

Modification of the human allergic immune response by allergen-DNA-transfected dendritic cells in vitro

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Background: Atopic-allergic diseases are characterized by T_H2-dominated immune responses, resulting in IgE production. DNA-based immunotherapies have been shown to shift the immune response toward a T_H1-type response in animal models. **Objective:** The aim of the study was to analyze whether dendritic cells (DCs) transfected with allergen-DNA conjugates are able to stimulate human autologous CD4⁺ T cells, CD8⁺ T cells, or both from atopic individuals to produce T_H1 cytokines instead of T_H2 cytokines.

Methods: For this purpose, human mature DCs from atopic donors were transfected with an adenovirus encoding the allergen Phl p 1. Autologous CD4⁺ and CD8⁺ T cells were stimulated with these transfected DCs, and proliferation and cytokine production were measured.

Results: By using an adenoviral vector, a transfection rate of 92% could be achieved. The proliferative response of CD4⁺ T cells stimulated with autologous transfected DCs was concentration dependent and almost as high as that of T cells stimulated with mature allergen-pulsed DCs. The proliferation of CD8⁺ T cells stimulated with transfected DCs, however, was higher than that of cells stimulated with allergen-pulsed DCs. The cytokine pattern showed a shift toward a T_H1 immune response compared with T cells stimulated with allergen-pulsed DCs.

Conclusions: Human DCs can be transfected with allergen-DNA conjugates very efficiently by using an adenoviral vector yielding DCs with high T-cell stimulatory capacities, directing the atopic-allergic immune response from T_H2 dominance toward T_H1 dominance. (*J Allergy Clin Immunol* 2004;113:327-33.)

Key words: Adenoviral transfection, allergy, dendritic cells, T_H1/T_H2

Atopic-allergic diseases, such as asthma, allergic rhinitis, and atopic eczema, are characterized by T_H2-dominated immune responses against allergens, such as

Abbreviations used

Ad-Phl p 1: Adenovirus-*Phleum pratense* 1
DC: Dendritic cell
IMDM: Iscove's modified Dulbecco's medium
MOI: Multiplicity of infection
ODN: Oligodeoxynucleotide
PE: Phycoerythrin

grass pollen. T_H2-dominated immune responses are responsible for IgE production, resulting in mast cell degranulation with release of inflammatory mediators after cross-linking of IgE on its receptor.¹

Antigen-specific modifications of the allergic immune response at the T-cell level are attractive objectives for immunotherapeutic interventions. Thus far, only allergen-specific immunotherapy has been shown to specifically modify a preexisting allergic T_H2-dominated immune response.^{2,3} Alternatives, modifications, or both of specific immunotherapy have been explored to increase the efficacy and to decrease the risk of potential adverse effects, such as anaphylactic reactions.

DNA-based immunotherapeutic approaches include immunomodulation with immunostimulatory CpG oligodeoxynucleotides (CpG-ODNs) and immunization with allergen-DNA conjugates. Allergen gene vaccines have been shown to be effective in modulating the allergic immune response in mouse models, especially shifting the immune response from a T_H2- to a T_H1-dominated response.⁴ Apart from that, dendritic cells are potent tools for the antigen-specific modulation of immune responses, including allergic immune responses.⁵

Furthermore, it has been shown in mouse studies that dendritic cells (DCs) transfected with adenovirus encoding human tyrosinase-related protein 2 induce a stronger anti-tumor immunity than direct injections of adenoviral vector encoding human tyrosinase-related protein 2. Delivery of adenovirus with DCs altered the character of the immune response, resulting in strong cellular immunity in the absence of humoral immunity.⁶ Therefore we used DCs transfected with adenovirus encoding allergen-DNA conjugates to investigate whether a stronger T_H1 immune response instead of an allergic T_H2-dominated immune

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response can be induced in vitro. We used an adenoviral vector because of the efficient transfection rate compared with other transfection methods, such as electroporation or lipofection.⁷ Possible advantages of allergen-DNA conjugate-transfected DCs compared with allergen protein-pulsed DCs are better responses from the CD8⁺ T cells because of MHC class I antigen presentation.

The present study explores the influence of allergen-DNA conjugate-pulsed human DCs on autologous T cells from sensitized atopic patients in vitro. We demonstrate that CD4⁺ T cells produce more T_H1 cytokines after stimulation with autologous adenovirus-transfected mature DCs and that allergen-DNA conjugate-transfected DCs are also able to activate IFN- γ -producing CD8⁺ T cells.

METHODS

Blood samples

Heparinized blood was obtained from atopic donors (mainly with allergic rhinitis) sensitized to grass pollen who had not undergone specific immunotherapy. Specific sensitization was documented by means of positive skin prick test responses to the allergen and detection of allergen-specific IgE in the sera (RAST class ≥ 2).

Cell-culture reagents

Iscove's modified Dulbecco's medium (IMDM) with L-glutamine and 25 mmol/L HEPES (Life Technologies GmbH, Karlsruhe, Germany) supplemented with 3024 mg/L sodium bicarbonate, 100 μ g/mL streptomycin, 100 U/mL penicillin, and 1% heat-inactivated autologous plasma was used for the culture of DCs and with 5% autologous plasma for the coculture of T cells and DCs.

Human recombinant IL-4, IL-1 β , and TNF- α were purchased from Strathmann Biotech GmbH (Hannover, Germany), GM-CSF (Leucomax) was obtained from Sandoz AG (Nürnberg, Germany), and prostaglandin E₂ (Minprostin) was obtained from Pharmacia & Upjohn GmbH (Erlangen, Germany). The grass-pollen allergen extract was obtained from ALK-Schering (Hamburg, Germany). Natural Phl p 1 was isolated from pollen extract according to the method of chromatographic separation of Suck et al.⁸ Phl p 1 allergen-DNA conjugate was taken from clone pPhlp1.p4.⁹

Antibodies

The following mAbs were used:

- mouse IgG: anti-CD80 (MAB104; Immunotech, Hamburg, Germany), anti-CD83 (HB15a, Immunotech), anti-CD86 (BU63; Camon, Wiesbaden, Germany), and mAb IG12 (directed against Phl p 1)⁹;
- rat IgG: anti-HLA-DR (YE2/36HLK, Camon) and mouse and rat subclass-specific isotypes (Immunotech);
- conjugated secondary antibodies: fluorescein (dichlorotriazinyl amino fluorescein [DTAF])–conjugated goat anti-rat IgG (Dianova, Hamburg, Germany) and phycoerythrin (PE)–conjugated donkey anti-mouse IgG (Dianova); and
- FITC- or PE-conjugated antibodies for staining of MACS-sorted T cells: anti-CD4 (RPA-T4), anti-CD8 (RPA-T8, all from BD PharMingen, San Diego, Calif), and FITC- and PE-conjugated mouse IgG isotype control (Immunotech).

Generation of monocyte-derived human DCs

PBMCs were isolated from heparinized blood by means of Ficoll-Paque 1.077 (Biochrom, Berlin, Germany) density centrifugation. To enrich CD14⁺ monocytes, 1×10^7 PBMCs per well were incubated for 45 minutes in a 6-well plate (Costar, Bodenheim, Ger-

many) in IMDM supplemented with 3% autologous plasma at 37°C. After washing the nonadherent cells with prewarmed PBS, the remaining monocytes (purity >90%) were incubated in 3 mL/well IMDM supplemented with 1% heat-inactivated autologous plasma, 1000 U/mL IL-4, and 800 U/mL GM-CSF. Cells were fed with fresh medium every other day. On day 7, the resulting immature DCs were pulsed with 5 μ g/mL whole grass-pollen extract or transfected with adenovirus-*Phleum pratense* 1 (Ad-Phl p 1; multiplicity of infection [MOI], 3–300) and further stimulated with 1000 U/mL TNF- α , 2000 U/mL IL-1 β , and 1 μ g/mL prostaglandin E₂ to induce their full maturation. Mature DCs were harvested 48 hours after stimulation, washed twice, and used for T cell-stimulation assays.

Purification of T cells

Autologous CD4⁺ and CD8⁺ T cells were obtained from PBMCs by using antibody-coated paramagnetic MicroBeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), according to the protocol of the manufacturer. Separation was controlled with flow cytometry (purity of >98% CD4⁺ T cells and >95% CD8⁺ T cells).

Coculture of T cells and autologous allergen-pulsed DCs

For proliferation assays, 1×10^5 CD4⁺ or CD8⁺ T cells were cocultured in 96-well plates (Costar) in triplicate with 1×10^4 autologous allergen-pulsed DCs in 200 μ L of IMDM supplemented with 5% heat-inactivated autologous plasma. After 5 days, the cells were pulsed with 37 kBq/well of tritiated thymidine ([methyl-³H]thymidine; ICN, Irvine, Calif) for 6 hours, and tritiated thymidine incorporation was evaluated in a beta counter (1205 Betaplate; LKB Wallac, Turku, Finland).

For cytokine production assays, 5×10^5 CD4⁺ or CD8⁺ T cells were cultured in 48-well plates in the presence of 5×10^4 autologous allergen-pulsed DCs in 1 mL of IMDM supplemented with 5% heat-inactivated autologous plasma. On day 7, T cells were restimulated with 5×10^4 newly generated autologous allergen-pulsed DCs, and supernatants were collected 24 hours later.

Flow cytometric analysis

Surface phenotyping was performed by staining 5×10^5 T cells or 5×10^4 DCs with specific mAbs for 20 minutes at 4°C. After being washed with PBS containing 0.1% BSA, the cells were incubated with DTAF- or PE-conjugated secondary antibodies for 20 minutes at 4°C, washed, and analyzed in a FACSCalibur (Becton Dickinson, Mountain View, Calif) equipped with CELLQuest Software.

Quantification of cytokine production by means of ELISA

Human IL-4, IL-5, IL-10, and IFN- γ levels were measured by means of ELISA according to the instructions of the distributors of the used pairs of antibodies (BD PharMingen), as described previously.¹⁰ The detection limit was 8 pg/mL for IL-4 and 32 pg/mL for all other cytokines.

Recombinant adenovirus and transduction of DCs

Recombinant E1-substituted, E3-deleted adenoviral vectors encoding green fluorescent protein were constructed through Cre-lox recombination, propagated on 293 cells, and purified by means of cesium-chloride gradient density centrifugation and subsequent dialysis according to standard protocols.¹¹

Recombinant Ad-Phl p 1 was purchased from QBioGene (Heidelberg, Germany) and expanded in 293 cells, purified by means of cesium-chloride gradient density centrifugation, and subsequently dialysed according to the instructions of the manufacturer.

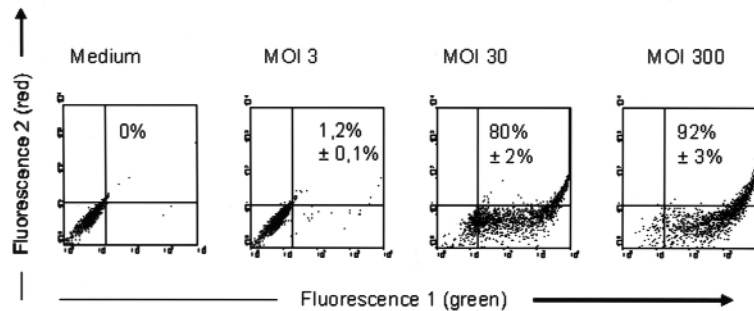


FIG 1. Efficiency of the adenoviral transfection of human mature DCs. DCs were generated as described and transfected at day 6 with adenoviral vectors encoding green fluorescent protein at different MOIs. One representative example of 8 independent experiments is shown.

Adenoviruses were added to DC cultures on day 7 at an MOI of 3, 30, or 300 (ie, 3×10^8 pfu/ 10^6 DCs per well).

SDS-PAGE and Western blotting

One hundred micrograms of cell lysates and 0.5 ng of the positive control nPhl p 1 were mixed with loading buffer (Roti Load, 4× concentrated; Roth, Karlsruhe, Germany), heated for 5 minutes at 96°C, and subjected to SDS-PAGE on a 15% polyacrylamide gel with 0.5% SDS by using standard procedures (45 mA; maximum, 500 V).

Proteins were blotted onto polyvinylidene difluoride membrane (Millipore, Bedford, Mass) by using a wet blotting unit (Minigel; Bio-Rad, Hercules, Calif) in a 10% methanol buffer for 2 hours and 30 V. After transfer, the membranes were blocked with Rotiblock (Roth) for 30 minutes.

For detection, the membranes were incubated with primary antibodies (IG12 anti-Phl p 1 and for loading control C-2 anti-actin; Santa Cruz Biotechnology, Santa Cruz, Calif) at a dilution of 1:200 for 2 hours and with horseradish peroxidase-conjugated secondary antibodies (New England Biolabs, Frankfurt, Germany) at a dilution of 1:1000 in Rotiblock for 1 hour. Blots were developed by using chemiluminescence (ECL system; Amersham, Buckinghamshire, United Kingdom). Detection of protein-loading control (actin) was achieved by incubating the membrane in stripping buffer (62.5 mmol/L Tris/HCL, 2% SDS, and 100 mmol/L 2-mercaptoethanol, pH 6.7) for 45 minutes at 50°C and washing in Tris-buffered saline plus Tween-10 to remove antibodies before further analysis.

RESULTS

High transfection efficiency of human DCs with an adenoviral vector

Enhanced green fluorescent protein DNA was transfected into these cells with the adenoviral vector enhanced green fluorescent protein to assess the efficiency of transfection of DCs. As shown in Fig 1, a good efficiency was achieved with this vector system. At an MOI of 3, the transfection rate was $1.5\% \pm 0.1\%$; at an MOI of 30, the transfection efficiency was $80\% \pm 2\%$; and at an MOI of 300, transfection was $92\% \pm 3\%$ (mean $\pm$ SD).

Expression of the protein Phl p 1 by mature DCs transfected with Phl p 1 DNA

Protein expression in mature DCs, which were transfected with Ad-Phl p 1 at an MOI of 300, was assessed

by means of Western blotting. DCs were lysed 2 days after transfection. As a positive control, we used 0.5 ng of the nPhl p 1 protein. As shown in Fig 2, mature transfected DCs expressed the protein Phl p 1. DCs that were not transfected did not express any amount of Phl p 1 protein (Fig 2). The protein-loading control actin was expressed at similar amounts by transfected and untransfected DCs.

Transfection of DCs does not change their surface marker expression compared with that of control DCs and allergen protein-pulsed DCs

Mature DCs were transfected with Ad-Phl p 1 at an MOI of 300 or, as a comparison, pulsed with allergen protein (5 μ g/mL) before final maturation with the cytokine cocktail. In Fig 3 it is shown that transfected mature DCs express equal levels of the costimulatory molecules CD80 and CD86 and the DC-specific marker CD83 compared with allergen-pulsed DCs. As a negative control, we used mature DCs stimulated with cytokine cocktail alone. The expression of MHC class I (HLA-A, HLA-B, and HLA-C) and class II (HLA-DR) molecules was also not influenced by transfection (not shown).

The viability of DCs after transduction at various MOIs was not influenced, as assessed by means of trypan blue exclusion (data not shown).

Allergen-transfected DCs stimulate proliferation of human autologous CD4⁺ and CD8⁺ T cells

As shown in Fig 4, transfected mature DCs were effective in inducing T-cell proliferation. DCs transfected only with adenovirus without allergen-DNA conjugate (Psi 5) or unpulsed DCs were used as negative controls, whereas allergen-pulsed mature DCs served as positive controls. The induction of the stimulation of CD4⁺ T cells by transfected DCs was concentration dependent and was almost as high as with allergen-pulsed mature DCs at an MOI of 30 (Fig 4, A). The lowest T-cell proliferation was measured with DCs transfected with adenovirus without

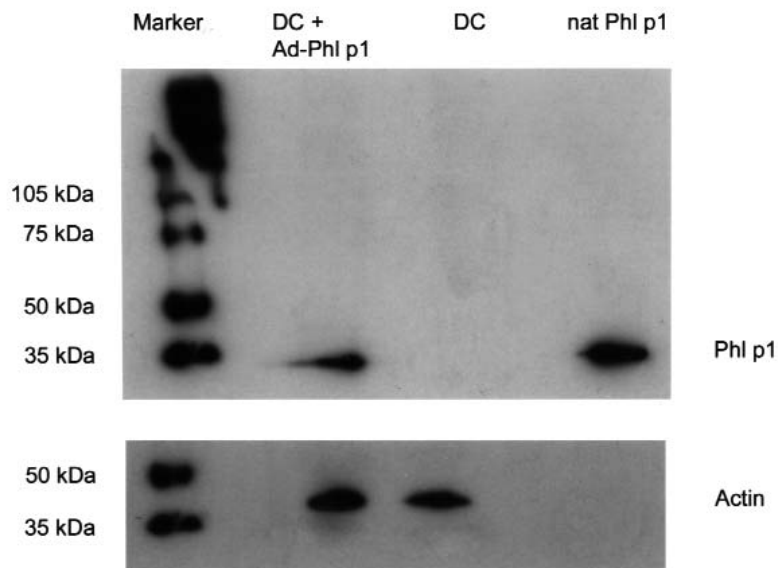


FIG 2. Expression of the protein Phl p 1 by transfected DCs. The cells were lysed 48 hours after transfection. Phl p 1 protein (0.5 ng) was used as a positive control, and as a negative control, we used untransfected mature DCs. A representative example of 3 separate experiments is shown.

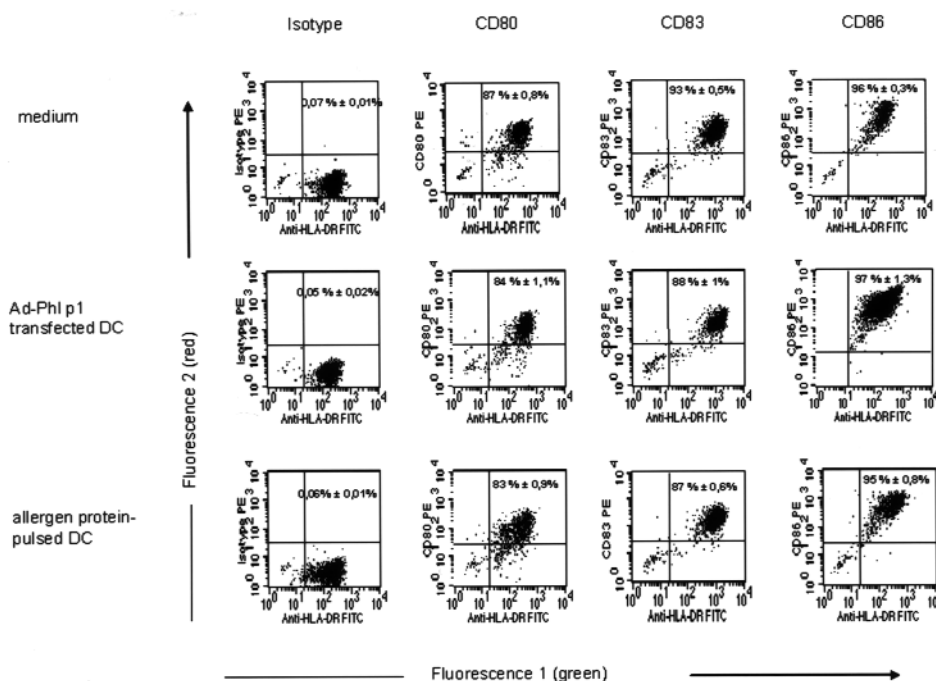


FIG 3. Surface marker expression of DCs after adenoviral transfection or exposure to allergen protein. DCs were generated as described and transfected with Ad-Phl p 1 at day 6. After 48 hours, cells were stained with the markers indicated. A representative example of 3 separate experiments is shown.

the allergen-DNA conjugate (Psi 5). CD8⁺ T cells also showed concentration-dependent proliferation after stimulation with transfected DCs, which was higher at the

optimal MOI of 300 than with allergen-pulsed DCs, although allergen was used at an optimal concentration, as determined in earlier experiments (Fig 4, B).

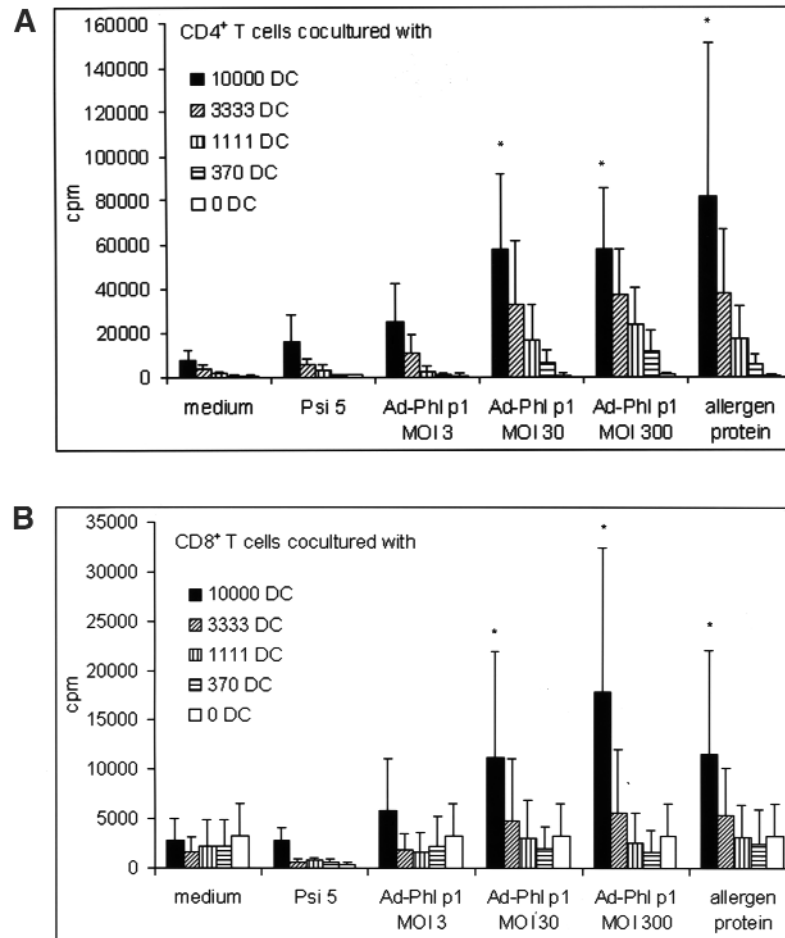


FIG 4. Proliferation of autologous human CD4⁺ (**A**) and CD8⁺ (**B**) T cells activated by transfected DCs. T cells were stimulated with allergen protein-pulsed, transfected, or control DCs. After 5 days, cells were pulsed with 1 μ Ci of tritiated thymidine. Results are expressed as the means $\pm$ SD from 8 atopic donors. * $P \leq .05$ compared with medium or Psi 5.

Allergen-transfected human DCs preferentially induce T_H1 cytokine production in human autologous CD4⁺ T cells and much stronger IFN- γ production in CD8⁺ T cells than allergen protein-pulsed DCs

Finally, we studied the cytokine pattern of CD4⁺ and CD8⁺ T cells cocultured with transfected autologous mature DCs. T cells were stimulated a second time with newly generated transfected autologous mature DCs after 7 days to achieve measurable amounts of cytokine production. As shown in Fig 5, A, transfected DCs induced a significantly higher amount of IFN- γ production in CD4⁺ T cells ($P \leq .02$) and CD8⁺ T cells ($P \leq .03$) than allergen protein-pulsed DCs (Fig 5, B). The production of the T_H2 cytokines IL-4, IL-5, and IL-10 was less than that in T cells stimulated with allergen protein-pulsed DCs. Production of IL-4, IL-5, and IL-10 in CD8⁺ T cells was not detectable (data not shown).

DISCUSSION

In the current study we have demonstrated that human DCs can be transfected with allergen-DNA conjugates very efficiently with the help of adenoviral vectors and that they also synthesize the corresponding protein. The transfection did not influence the viability, morphology, surface marker expression, and general capability to present antigen. As expected, CD8⁺ T cells were activated more strongly by allergen-DNA conjugate-transfected DCs than by allergen protein-pulsed DCs, but CD4⁺ T cells were also activated. We found a lower production of IL-4, IL-5, and IL-10 and a stronger production of IFN- γ compared with stimulation with allergen protein-pulsed DCs, indicating that the route of the antigen presentation has been changed, resulting in stimulation of CD8⁺ T cells rather than CD4⁺ T cells and a shift in the CD4⁺ compartment from T_H2 to T_H1 expression.

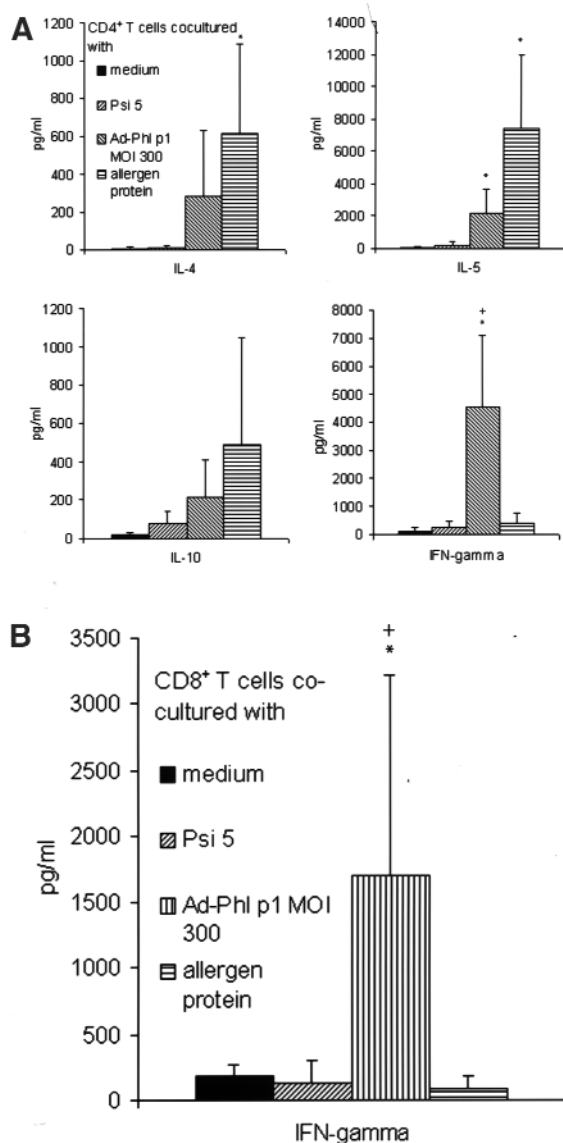


FIG 5. Cytokine production by CD4⁺ (**A**) and CD8⁺ (**B**) T cells after stimulation with autologous DCs. T cells were stimulated twice with allergen protein-pulsed, transfected, or control DCs, and cytokines were measured by means of ELISA. Results are expressed as means $\pm$ SD from 6 donors. * $P \leq .05$ compared with medium or Psi 5. + $P \leq .03$ comparing allergen-DNA conjugate-transfected and allergen protein-pulsed DCs.

Atopic-allergic diseases are characterized by T_H2 -dominated immune responses, resulting in specific IgE production.¹ Therefore inhibition of the function of allergen-specific T cells, especially T_H2 cells, is an attractive target for immunologically based therapies. Thus far, allergen-specific immunotherapy is the only therapy that changes a preexisting allergic T_H2 immune response.² However, the efficacy and safety of this form of therapy might be enhanced by modifications. Studies have shown that higher allergen doses improve the clinical efficacy of specific immunotherapy. However, with higher allergen doses, the risk of adverse effects also increases.¹² There-

fore reagents with higher immunogenic and lower allergenic potentials than traditional allergen extracts would be attractive for use in specific immunotherapy.¹³

By using immunostimulatory sequences with ODNs, both gene and protein vaccinations are able to prevent subsequent T_H2 -biased immune responses, including IgE production in mice.¹² However, DNA-based vaccination has been shown to be significantly more effective than protein-based vaccination concerning protection against the development of T_H2 -biased immune responses and lethal anaphylactic reactions.¹² The finding that plasmid DNA immunization predominantly induces a T_H1 immune response whereas protein immunization induces a T_H2 -dominated immune response in rodents was primarily demonstrated by Raz et al,¹⁴ who have shown that splenic CD4⁺ T cells isolated from plasmid DNA-immunized mice secreted IFN- γ but not IL-4 and IL-5, whereas protein-injected mice secreted IL-4 and IL-5 but not IFN- γ .¹⁴ Furthermore, Hochreiter et al¹⁵ have reported that gene gun immunization and protein immunization alone induced a T_H2 -dominated immune response for different antigens in mice. Coinjection from CpG-ODNs together with gene gun immunization resulted in an antigen-specific T_H1 immune response. However, the effect of CpG motifs in vivo depends on a variety of parameters, such as the nature of the antigen, the immunization modality, and the species. They found a clear effect in mice coinjected with β -galactosidase and CpG motifs but not in mice injected with Bet v 1a or Phl p 2 and CpG-ODNs.¹⁵ Additionally, this stimulation of the immune system was not antigen specific. Finally, Sudowe et al¹⁶ have shown that vaccination of mice with replication-defective adenovirus encoding betagalactosidase with CMV-promoter prevents the development of specific IgE responses, shifting the immune response from T_H2 to T_H1 domination and generating IFN- γ -producing CD8⁺ T cells.

Our study is the first to use human immune cells, allergen-DNA conjugates, and transfection of DCs. The use of DCs might be of additional advantage because gene transfer to DCs ensures that gene products are endogenously processed, leading to the generation of MHC class I-restricted CD8⁺ T-cell responses,¹⁷ which are long lasting.¹⁸ The reasons why we used viral vectors in our study are that they allow the achievement of high transfection rates and that they might also enhance the immunogenicity of a vaccine because of the adjuvant properties of some of the viral products.¹⁹ However, the development of neutralizing antiadenoviral antibodies after injection of viral vectors is a severe problem. Again, this problem is more limited by the use of DCs because the adenoviral proteins are expressed inside the DCs, and this reduces the exposure to proteins of the viral envelope to the host and hence weakens the possible neutralization of transfected cells by antiviral antibodies.⁷ However, a nonviral transfection would be clinically preferred, avoiding the potential pathogenicity and immunogenicity of viral vectors. However, nonviral gene transfer systems, such as electroporation, are limited by their low efficiency for transfection of DCs.²⁰

Taken together, adenoviral transfection of human DCs with allergen-DNA conjugates represents a potential new form of immunotherapy that is already used for immunotherapy in cancer with tumor peptides or DNA²¹ and might also be explored in severe cases of atopic-allergic diseases.

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