

Allergen-specific immune deviation from a T_H2 to a T_H1 response induced by dendritic cells and collagen type I

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Background: Atopy and IgE production are associated with enhanced allergen-specific T_H2 responses. Therefore a causative treatment may result from the deviation of this T_H2-dominated immune response toward a T_H1 response.

Objective: This study was carried out to analyze whether dendritic cells, the most potent antigen-presenting cells that are also known to induce antigen-specific T_H1 responses, are suitable for therapy of atopic diseases by shifting the allergen-specific T_H2 response toward a T_H1 response.

Methods: Monocyte-derived dendritic cells were used to present allergens in vitro to autologous CD4⁺ T cells of allergic persons. Because collagen type I activates dendritic cells and enhances the secretion of IL-12, we performed allergen presentation assays also in the presence of collagen type I.

Results: After stimulation with allergen-pulsed dendritic cells the production of IFN- γ as well as that of IL-4 and IL-5 by CD4⁺ T cells was enhanced. In the presence of collagen type I, however, a significant shift toward a T_H1 response with increased production of IFN- γ and a decreased production of IL-5 could be observed. When T cells were stimulated directly with anti-CD3 and anti-CD28 in the absence of antigen-presenting cells, it was demonstrated that collagen type I also exerted a direct effect on T cells, increasing their IFN- γ production.

Conclusion: These data indicate that collagen type I influences dendritic cells as well as T cells in a way that a shift in cytokine production results in a T_H1 response even in already-sensitized atopic individuals. (*J Allergy Clin Immunol* 1999;104:1052-9.)

Key words: T-cell differentiation, dendritic cells, collagen type I

The immune response of atopic individuals against allergens is characterized by an increased release of T_H2 cytokines by allergen-specific T-helper cells.^{1,2} The enhanced IL-4 production in turn leads to the production of IgE by B cells, which binds to high-affinity IgE receptors on mast cells and basophils.^{3,4} During the next allergen contact cell-bound IgE is cross-linked, inducing a release of inflammatory mediators such as histamine and leukotriene.⁵ IL-5 produced by T_H2 cells participates in

Abbreviations used:

DC: Dendritic cells

MIP-1 α : Macrophage inflammatory protein-1 α

the development of eosinophilic allergic inflammation.⁶ Therefore deviation of this T_H2-dominated immune response toward a T_H1 response as it occurs during specific immunotherapy appears to be a promising approach for the causative treatment of atopic diseases.⁷⁻¹⁰

Presentation of allergens by dendritic cells (DC) could be a new form of immunotherapy. Characteristic for these most potent antigen-presenting cells is their ability to induce an immune response de novo.¹¹⁻¹³ Because of their secretion of IL-12, these cells are well suited to induce T_H1 responses.^{14,15} Today, immature DC can be obtained by culturing CD14⁺ monocytes for 7 days in GM-CSF and IL-4.¹⁶ Antigens added at this time of culture are processed and presented. Full maturation is obtained by adding inflammatory cytokines such as IL-1 β , TNF- α , IL-6, and PGE₂.^{17,18} These cultured DC are already used in clinical trials to induce immune responses against tumor antigens in vivo.¹⁹

Our study was set up to analyze the immune response-presenting allergens by DC to autologous CD4⁺ T cells of atopic donors. As we had observed that cultivation of DC on collagen type I enhances the production of IL-12, we further investigated the influence of DC on allergen-specific T cells in the presence of collagen type I.

METHODS

Subjects

Heparinized blood was obtained from atopic donors with grass pollen, birch pollen, rye pollen, or house dust mite allergy. Specific sensitization was documented by positive skin prick test to the respective allergen or detection of allergen-specific IgE in the sera (radioallergosorbent test class ≥ 2).

Preparation of monocyte-derived DC and allergen pulse

PBMCs were isolated from 40 mL of heparinized blood by Ficoll-Paque 1.077 (Biochrom, Berlin, Germany) density centrifugation. To enrich CD14⁺ monocytes, 1×10^7 cells per well were incubated for 45 minutes in a 6-well plate (Costar, Bodenheim, Germany) with RPMI 1640 (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 3% autologous serum at 37°C. Preincubation of the wells with medium for 5 minutes was required to reduce B-cell contamination. After monocytes were washed with pre-

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warmed PBS, the purity of CD14⁺ monocytes was 85% to 90%, as measured by flow cytometry. The isolated cells were incubated in 3 mL/well X-VIVO 15 (Bio-Whittaker, Walkersville, Md) supplemented with 1% heat-inactivated autologous serum, 1000 U/mL IL-4 (Pharma Biotechnologie Hannover, Hannover, Germany), and 1000 U/mL GM-CSF (Leucomax, Sandoz AG, Nürnberg, Germany). Every other day 1 mL of the medium was replaced by fresh medium containing 1000 U/mL IL-4 and 2000 U/mL GM-CSF. After 7 days of culture 10 µg/mL grass pollen, birch pollen, rye pollen, or house dust mite allergen extract (*Dermatophagoides pteronyssinus*) (Alk-Scherax, Hamburg, Germany) was added. Supplementing 1000 U/mL TNF-α, 2000 U/mL IL-1β (Pharma Biotechnologie Hannover), and 1 µg/mL PGE₂ (Minprostin E₂, Pharmacia and Upjohn GmbH, Erlangen, Germany) induced the full maturation of the DC. After an additional 2 days the nonadherent mature DC were harvested and used for T-cell stimulation assays.

Coculture of allergen-pulsed DC with autologous CD4⁺ T cells

Eight days after initiation of the DC culture, autologous CD4⁺ T cells were isolated from heparinized blood of the same donor. The T cells were enriched with use of nylon wool columns as described elsewhere.²⁰ The resulting cells were incubated for 30 minutes on ice with antihuman CD8 (OKT8 supernatant, American Type Culture Collection, Rockville, Md) and antihuman CD14 (3C10 supernatant, American Type Culture Collection). After the cells were washed 3 times with PBS, CD8⁺, CD14⁺, and CD19⁺ cells were depleted with use of antimouse IgG and Pan B-Dynabeads (Dyna, Hamburg, Germany).

The presentation of allergens was performed in 48-well plates (Costar). A total of 5×10^4 DC and 5×10^5 T cells were added to each well in 1 mL of X-VIVO 15. Before the coculture the DC were washed thoroughly 3 times with PBS to remove all interfering cytokines such as IL-4 and PGE₂. Because the cytokine production of the T cells was rather weak after the primary stimulation, we performed a secondary stimulation after an additional 5 days. Therefore we used freshly generated mature DC from the same donor, which were cultured in the same way as the DC for the primary stimulation. Forty-eight hours after the secondary stimulation, 500 µL supernatant was collected and analyzed.

Assessment of the influence of collagen type I

To analyze the effects of collagen type I on the presentation of allergens, each well of a 48-well plate was coated with 50 µg per well of collagen type I from human placenta (Sigma Aldrich, Deisenhofen, Germany). Only batches with a contamination of endotoxin <1 ng/mL were used, tested with QCL-1000 chromogenic *Limulus* amoebocyte lysate (Bio-Whittaker) according to the instructions of the manufacturer. To eliminate the possibility of endotoxin being responsible for measured effects of collagen, we further added polymyxin B sulfate (Sigma) at a concentration of 10 µg/mL in some experiments.²¹ Contamination with TNF-α, IL-1β, IL-4, IL-5, or IFN-γ could not be measured in the collagen-preparations with ELISA. The coculture of DC and T cells was performed as described above. To determine whether collagen-induced IL-12 secretion of the DC is responsible for the increased IFN-γ production, 2 µg/mL of anti-IL-12 neutralizing mAb was added (R&D systems, Wiesbaden, Germany) to the cultures. To investigate the direct influence of collagen type I on the T cells independently from DC, T cells were also activated directly with antihuman CD3 in a titrated dilution of 1:20 (OKT3, supernatant, American Type Culture Collection) and 0.5 to 1 µg/mL antihuman CD28 (Pharmingen, San Diego, Calif). Supernatants were collected 48 hours after stimula-

tion. To determine the effect of collagen on memory and naive T cells, CD45RA⁺ and CD45RO⁺ CD4⁺ T cells were purified from PBMCs with use of antibody-coated paramagnetic MicroBeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the protocol of the manufacturer.

Determination of surface marker expression by flow cytometry

Surface marker expression was measured by staining the cells with specific mouse antihuman mAbs for 20 minutes on ice. Antibodies used were CD2 (OKT11, supernatant), CD4 (OKT4, supernatant), CD8 (OKT8, supernatant), CD14 (3C10, supernatant) (American Type Culture Collection), CD19 (HIB19, Pharmingen), CD80 (MAB104), CD83 (HB15) (Immunotech, Hamburg, Germany), CD86 (BU63), and rat antihuman HLA-DR (YE2/36HLK) (Serotec, Oxford, UK). After being washed with PBS containing 2% FCS, the cells were stained with phycoerythrin-conjugated donkey antimouse IgG (Dianova, Hamburg, Germany) and dichlorotriazinyl-amino-fluorescein-conjugated goat antirat IgG (Dianova). After 15 minutes the cells were washed and analyzed in a FAC-SCalibur (Becton Dickinson, Mountain View, Calif) equipped with CELLQuest software.

Determination of cytokine production by ELISA

Human IL-4, IL-5, IL-12 (p40), IFN-γ (Pharmingen), IL-6, TNF-α, and macrophage inflammatory protein-1α (MIP-1α) (R&D Systems) were measured by ELISA according to the instructions of the distributors of the pairs of antibodies used. Briefly, Nunc Maxisorp Immunoplates (Nunc GmbH, Wiesbaden, Germany) were coated overnight with 2 µg/mL capture antibody at 4°C. The plates were blocked with PBS containing 1% BSA (Sigma) for 2 hours at 37°C. Serial dilutions of the standard cytokines (Pharmingen and R&D Systems) and culture supernatants were incubated overnight at 4°C. Next day the plates were incubated at 37°C for 1 hour with 1 µg/mL biotinylated detection antibody, followed by a 30-minute incubation with peroxidase-conjugated streptavidin (Zymed, San Francisco, Calif). After development with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) the plates were read at 405 nm. Cytokine concentrations were calculated according to the standard curves. The detection limit of all cytokines was 32 pg/mL, except for that of IL-4, which reached a sensitivity of 8 pg/mL.

Statistics

The Student *t* test was used to test the statistical significance of the results; *P* < .05 was considered significant.

RESULTS

Induction of IFN-γ, IL-4, and IL-5 production by allergen-pulsed DC

In our experiments we used DC derived from CD14⁺ monocytes for the presentation of allergens. The allergens were added at a concentration of 10 µg/mL to CD80^{low}, CD86^{low}, CD83⁻, and CD14⁻ immature DC, which are able to take up and process antigens. After addition of inflammatory cytokines, >70% of the cells matured into CD83⁺ DC (Fig 1). To obtain measurable cytokine quantities, autologous CD4⁺ T-helper cells had to be stimulated twice with antigen-pulsed DC in a 7-day interval. Forty-eight hours after the secondary stimulation we observed an increased IFN-γ as well as IL-5 and IL-4 production compared with cultures with unpulsed

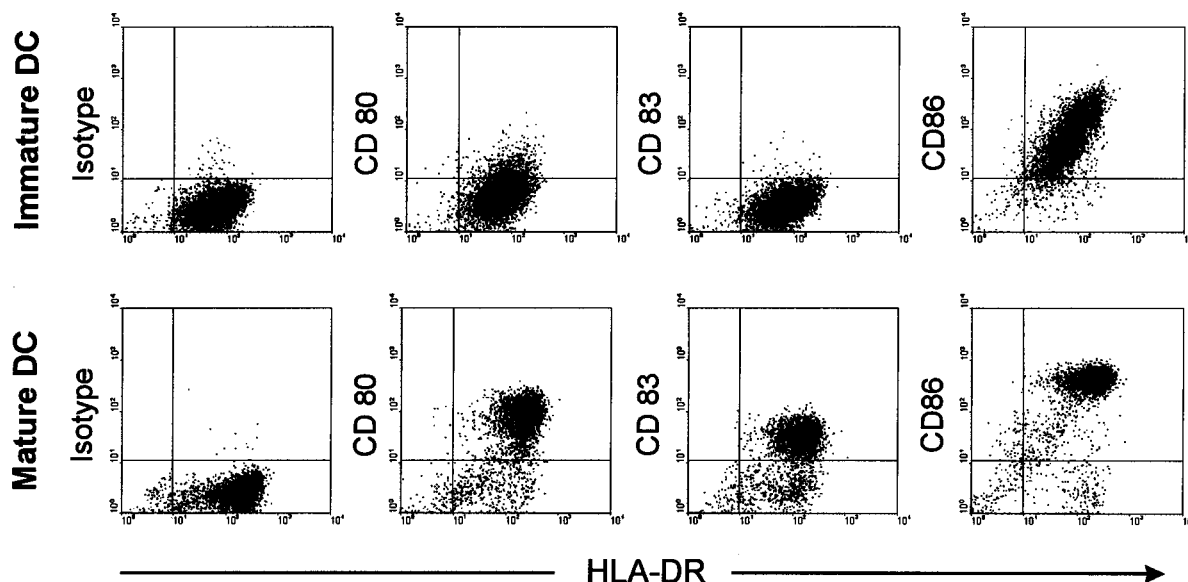


FIG 1. Phenotype of immature DC cultured for 6 days and mature DC cultured for 9 days. Cells were double stained for HLA-DR and additional marker as indicated.

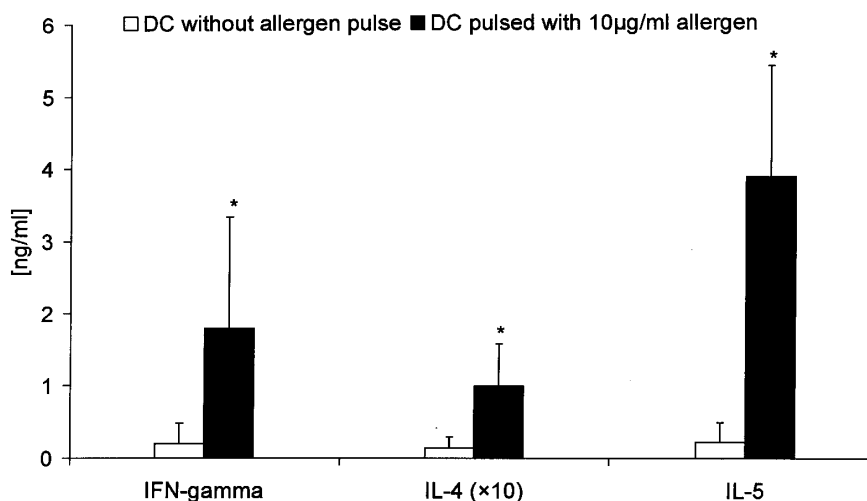


FIG 2. Cytokine expression of CD4⁺ T cells from allergic donors after stimulation with allergen-pulsed DC. Supernatants were collected 48 hours after second stimulation with DC, which were pulsed with 10 µg/mL allergen. Data represent mean $\pm$ SD of 8 experiments. (Asterisk, $P < .05$).

DC (Fig 2). IL-4 production was often low but we found IL-4 quantities up to 187 pg/mL when the allergen-pulsed DC were used. No IL-4 could be detected by measuring the supernatants immediately after starting the cocultivation, confirming that IL-4 was not transferred from the preceding DC cultures. Variation of DC numbers from 5×10^3 to 5×10^5 or the allergen concentrations from 25 ng to 25 µg altered the quantity of cytokines produced with increased cytokine production with higher numbers of DC or higher amounts of allergen but did not significantly change the IFN- γ /IL-5 ratio (data not shown). Cultivating the DC without PGE₂, which is known to induce a T_H2 response,²² decreased

the percentage of mature CD83⁺ DC but did not change the cytokine pattern.

Enhancement of T_H1 cytokine production and inhibition of T_H2 cytokine production performing antigen presentation on collagen type I

In earlier experiments we demonstrated that collagen type I has an effect on the maturation of DC, inducing an increased TNF- α production.²³ In following experiments we extended our investigations and observed an increased production of IL-6, IL-12, and MIP-1 α pro-

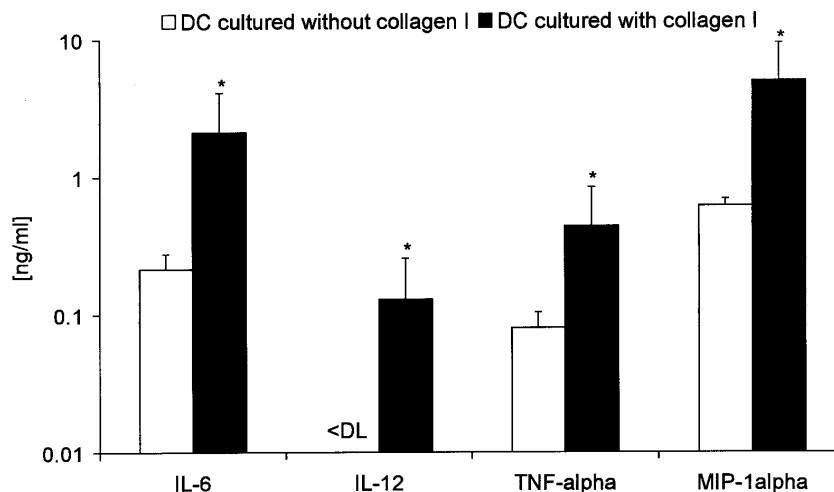


FIG 3. Cytokine expression of DC cultivated on collagen type I. DC cultured for 6 days were transferred into 6-well plates, which were coated with 200 μ g per well of collagen type I. Supernatants were collected after additional 48 hours and analyzed by ELISA (asterisk, $P < .05$; $n = 3$, $\pm$ SD). DL, Detection limit.

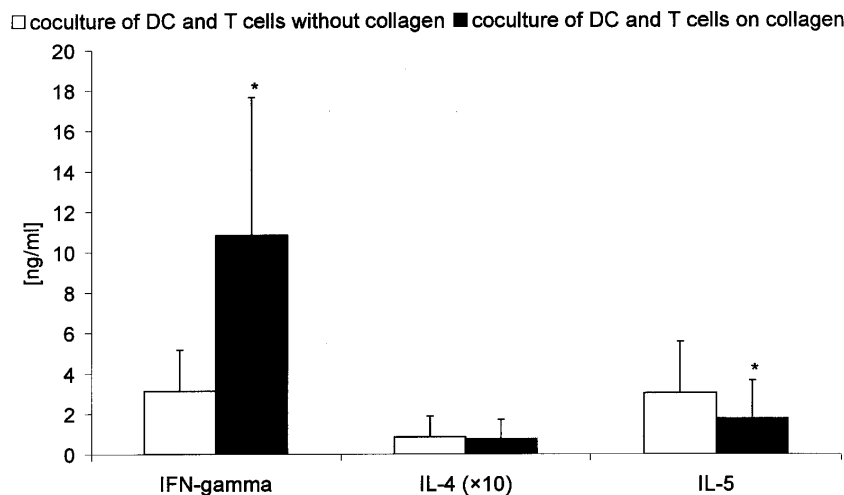


FIG 4. Influence of collagen type I on cytokine production of T cells stimulated with allergen-pulsed DC. Allergen presentation was performed in 48-well plate coated with 50 μ g per well of collagen type I. Cytokines were measured 48 hours after second stimulation with allergen pulsed DC. Asterisk, $P < .05$; $n = 15$; $\pm$ SD.

duction when DC were cultured on collagen type I (Fig 3). Because the presentation of allergens with DC did not lead to an exclusive T_H1 response as described above, we performed the allergen presentation in collagen type I-coated wells to obtain a further activation of the DC. This way the production of IFN- γ was strongly increased ($P = .0023$) and IL-5 production was decreased ($P = .041$) (Fig 4). The IL-4 decrease was not significant in these experiments. This effect was collagen type I specific because collagen type IV or fibronectin did not influence the IFN- γ quantities (data not shown). Because LPS contamination in collagen has been described to have strong effects on monocytes and dendritic cells,²⁴ we only used collagen type I batches with low contamination of endotoxin (<1 ng/mL) and excluded the respon-

sibility of endotoxin for IFN- γ induction with further experiments. Addition of 10 ng/mL LPS to cultures of DC with T cells without collagen also increased the IFN- γ production; however, this increase could be inhibited significantly by addition of polymyxin B sulfate. Addition of this compound to the collagen coated wells had no significant effect on the IFN- γ production induced in the presence of collagen type I (Fig 5).

Neutralization of IL-12 partly inhibits the T_H2/T_H1 -shift induced by DC in the presence of collagen type I

To ensure that the increased production of IL-12 by DC during exposure to collagen type I is responsible for the

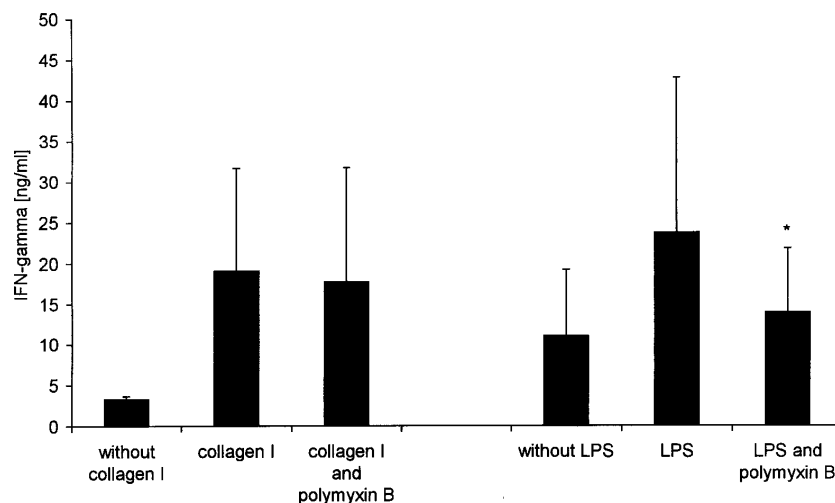


FIG 5. Inhibition of LPS effects on DC by polymyxin B. To rule out possibility that contaminating endotoxin in collagen batches is responsible for increased IFN- γ production, 10 μ g/mL polymyxin B was added to cocultures. Supernatants were collected 48 hours after second stimulation with allergen-pulsed DC. Polymyxin inhibited LPS-induced increase of IFN- γ production significantly (asterisk, $P < .05$), whereas no significant inhibition of collagen-induced production of IFN- γ could be observed ($n = 4$, $\pm$ SD).

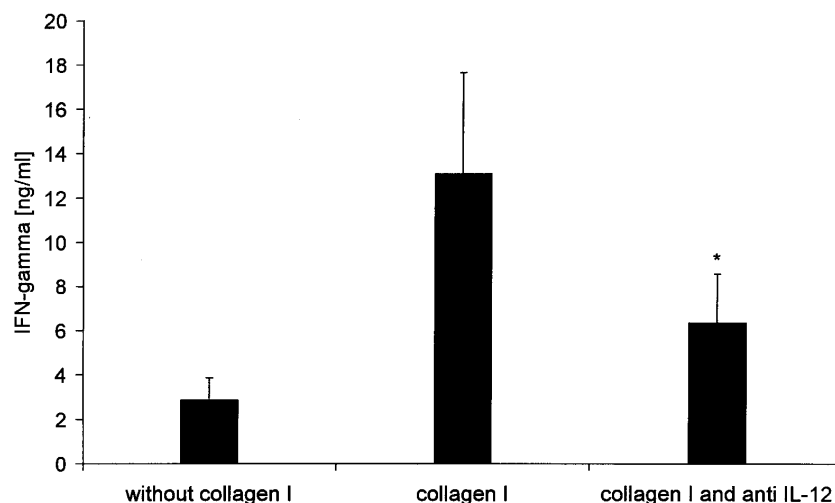


FIG 6. Inhibition of effects of collagen type I by blocking IL-12. To determine whether enhanced IL-12 secretion by DC on collagen is responsible for IFN- γ induction, IL-12 was blocked by adding 2 μ g/mL neutralizing anti-IL-12 mAb to experiments. Asterisk, $P < .05$; $n = 3$; $\pm$ SD.

enhanced secretion of IFN- γ , we added blocking anti-IL-12 mAb to the cocultures of DC and T cells. This resulted in a partial inhibition of the enhanced IFN- γ secretion of the T cells in the presence of collagen type I (Fig 6).

Direct enhancement of IFN- γ production by activation of T cells in the presence of collagen type I

Because we have shown above that the enhanced IL-12 production of DC is not exclusively responsible for

the induced cytokine shift with increased IFN- γ production, we determined whether T cells are also influenced by collagen type I directly. In these experiments T cells were activated with mAbs against CD3 and CD28 in the absence of antigen-presenting cells. As a result of the unspecific activation of all T cells, the secretion of IL-5 was much lower compared with prior experiments with activated allergen-specific T cells, and IL-4 was below the detection limit. Nevertheless the activation of T cells in collagen type I-coated wells again induced a significantly higher IFN- γ production compared with the acti-

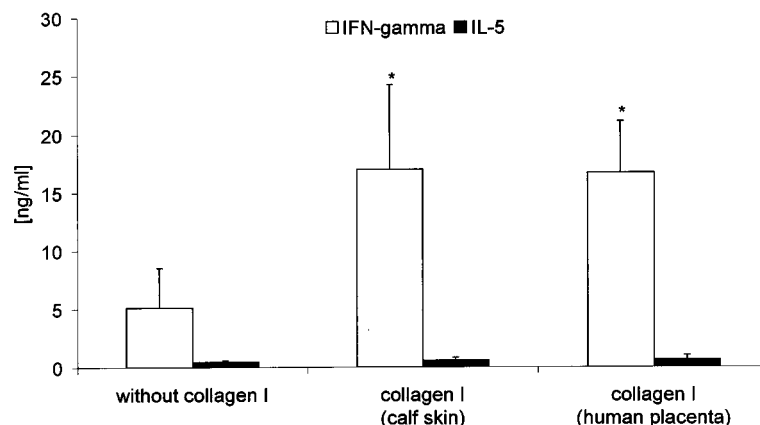


FIG 7. Activation of T cells on collagen type I in the absence of antigen-presenting cells. To evaluate whether collagen type I reacts directly with CD4⁺ T cells and thus induces an enhanced secretion of IFN- γ , activation was performed by adding α -CD3 and α -CD28 mAbs to cultures in absence of antigen-presenting cells. Wells were either coated with human collagen or collagen of calf skin. Significant induction of IFN- γ (asterisk, $P < .05$) was compared with that of uncoated wells ($n = 3$, $\pm$ SD).

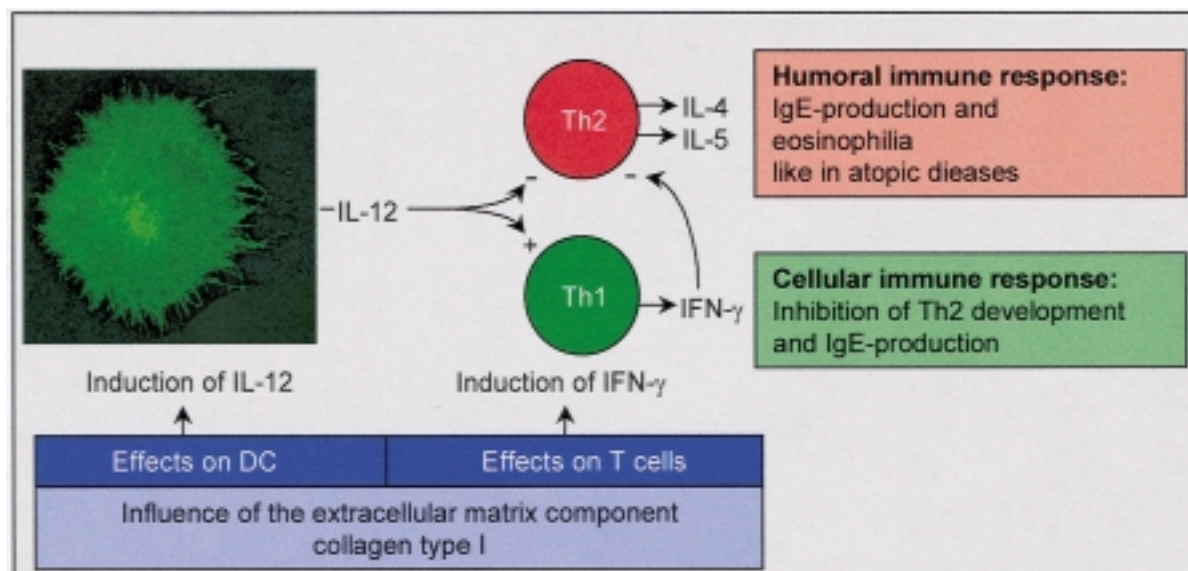


FIG 8. Effects of collagen type I on T_H1/T_H2 development of T cells. Collagen type I induces secretion of IL-12 by dendritic cells and of IFN- γ by T cells. Both cytokines suppress T_H2 development of CD4⁺ T-helper cells.

vation in uncoated wells (Fig 7). With use of highly purified CD45RA⁺ naive or CD45RO⁺ memory CD4⁺ T cells (purity >95%) we could determine that both subpopulations were stimulated by collagen type I, leading to an increased IFN- γ secretion (data not shown). This effect was induced by human as well as bovine collagen type I.

DISCUSSION

DC as potent antigen-presenting cells are known to be able to induce T_H1 responses in mixed lymphocyte and antigen-specific reactions.^{14,15,18} The goal of our experiments was to evaluate the capacity of DC to induce a T_H1

shift presenting allergens to CD4⁺ T cells of allergic persons who have an established T_H2-dominated immune response. The results presented in this study, however, indicate that a simple presentation of allergens by DC is not sufficient to reverse an established T_H2 response. These results are in agreement with the observation that monocyte-derived DC not only produce T cell-activating cytokines (IL-1 α , IL-6, IL-15, and TNF- α) but also cytokines that modulate T_H1 (IL-12 and IL-18) and T_H2 (IL-13, IL-10) cell development.²⁵ The secretion of these factors and subsequent induction of T_H1/T_H2 is dependent on further signals such as CD40 engagement, which induces IL-12.²⁶ In prior experiments we could show that collagen type I, a protein of the extracellular matrix,

might also be one of these signals inducing the secretion of several cytokines, including IL-12.²³ Other extracellular matrix proteins we tested, such as collagen type IV and fibronectin, did not have this distinct potential. In the study presented here we show that the presentation of allergens on collagen type I surfaces increases the production of IFN- γ by T cells. This effect was partially the result of enhanced IL-12 production of collagen-activated DC because it could be inhibited significantly by addition of blocking IL-12 mAb. The possibility that contaminating endotoxin (<1 ng) in the collagen preparations is responsible for these effects could be excluded.²⁴ Adding polymyxin sulfate, which is known to inhibit endotoxin effects, did not reduce the increased IFN- γ production in the presence of collagen type I, whereas it inhibited the LPS-induced IFN- γ production significantly. Contaminating cytokines in the collagen preparations could not be detected. It was also determined in proliferation assays that collagen from human placenta was not recognized as an antigen inducing proliferation of T cells and thereby altering the cytokine pattern (data not shown).

Inducing an activation of T cells by adding mAbs against CD3 and CD28 in the absence of antigen-presenting cells indicated that collagen type I also interacts directly with CD4⁺ T cells, enhancing the secretion of IFN- γ . Experiments with CD45RA⁺ or CD45RO⁺ T cells showed that naive as well as memory T cells are influenced by this matrix protein. Both subpopulations demonstrated an increased IFN- γ secretion after stimulation with anti-CD3 and anti-CD28. This indicates that collagen type I induces a preferential development of T_H1 cells from naive T cells but also induces a T_H2/T_H1 shift in mature T cells that is in line with observations that human T_H2 cells are not stable in their cytokine pattern and can be differentiated into T_H1 cells.²⁷ The results are also in accordance with observations that CD4⁺ T cells can be influenced directly by collagen type I.²⁸ Taken together, our experiments indicate that collagen acts in various ways inducing a T_H1 shift: (1) activating DC to produce IL-12 and (2) directly activating T cells to increase IFN- γ production (Fig 8).

The specific composition of the extracellular matrix of different tissues might therefore influence the polarization of natural immune responses and may be exploited to direct artificially induced immune responses with therapeutic intentions such as in vaccinations or allergen immunotherapy. Alternatively, antigen-pulsed DC or antigen-specific T cells may be differentiated in vitro under the influence of factors such as collagen until they express a stable phenotype, which appears to be appropriate for the desired therapeutic effect.

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