduces the risk of anaphylactic shock (1, 2, 16). In addition to the decreased IgE binding, allergoids also display reduced antigenicity towards Th cells (17–19), the mechanisms of which are not entirely clear. It may result from a reduced allergen-specific proliferation of all individual Th cells, e.g., as a consequence of reduced uptake of allergoids by APC, their impaired processing, reduced ability to activate APC, or inhibition of APC functions. Alternatively, reduced allergen-specific proliferation may result from the unresponsiveness of a part of allergen-specific T cells, as a consequence of the degradation of certain Th cell epitopes.

Abbreviations: DC, dendritic cell; HDM, native house dust mite extract; HDM-GA, glutaraldehyde-modified HDM allergoid; TCC, T helper cell clones; Th cell, T helper cell.

A single study on this issue, previously performed with birch pollen allergoid, showed a loss of the reactivity of selected allergen-specific T cells, indicating that some T cell epitopes have been lost upon paraformaldehyde treatment (17). Another study addressing this issue showed that the presentation of allergoid by dendritic cells (DCs), rather than nonprofessional APC, can restore the reactivity of grass pollen (Phl p)-specific T cell clones (19). Here, we present a similar study on HDM allergoid, comparing the Th cell-stimulatory activity of native HDM and glutaraldehyde-modified HDM (HDM-GA) at the polyclonal and clonal levels, which is extended by an analysis of the ability of these compounds to induce the production of the Th1, Th2 and Tr1 signature cytokines IFN- γ , IL-4 and IL-10.

Materials and methods

Allergens

House dust mite (*Dermatophagoides pteronyssinus*) bodies were obtained from Biopol (Spokane, USA) and contained >99% pure adult mites and mite stages. Mite extract was obtained by extracting 100 g crushed mite bodies for 24 h at 4°C in 2 l of buffer containing 5 g NaCl, 2.5 g NaHCO₃ and 1 g NaN₃ per litre of distilled water. The extract was filtered using filters ranging from 8 to 0.2 μ m and subsequently diafiltrated against distilled water using a Millipore set-up with a Centrasette Nova 5 K membrane (Filtron Technology Corporation, Northborough, USA). After diafiltration the extract was lyophilized. Modified mite extract was obtained by preparing a 10 mg/ml solution of lyophilized mite material in 0.1 M phosphate buffered at pH 6.5. Subsequently, 50% glutaraldehyde (6 g per litre solution) was added to the mite solution and the mixture was stirred for 15 min and stored overnight at room

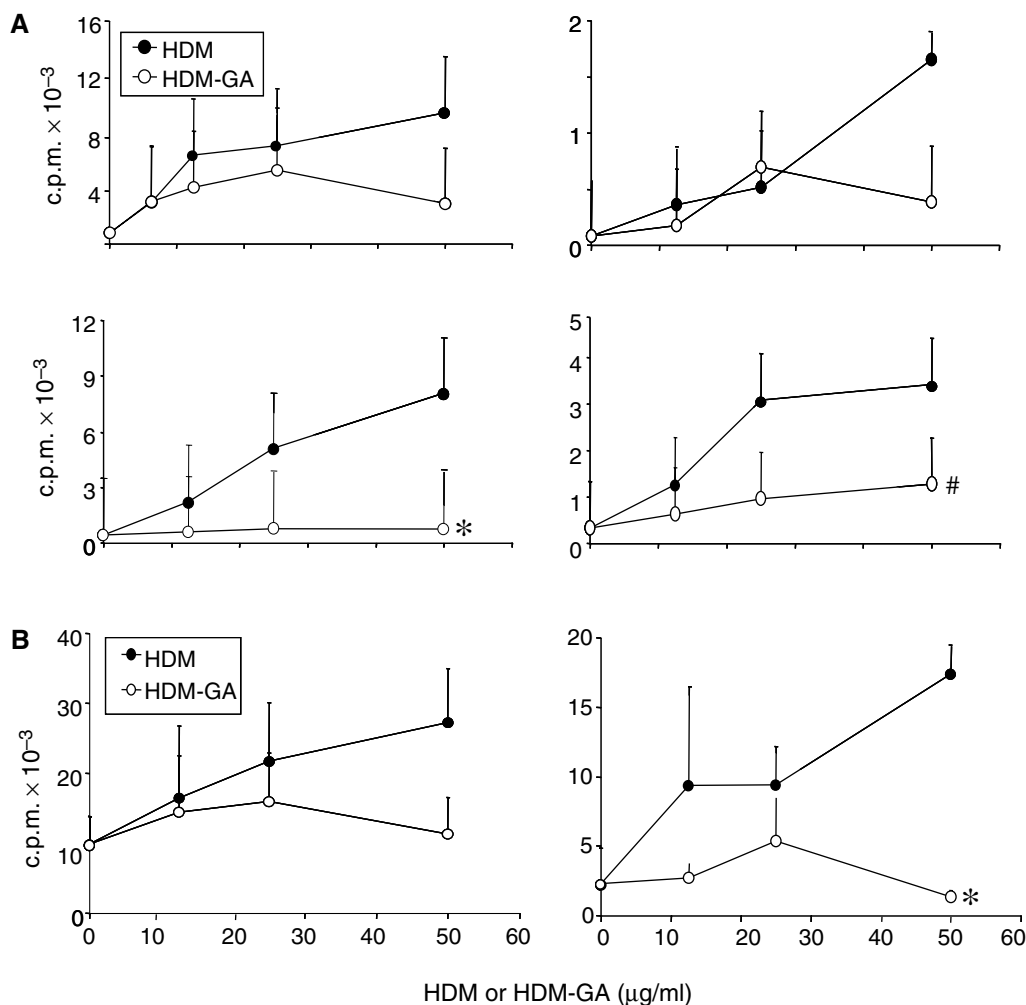


Figure 1. Decreased proliferation of PBMCs and CD4⁺ Th cells to HDM-GA. (A) PBMCs from distinct donors were stimulated with indicated doses of HDM or HDM-GA. Proliferation was measured by adding ³H-labelled thymidine at day 6 and harvesting the radio-labelled cells 18 h later. (B) Purified CD4⁺ Th cells (10^5 cells/well) were cocultured with DCs (2×10^4) in the presence of increasing concentrations of HDM or HDM-GA protein (Bradford). Proliferation was measured by adding ³H-labelled thymidine at day 4 and harvesting the cells 18 h later. * $P < 0.05$, # $P < 0.06$. In both types of experiments, the differences in the magnitude of the response induced by HDM vs HDM-GA were determined by the Kruskal–Wallis test.

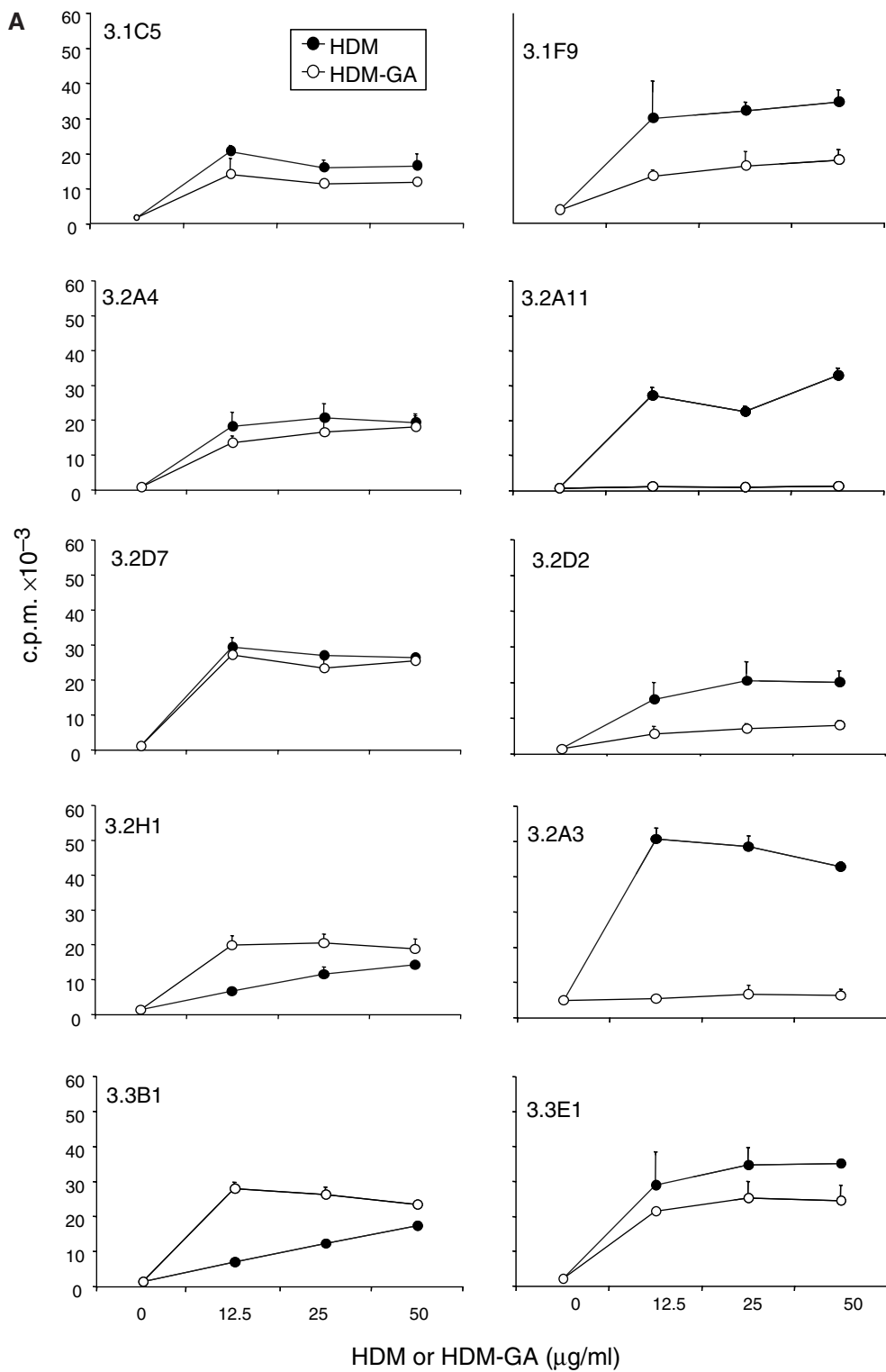


Figure 2. Selective loss of reactivity to modified HDM in 23 HDM-specific TCCs. Resting (days 10–14 after the last stimulation with feeder cells) HDM-reactive TCCs at 3×10^4 cells per well were cocultured with DCs at 2×10^4 cells per well in the presence of increasing concentrations of HDM or HDM-GA. Proliferation was measured by adding ^3H thymidine at 24 h and harvesting the radio-labelled cells 18 h later. The results shown were obtained with TCC from three different donors (A–C).

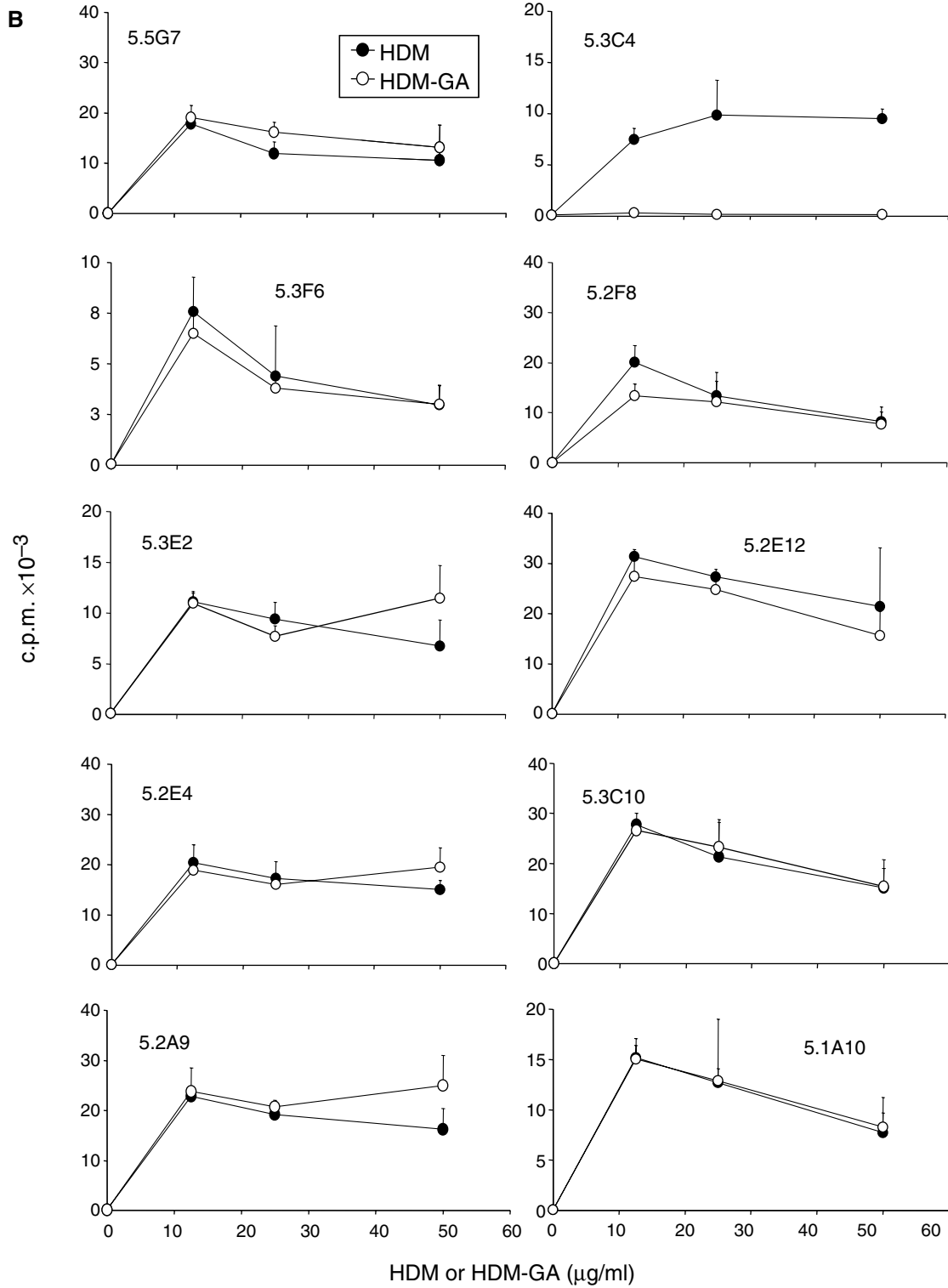


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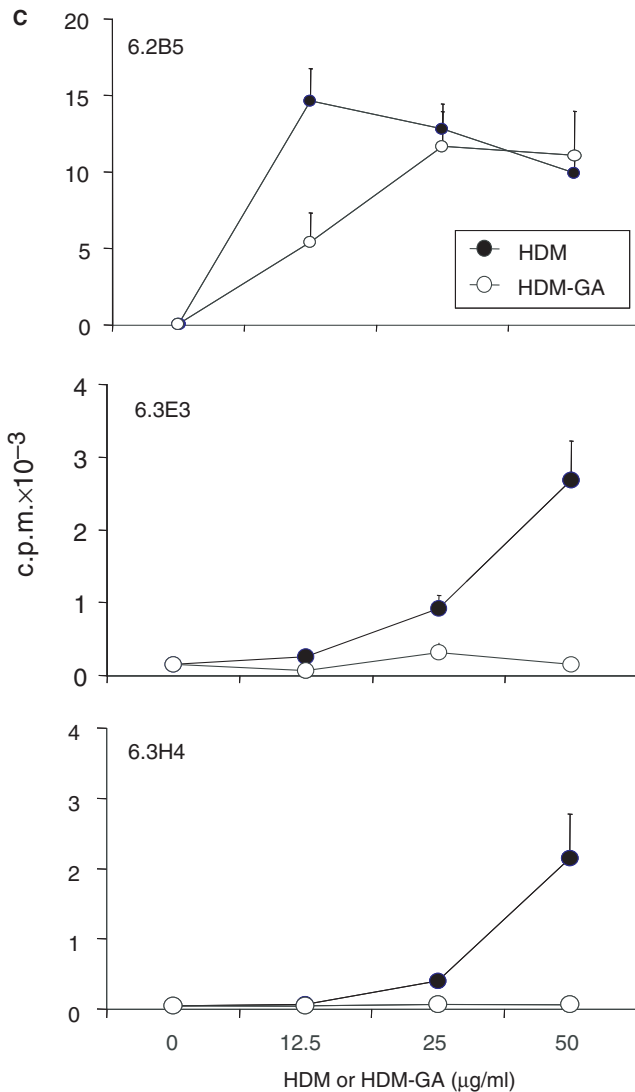


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temperature. The solution was diafiltrated against distilled water using a Pall Filtron mini Ultrasette 5 K diafiltration cassette (Filtron Technology Corporation) after which glycine (15 g per litre solution) was added. After 30 min of stirring, phosphate salts were added to obtain a 0.1 M phosphate buffer of pH 6.5 and the solution was stored overnight at room temperature. The solution was subsequently diafiltrated against distilled water, using a Pall Filtron mini Ultrasette 100 K diafiltration cassette (Filtron Technology Corporation). All chemicals used were from Merck (Darmstadt, Germany).

Isolation of PBMC, bulk and naïve CD4^+ T cells and monocytes

Peripheral blood mononuclear cells (PBMC) from allergic individuals with a history of allergic rhinitis to house dust and positive skin tests to HDM, not receiving any form of allergen-based immunotherapy, were obtained by Ficoll (Nycomed, Oslo, Norway) centrifugation (20–25), and either analysed directly thereafter or used to generate HDM-specific lines and clones. Autologous monocytes

were purified (>90% purity) from PBMC by density-gradient centrifugation using Percoll (Pharmacia, Uppsala, Sweden). CD4^+ Th cells were purified (>98% purity) from the remaining fraction using negative selection columns (R & D Systems Europe Ltd., Abingdon, UK).

Generation of monocyte-derived DCs

To obtain autologous DCs, monocytes (5×10^5 cells/ml) from individual donors were cultured for 6 days in 24-well plates (Costar, Cambridge, MA) in IMDM with 10% FCS (Hyclone) and gentamycin (80 $\mu\text{g/ml}$; Duchefa, Haarlem, The Netherlands) supplemented with rhuGM-CSF (500 U/ml; a gift of Schering-Plough, Uden, The Netherlands) and rhuIL-4 (250 U/ml; PBH), as described earlier (20, 21). At day 6, the cultured cells consisted of uniformly HLA-DR^+ , CD83^- , $\text{CD40}^{\text{high}}$ DCs, without detectable CD3^+ cells. Over 90% of the cells expressed high levels of CD1a. Cell-surface phenotype was analysed by flow cytometry, after labelling of cells with appropriate mAb, as described earlier (20, 21).

Generation of allergen-specific Th cell clones (TCCs)

HDM-reactive TCCs were generated from the peripheral blood as described earlier (22–24). In brief, PBMC were cultured for 14 days in IMDM supplemented with 5% normal human serum (Bio-Whittaker, Walkersville, MD) and gentamycin (80 $\mu\text{g/ml}$; Duchefa, Haarlem, The Netherlands) in the presence of native HDM (50 $\mu\text{g/ml}$ Bradford) for 14 days. rIL-2 (10 U/ml; Chiron, Emeryville, CA) was first added on day 5, followed by addition on days 8 and 11. HDM-specific cell lines were subsequently cloned by limiting dilution and expanded in the presence of the IL-2- and PHA-containing feeder mix (22–24). They were tested for specificity using 3000R-irradiated autologous PBMC as a source of APC. The clones responding to native HDM with a stimulation index of ≥ 10 were considered HDM-reactive and were used in subsequent experiments. All these clones expressed CD4.

Allergen-specific proliferation of freshly isolated PBMCs, Th cells or TCCs

Proliferation of PBMC to HDM and HDM-GA was determined upon culture of 2×10^5 cells per well in IMDM supplemented with 5% normal human serum (Bio-Whittaker, Walkersville, MD) and gentamycin (80 $\mu\text{g/ml}$; Duchefa, Haarlem, The Netherlands). The indicated amount of allergen or allergoid was added at day 0, followed by addition of ^3H -labelled thymidine at day 6 and harvesting the radioactively labelled cells at day 7 to measure the Ag-induced proliferation. To determine the proliferation of pure Th cells to HDM or HDM-GA, 1×10^5 freshly isolated CD4^+ Th cells/well or 3×10^4 cells HDM-reactive TCC/well (used at days 10–14 after last stimulation with feeder cells) were cocultured with DCs (2×10^4) or monocytes (6×10^4) as APC in a final volume of 0.2 ml (20, 21), in the presence of increasing concentrations of HDM or HDM-GA, as indicated. Proliferation was determined by addition of tritiated thymidine 18 h before the termination of the assay.

Cytokine production of HDM-reactive TCCs

The cytokine profiles of TCCs were initially screened after stimulation for 24 h with CD3 mAb (1 $\mu\text{g/ml}$; CLB-T3/3, CLB, Amsterdam, The Netherlands) and CD28 mAb (2 $\mu\text{g/ml}$; CLB-CD28/1, CLB), as described earlier (20, 21). To analyse the cytokine production of TCCs upon stimulation by HDM or HDM-GA

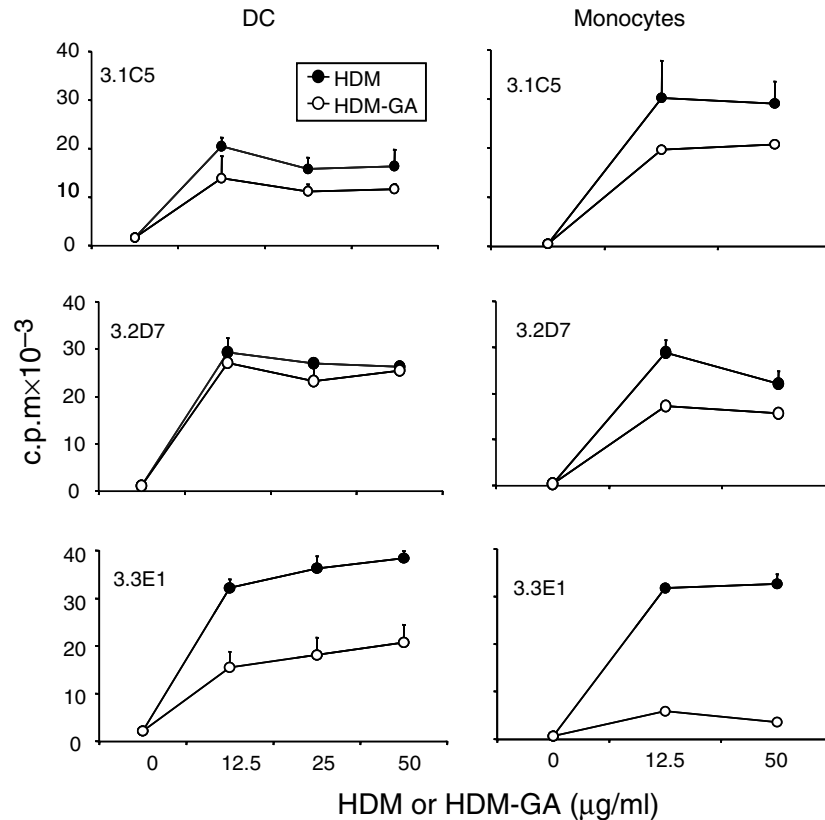


Figure 3. Allergoid-reactive clones respond to the antigen presented by DCs or monocytes. Resting (days 10–14 after last stimulation with feeder cells) HDM-specific TCCs at 3×10^4 cells per well were cocultured with DCs at 2×10^4 cells per well or monocytes at 6×10^4 cells per well in the presence of increasing concentrations of HDM or HDM-GA. Proliferation was measured by adding ^3H thymidine at 24 h (DC) or 48 h (monocytes) and harvesting the radio-labelled cells 18 h later. Results are shown from a representative experiment out of three.

presented by DC, 1×10^5 TCCs (Th0/Th2-type: 6.4B9 and 6.1G2, and Th2/Tr1-type: 6.2G9) were cocultured with 2×10^4 DCs for 48 h. The levels of IFN- γ , IL-4 and IL-10 were determined in supernatants by specific ELISAs, as described before (21, 26).

Statistical analysis

Data were analysed for statistical significance with the SPSS software (version 11.5; SPSS Inc., Chicago, IL). The responses of PBMCs and polyclonal CD4⁺ T cells to increasing concentrations of HDM and HDM-PFA were compared using the nonparametric Kruskal–Wallis test. The IL-4/IFN- γ ratios observed in TCCs stimulated by DCs presenting HDM or HDM-GA, or by the combination of CD3 and CD28 mAbs, were compared using paired samples *t*-test. A *P* value < 0.05 was considered as the level of significance.

Results

HDM-GA shows partially reduced stimulatory activity for PBMCs and its CD4⁺ Th cell fraction

To compare the T cell stimulatory capacity of native HDM and HDM-GA, PBMCs of four donors were stimulated with increasing concentrations of these antigen

forms. In accordance with previous reports (17–19), the modification of allergen resulted in reduced proliferation of PBMCs (Fig. 1A). The degree of this reduction was highly variable between individual donors tested, ranging from a nearly complete to only marginal inhibition. Reduced stimulatory capacity of the allergoid was also observed in the cultures of freshly isolated CD4⁺ Th cells stimulated by autologous DCs (Fig. 1B).

Most of the individual HDM-specific clones show either an undisturbed responsiveness to HDM-GA or a complete lack of responsiveness

To address the mechanism underlying the reduced proliferative response of bulk populations of Th cells to HDM allergoid, HDM-reactive Th cells were cloned (22–24) from three different donors. The proliferation of the clones (TCCs) was tested using autologous DCs as APC. As shown in Fig. 2, the majority of the HDM-reactive TCCs displayed one of two patterns of responsiveness: fully preserved reactivity against HDM-GA or a complete lack of reactivity. The frequency of allergoid-nonreactive clones showed high variability in the three

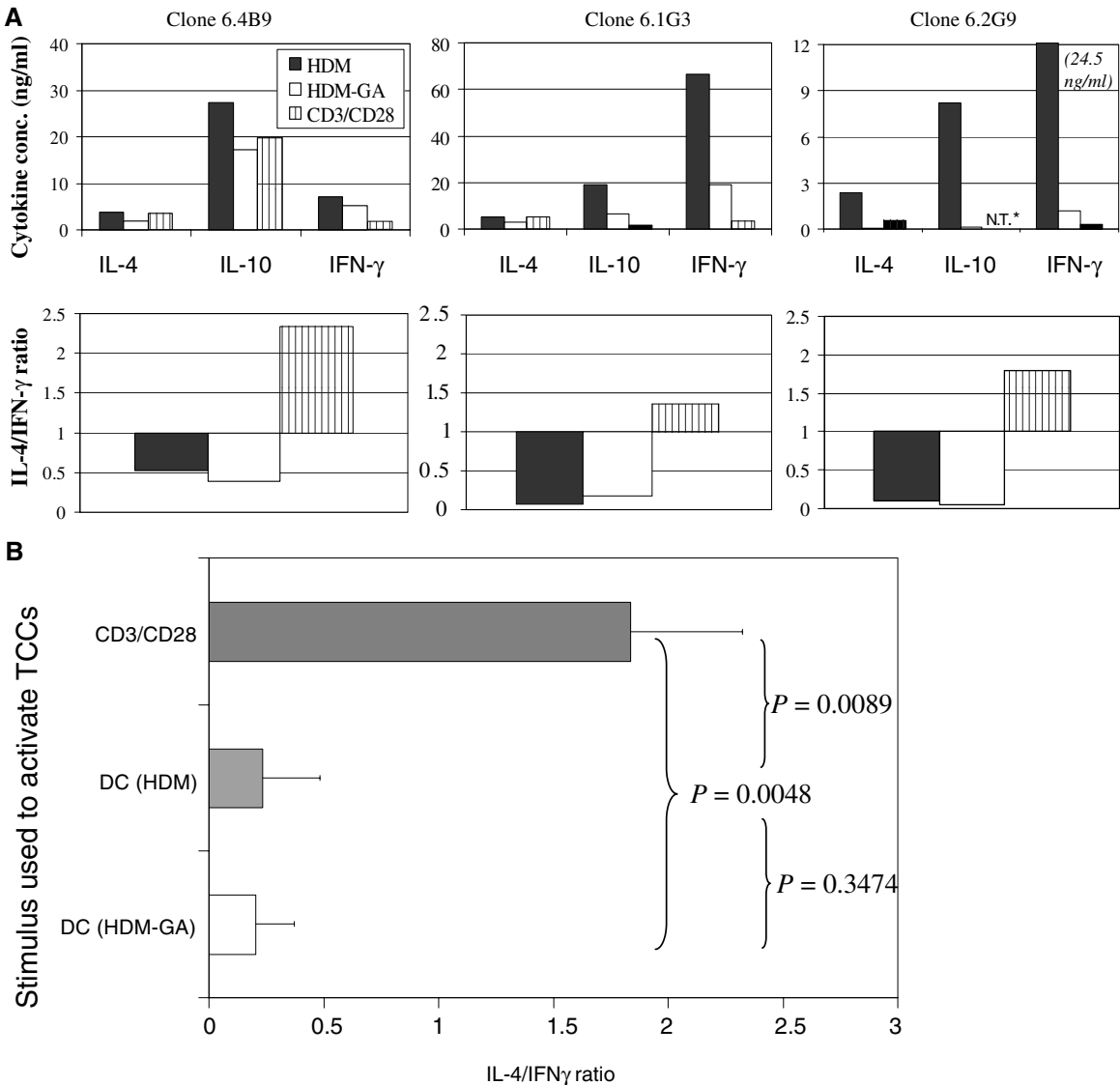


Figure 4. HDM and HDM-GA induce similar cytokine production profiles in double-reactive TCCs. Resting (days 10–14 after the last stimulation with feeder cells) HDM-reactive TCCs (clones 6.4B9, 6.1G3, and 6.2G9) at 1×10^5 cells per well were cocultured with DCs at 2×10^4 cells per well in the presence of 25 $\mu\text{g/ml}$ of native HDM or HDM-GA, or alternatively, they were stimulated with the combination of CD3 and CD28 mAbs (20, 21, 25). Supernatants were harvested after 48 h and the concentrations of IFN- γ , IL-4 and IL-10 were measured with specific ELISA. (A) Cytokine production pattern in individual TCCs. *IL-10 data not available; clone 6.2G9 has been classified as Th2/Tr1-like (with the IL-10 production of 12.8 ng/ml, as determined in the earlier screening experiment). (B) Cumulative analysis of the responses of the three HDM-specific clones (6.4B9, 6.1G3 and 6.2G9). The IL-4/IFN- γ ratios induced by DCs presenting either native HDM or HDM-GA were significantly lower than those induced by CD3/CD28 stimulation (21), and similar to each other, as determined by paired *t*-test, analysing the responses of the three clones.

donors tested: three (3.2A11, 3.2A3 and 3.2D2) out of 10 (Fig. 2A); one (5.3C4) out of 10 (Fig. 2B); and two (6.2E3 and 6.3H4) out of three (Fig. 2C). The complete loss of reactivity in part of the clones is consistent with a selective loss of some Th cell epitopes as a result of glutaraldehyde treatment of HDM, a possibility previously demonstrated in the case of birch pollen (17). Only two of 23 clones (3.1F9 and 3.2D2) demonstrated a

partial loss of responsiveness to the allergoid (Fig. 2A). The preserved or lost reactivity to HDM-GA did not correlate with the cytokine production profiles of TCCs, which were predominantly Th2 and Th0 type, with lesser frequencies of Th1- and Tr1-like clones (data not shown).

Using two double-reactive clones, we compared the presentation of HDM and HDM-GA by different APC

types, i.e. freshly isolated peripheral blood monocytes and monocyte-derived DCs. Although the kinetics of T cell responses to monocyte-presented antigens was delayed compared to their responses to DC-presented antigen (data not shown), monocytes proved to be able to efficiently present both allergen and allergoid (Fig. 3).

Native and modified HDMs induce similar cytokine profiles

We have previously demonstrated that IL-12-producing DCs can revert the Th2-dominated cytokine production profiles (21), a phenomenon with a potential relevance for the immunotherapy of atopic diseases. To test whether the DC presentation of the native allergen and allergoid can both result in such desirable Th1-skewing effect, we analysed the production of Th1 and Th2 cytokines by two Th0/Th2-type and one Th2/Tr1-type TCCs. As shown in Fig. 4, the stimulation of TCCs by allergen and allergoid presented by DCs resulted in similar production ratios of Th1-, Th2- and Tr1-type cytokines: IL-4, IFN- γ and IL-10. DC presentation of both HDM and HDM-GA resulted in strongly reduced IL-4/IFN- γ ratios, compared to those observed after the stimulation of TCCs with CD3 and CD28 mAbs.

Discussion

Although allergoids are successfully used in immunotherapy of atopic allergy (27, 28), they show a reduced potential to stimulate Th cells, the population of immune cells believed to be a pivotal target of immunotherapy. Studies on the mechanism of this reduced T cell stimulatory capacity of allergoids are sparse. The results of our current analysis, showing the loss of responsiveness to allergoid in part of the TCCs with a simultaneous unchanged responsiveness of remaining clones, indicate that the reduced stimulatory capacity of HDM-GA primarily results from a selective loss of a part of Th cell epitopes in the course of glutaraldehyde treatment. The unchanged reactivity of the majority of the remaining clones to allergoid argues against the possibility that the decreased stimulatory activity of allergoid results from a defective uptake and/or processing of allergoid by APC, or from the induction of any suppressive factor(s). These data support a previous study on the mechanism of reduced T cell stimulatory capacity of modified allergen, reporting the selective loss of Th cell epitopes in birch pollen allergoid (17). Among the 23 TCCs tested, only two clones showed a quantitatively reduced ability to react to HDM-GA. This partial reactivity was independent of the dose of allergoid. The mechanism underlying this intermediate reactivity on the clonal level is currently unclear but may result from an incomplete destruction of the relevant epitope.

In contrast to the current findings and the study by Dormann et al. (17), all allergen-specific clones tested in a study by Kahlert et al. (18) showed full responsiveness to allergoid when it was presented by DCs, in contrast to nonprofessional APC. However, the low number of clones (five) tested in their study may have been too low to reveal the occurrence of allergoid nonresponsive cells. In the current study, although the proliferative responses to HDM and HDM-GA presented by monocytes showed a delayed kinetics compared to the responses mediated by DCs, both the native allergen and allergoid were successfully presented to the three clones tested. Interestingly, in the case of one clone (3.3E1) showing a partially reduced responsiveness to the allergoid presented by DCs, this reduction was even more pronounced when the allergoid was presented by monocytes, reminiscent of the data from Kahlert et al. (18).

Interestingly, the loss of reactivity to allergoid in a proportion allergen-specific TCC apparently does not hamper its clinical effectiveness, as judged by the clinical effectiveness of this form of immunotherapy (27, 28). The mechanism of suppression of the pathogenic activity of any allergoid-nonresponsive clones may be analogous to the 'infectious tolerance' demonstrated in several animal models (29–31).

When tested as the antigens presented by DCs, both these compounds induced a similar cytokine profile in the HDM-specific TCCs. These data strongly suggest that glutaraldehyde modification of HDM does not induce the capacity to polarize the cytokine profile in Th cells. Interestingly, the presentation of both forms of allergen by DCs was similarly effective in reducing the IL-4/IFN- γ ratios in HDM-specific TCCs, supporting the notion that IL-12-producing DCs (as opposed to B cells) are able to reverse the IL-4-dominated cytokine profiles of initially Th2-type cells (21).

In summary, the present results indicate that the reduced T cell-stimulatory activity of HDM allergoid results from a lower frequency of responding Th cells rather than from any defect of all HDM-reactive Th cells to respond to the allergoid. This suggests that allergen and allergoid have comparable immunoregulatory activities and that the beneficial impact of allergoid-based immunotherapy results from repetitive stimulation of pathogenic HDM-reactive Th cells, rather than from any intrinsic ability of allergoid to induce a different type of immune response than the native allergen.

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