

The Toll-Like Receptor-2/6 Agonist Macrophage-Activating Lipopeptide-2 Cooperates with IFN- γ to Reverse the Th2 Skew in an In Vitro Allergy Model¹

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Dendritic cells (DC) are the most potent APCs with the capacity to induce, modulate, or shut down immune function. These features make them potentially useful for treating diseases associated with misled immunologic responses. Therefore, it was the aim of this study to reverse the allergen-dependent Th2 reaction responsible for allergic symptoms by modulating DC function. This issue was addressed in an in vitro test system consisting of human monocyte-derived allergen-pulsed DC from allergics cocultured with autologous lymphocytes. A Th2 reaction judged by the amplification of IL-4 and the down-regulation of IFN- γ was induced by pulsing DC with the relevant allergen. To modulate this reaction, the Toll-like receptor 2/6 engaging mycoplasmal lipopeptide macrophage-activating lipopeptide 2 kDa was combined with IFN- γ to stimulate allergen-pulsed DC. Such treatment resulted in a 500-fold increase in IFN- γ production in the supernatant of cocultured autologous lymphocytes, while the Th2 marker IL-4 was not affected. This phenomenon was associated with an increase in proliferation and the number of IFN- γ -producing lymphocytes. Phenotype and function of thus treated DC remained stable. These data indicate that a former allergen-dependent Th2 reaction can be reversed toward a Th1-type response by an appropriate treatment of DC. *The Journal of Immunology*, 2004, 172: 6080–6086.

Dendritic cells (DC)³ are considered to be the most important APCs of the immune system. They sample Ag at the body's environmental interface and, under appropriate conditions, mature and migrate to the lymphatic organs, where they induce primary and enhance secondary immune responses (1). The conditions under which DC maturation is induced determine the outcome of the immune response. For example, bacterial infections lead to a Th1 response characterized by IFN- γ -producing Th cells, whereas parasite infections instruct DC to mount a Th2 response with IL-4-producing Th cells (2). Furthermore, DC can induce an unpolarized Th0 response or shut down T cell function, resulting in tolerance. The mechanisms involved in this plasticity are affinity and duration of MHC II-TCR interaction, expression pattern of costimulatory molecules, and production of cytokines (3), which can be referred to as micromilieu in the in vivo situation.

The lack of Th1-inducing factors, e.g., the reduction of pathogen-associated molecular patterns (PAMP), which are detected by DC expressing Toll-like receptors (TLR), is associated with the germ-reduced living standard in the cities of the so-called Western world. This coincides with an increase of Th2-mediated disorders such as allergic asthma bronchiale and atopic dermatitis in the

urban population, which are not detected in the rural population. The inverse association of microbial load and Th2 disorders has led to the formulation of the hygiene hypothesis (4, 5). According to this hypothesis, a constant Th1 triggering balances the immune system, and the removal of these triggers skews the system toward Th2 (6).

Because of these findings, we hypothesized that treatment of DC with PAMP and with Th1-driving cytokines might convert a Th2 into a Th1 response. We have previously shown that the TLR2 and TLR6 engaging (7, 8) *Mycoplasma fermentans*-derived PAMP macrophage-activating lipopeptide 2 kDa (MALP-2) (9) matures DC, but does not influence Th skewing (10). In continuation of these studies, we evaluated the effect of a combined MALP-2 and IFN- γ pretreatment of DC on their lymphocyte-modulating potential. Our data indicate that a combination of TLR and IFN- γ receptor stimulation of DC results in a shift from a Th2- to a Th1-like response in a coculture system with appropriately pretreated DC and autologous lymphocytes from allergic subjects.

Materials and Methods

Patients

Citrate phosphate dextrose adenine-preserved blood was collected by venous puncture from, if not otherwise mentioned, six allergic volunteers. Proband reacted with a positive skin-prick test response to affinity-purified Der p 1 (ALK Scherax, Hamburg, Germany), the major allergen of the house dust mite *Dermatophagoides pteronyssinus*. A positive response was defined by a wheal of 3 mm or greater. A 0.9% NaCl solution served as negative control and histamine as positive control. Blood from donors with a negative test was used in control experiments. All donors gave their informed consent. The study was approved by the ethics committee of Hannover Medical School (Hannover, Germany).

Generation of DC

Monocyte-derived DC were generated according to principles brought forward by Peters et al. (11) and Sallusto and Lanzavecchia (12), with modifications as previously described (10). Briefly, blood was centrifuged to remove platelets, diluted, and layered over Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden). After density gradient centrifugation, PBMC

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³ Abbreviations used in this paper: DC, dendritic cell; Der p 1, major allergen of house dust mite *D. pteronyssinus*; MALP-2, macrophage-activating lipopeptide 2 kDa; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor.

were collected and monocytes were enriched using the MACS system and anti-CD14 beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD14-positive fraction (monocytes) was cultured in the serum-free medium X-VIVO 15 supplemented with 100 U/ml penicillin and 100 ng/ml streptomycin (all BioWhittaker, Verviers, Belgium). The CD14-negative fraction was cryopreserved in 90% FCS (Life Technologies, Eggenstein-Leopoldshafen, Germany) and 10% DMSO (Sigma-Aldrich, Taufkirchen, Germany). At days 0 and 5 after isolation, 800 U/ml rGM-CSF and 500 U/ml rIL-4 (Strathmann Biotec, Hamburg, Germany) were added to the monocyte culture to induce DC (see Fig. 1a). We have previously demonstrated that immature DC (day 5) generated according to this protocol endocytose FITC-labeled dextran and express the TLR 2 and 6 necessary for MALP-2 signaling (10).

Stimulation of DC

On day 5 of the culture, DC were pulsed with affinity-purified house dust mite allergen adjusted to a concentration of 1000 SQ-U/ml (corresponding to 100 ng/ml Der p 1) (ALK Scherax) or with PBS as control. DC were further treated with either 100 pg/ml MALP-2 synthesized and purified as described (7), 5000 U/ml IFN- γ (Strathmann Biotec), or a combination of both for 48 h. Control cultures were left untreated. The respective optimal concentrations were experimentally determined using the highest expression of stimulatory cell surface molecules as readout system (data not shown).

Washout experiments

A constant, stable phenotype and function of DC is a prerequisite for their therapeutical application (13, 14). To test the quality of DC stimulated with MALP-2 and IFN- γ in terms of their stability, DC were subjected to a 4-day washout period ($n = 3$). On day 7, cells were not harvested, but cell culture medium was removed and substituted for fresh medium without any cytokines or stimuli. After further cultivation for 4 days, cells were eventually harvested and subjected to flow cytometry and coculture (Fig. 1b).

Flow cytometric analysis of DC

For immunophenotyping, 2×10^5 DC per measurement were harvested after 7 days of culture or after the washout period and washed in PBS supplemented with 0.5% FCS and 10 mM Na $_3$ (Sigma-Aldrich). Cells were incubated for 30 min at 4°C with one of the following combinations of labeled murine mAbs (isotype control FITC/isotype control PE, anti-CD83 FITC, anti-CD86 FITC/anti-CD40 PE, anti-CD80 FITC/anti-HLA-DR PE (all BD Biosciences, Heidelberg, Germany)), washed, and analyzed on an EPICS XL-MCL (Beckman Coulter, Krefeld, Germany) flow cytometer. Data were processed with the Expo 32 cytometer software (Beckman Coulter). The expression of the cell surface molecules was evaluated by using the median of fluorescence intensity after subtraction of the values of the isotype control (Table I), or compared by overlaying the fluorescence profiles of the respective stained molecules in the washout experiments (Fig. 3).

Coculture of DC with autologous lymphocytes

As previously described (10), a portion of DC was used for coculture experiments with autologous lymphocytes from day 7 to 13, or from day 11 to 14 in the washout experiments. To remove free stimulants, DC were harvested and washed twice. The cells were titrated at a ratio of 1:3 in fresh medium in fresh round-bottom microtiterplates from concentrations of 3×10^4 to 4.1×10^2 per well. A total of 1×10^5 vital cryopreserved autologous lymphocytes was added, resulting in ratios of DC to lymphocytes from 1:3 to 1:2187 in a final volume of 200 μ l in serum-free X-VIVO-15. No further stimulants were given.

Determination of cytokines

Cytokine concentrations were determined in DC culture supernatant collected on day 7 (TNF- α , IL-10, IL-12p70) and in the supernatant of the coculture of DC and autologous lymphocytes collected on day 10 or 14 in the washout experiments (IFN- γ , IL-4). Duo-Set ELISA kits for the detection of the mentioned cytokines were used (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

Determination of IL-4- and IFN- γ -producing lymphocytes

IL-4- or IFN- γ -producing lymphocytes were determined in the coculture of DC and autologous lymphocytes on day 9 (IL-4) or 10 (IFN- γ) ($n = 3$). ELISPOT kits were used (IL-4, R&D Systems; IFN- γ , BD Bioscience) according to the manufacturer's instructions. The frequency of IL-4- or IFN- γ -producing cells in the coculture of Der p 1-loaded DC with lymphocytes was compared with prestimulation of DC with Der p 1, MALP-2, and IFN- γ . Controls with either cell population alone were included. A cell concentration of 3.3×10^4 DC and 1×10^5 lymphocytes for determination of IL-4 and of 1×10^4 DC and 3×10^4 lymphocytes for determination of IFN- γ -producing cells in a total volume of 200 μ l of cell culture medium gave a spot frequency that could be counted reliably.

Proliferation assay

The coculture was pulsed with 5 μ Ci/ml [^{3} H]thymidine (Amersham Buchler, Braunschweig, Germany) on day 12, or in washout experiments on day 16. After 18 h, the cells were harvested on filtermats (Canberra-Packard, Dreieich, Germany). After drying, 20 μ l of liquid scintillator (Canberra-Packard) was added, and the plates were sealed. The cpm were determined on a Topcount microplate scintillation counter (Canberra-Packard).

Blocking of IL-12p70 in the coculture

Directly after preparing the coculture (day 7) and additionally on day 10, 3 μ g/ml IL-12p70-blocking Ab (R&D Systems) was added. Controls using an unspecific isotype control Ab (R&D Systems) were included. IL-12p70 blocking was performed in the coculture of DC pretreated with Der p 1, MALP-2, and IFN- γ with autologous lymphocytes from three separate experiments with cells from different donors.

Statistical analysis

Data are presented as the mean $\pm$ SEM. The paired Student's t test was applied for statistical analysis. Values of $p \leq 0.05$ were considered significant.

Results

DC pulsed with Der p 1 induce a Th2-skewed immune response in autologous lymphocytes of allergic subjects

To mimic an allergic vs a normal Th response, DC from 18 house dust mite allergic subjects and 6 nonallergic controls were pulsed with Der p 1 or PBS at day 5 of DC generation for 48 h. After 3 days of coculturing thus treated DC with autologous lymphocytes, the cytokines IL-4 and IFN- γ were measured in the supernatant. The experimental system is outlined in Fig. 1a.

In allergic subjects, pretreatment of DC with Der p 1 resulted in a reduction of IFN- γ near the detection limit (3.6 pg/ml $\pm$ 5.1) compared with PBS-treated DC (7.7 pg/ml $\pm$ 3.7). Such Ag-dependent suppression of IFN- γ production was not observed in

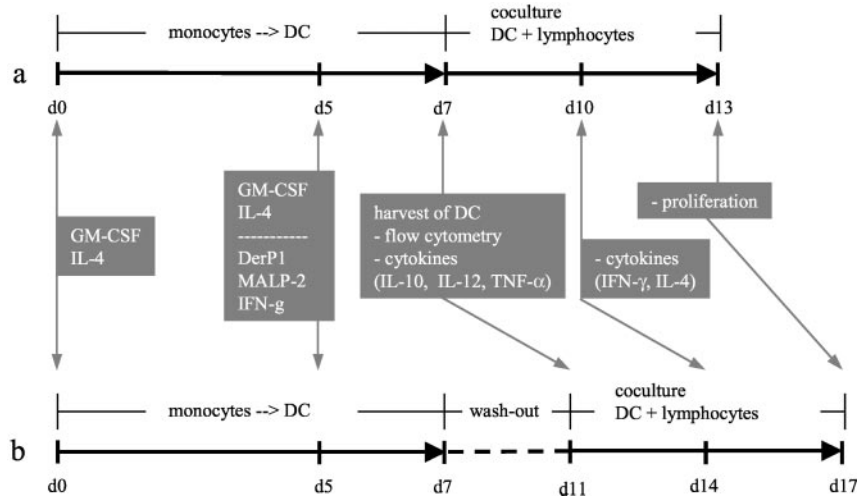
Table I. Flow cytometric analysis of DC after a 48-h treatment with PBS, Der p 1, Der p 1 + MALP-2, Der p 1 + IFN- γ , or Der p 1 + MALP-2 + IFN- γ

	CD40	CD80	CD83	CD86	HLA-DR
PBS	670.6 $\pm$ 153.6	44.4 $\pm$ 17.9	55.6 $\pm$ 8.3	173.1 $\pm$ 29.1	390.9 $\pm$ 109.9
Der p 1	733 $\pm$ 160.5	47.2 $\pm$ 18.9	47.3 $\pm$ 9	159.8 $\pm$ 29.3	426.9 $\pm$ 131.9
Der p 1 + MALP-2	<u>1389.6 $\pm$ 269.9</u>	<u>103.7 $\pm$ 31.2</u>	<u>72.4 $\pm$ 8.8</u>	<u>260.2 $\pm$ 53.8</u>	746.1 $\pm$ 295
Der p 1 + IFN- γ	<u>1769.4 $\pm$ 519.9</u>	69.3 $\pm$ 17.2	38.6 $\pm$ 6.2	123.9 $\pm$ 25.7	<u>669.1 $\pm$ 204.2</u>
Der p 1 + MALP-2 + IFN- γ	<u>2673.9 $\pm$ 717.5</u>	<u>248.5 $\pm$ 94.3</u>	<u>90.7 $\pm$ 14.6</u>	<u>358.9 $\pm$ 63.7</u>	<u>1253.8 $\pm$ 417.6</u>

^a Values are means $\pm$ SEM of the median fluorescence intensities from six individual experiments.

^b Underlined numbers indicate significant differences ($p \leq 0.05$) compared with the respective Der p 1-treated group.

FIGURE 1. Schematic presentation of the study design. *a*, Standard protocol of generating DC; *b*, the washout protocol. Monocytes were isolated on day 0, and GM-CSF and IL-4 were added to induce DC. Second addition of GM-CSF and IL-4, Der p 1 pulse, and stimulation with PBS, IFN- γ , MALP-2, or combinations thereof on day 5. DC were harvested on day 7 (standard protocol) or on day 11 after removal of all stimulants on day 7 (washout protocol). DC were analyzed by flow cytometry, and IL-12, IL-10, and TNF- α were determined from the cultured medium. DC were cocultured with autologous lymphocytes from day 7 or 11, respectively. IFN- γ and IL-4 were measured in the supernatant after 3 days of coculture, and proliferation in the coculture was determined after 6 days by [^{3} H]thymidine incorporation.



healthy controls ($8.7 \text{ pg/ml} \pm 6.6$ vs $11.6 \text{ pg/ml} \pm 2.5$). In contrast, Der p 1-pretreated DC from allergic subjects induced a significant up-regulation of IL-4 ($13.6 \text{ pg/ml} \pm 7.3$) compared with unpulsed DC ($9.0 \text{ pg/ml} \pm 5.8$) ($p = 0.016$). IL-4 concentration was below the quantification limit of 5 pg/ml ELISA in healthy donors, no matter whether DC had been pulsed or not (Fig. 2).

MALP-2 and IFN- γ synergistically increase the expression of stimulatory cell surface molecules of allergen-pulsed DC from allergic subjects

To evaluate the effect of the test substances on the DC phenotype, Der p 1, MALP-2, and IFN- γ or PBS were added at day 5 of the DC culture, and the surface expressions of CD40, CD80, CD83, CD86, and HLA-DR were determined 48 h later. The experimental system is shown in Fig. 1, and the results in Table I. Pulsing of DC with Der p 1 alone had no effect on the expression of the surface molecules compared with untreated DC. MALP-2 combined with Der p 1 significantly up-regulated CD40, CD80, CD83, and CD86, while IFN- γ only up-regulated HLA-DR and CD40. Using a combination of MALP-2 and IFN- γ to stimulate Der p 1-pulsed DC, a strong synergistic amplification of CD40, CD80, CD83, CD86,

and HLA-DR was observed (Table I). The expression of CD40 and CD83 was stable for a period of at least 4 days after removal of stimulants; the expression of CD80, CD86, and HLA-DR was increased even further after this time (Fig. 3).

MALP-2 and IFN- γ induce IL-12p70 production of allergen-pulsed DC from allergic subjects

To further analyze the effect of MALP-2 and IFN- γ on DC, the production of TNF- α , IL-10, and IL-12p70 was determined in the cell culture supernatants by ELISA at day 7. Neither stimulation with Der p 1 alone nor in combination with IFN- γ influenced the production of IL-10 and IL-12p70 compared with untreated DC (Fig. 4, *a* and *b*). The addition of MALP-2 alone to allergen-pulsed DC only induced IL-10 and TNF- α production, but had no effect

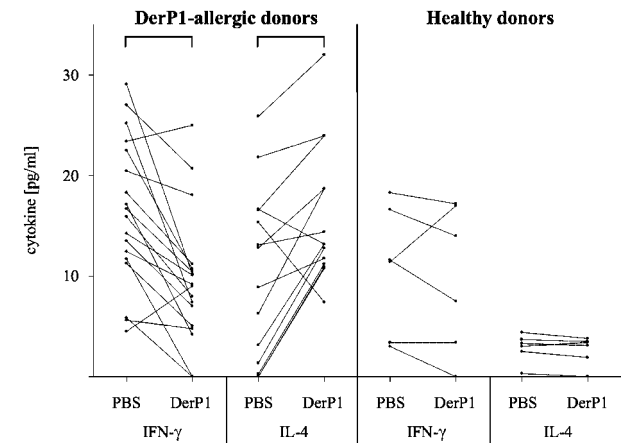


FIGURE 2. Determination of IFN- γ vs IL-4 production in the in vitro allergy model. DC from 18 Der p 1 allergic and 6 healthy donors were incubated with Der p 1 vs PBS as control. After thorough washing, DC were cocultured with autologous lymphocytes, and supernatant was assayed for the given cytokines after 3 days. Connected samples of one donor are linked by lines. The bracket indicates a significant difference ($p \leq 0.05$) between the compared groups.

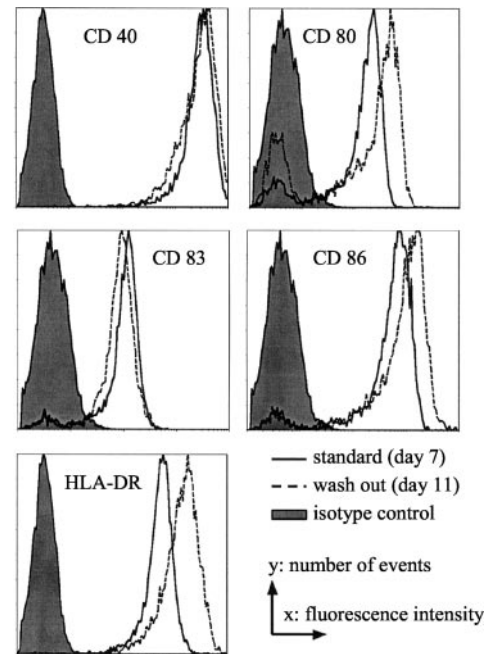


FIGURE 3. Flow cytometric analysis of DC from allergic subjects harvested after 7 days according to the standard protocol, or after a further 4-day period in the washout protocol. The Ag expression profiles from one representative experiment are compared and shown with the appropriate isotype controls.

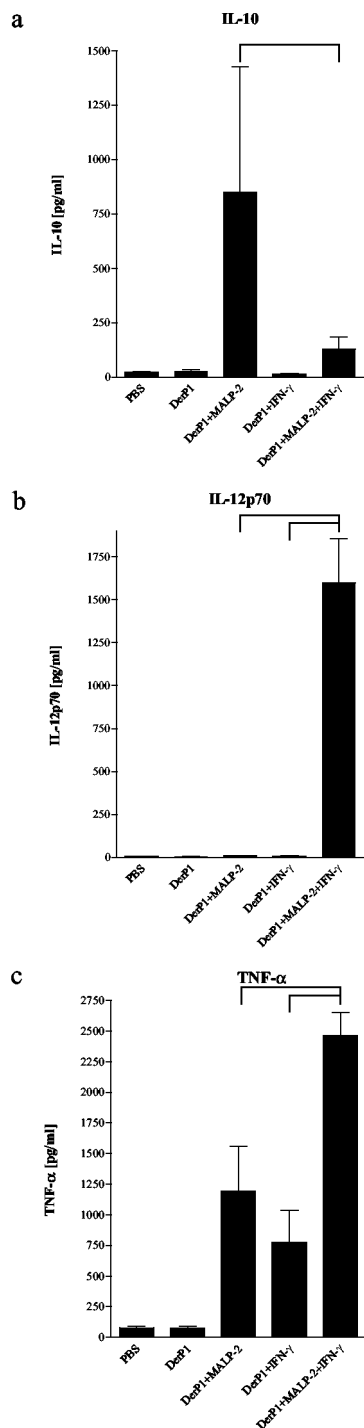


FIGURE 4. Production of IL-10 (a), IL-12p70 (b), and TNF- α (c) after treatment of immature DC with PBS, Der p 1, Der p 1 + MALP-2, Der p 1 + IFN- γ , or Der p 1 + MALP-2 + IFN- γ for 2 days. The bracket indicates a significant difference ($p \leq 0.05$) between the compared groups.

on IL-12p70 release. However, when stimulating with a combination of MALP-2 and IFN- γ , the production of IL-12p70 was massively increased, accompanied by a reduction of IL-10 to almost basal levels (no significant difference to PBS- and Der p 1-treated DC). TNF- α was produced at low levels in the unstimulated and the Der p 1-pulsed group (Fig. 4c). Either MALP-2 or IFN- γ induced a comparable moderate TNF- α release, while the stimulation with both substances together more than doubled the concentration of TNF- α compared with the stimulation with the single

substances. The absence of allergen did not significantly alter these results (data not shown). These data suggest the modulation of DC from allergics toward a phenotype that triggers a Th1 response (15) by a combined treatment with MALP-2 and IFN- γ even in the presence of allergen.

MALP-2- and IFN- γ -treated DC from allergic subjects increase allergen-associated proliferation of autologous lymphocytes

To further assess the functional effects of MALP-2 and IFN- γ treatment, DC were cocultured with autologous lymphocytes after their harvest on day 7 or 11 in the washout experiments, respectively. For this purpose, DC were seeded at various cell numbers in fresh medium and new cell culture plates to remove the stimuli, and a constant number of the cryopreserved autologous lymphocytes was added. [^3H]Thymidine was added to the coculture on day 12 for an additional 18 h. Allergen-pulsed DC exerted a very weak effect on lymphocyte proliferation (Fig. 5). Pretreatment of pulsed DC with IFN- γ alone increased proliferation, which was further augmented by MALP-2 alone. The strongest response, however, was measured when allergen-pulsed DC were treated with a combination of both substances. This activated state of the DC was stable for at least 4 days, as lymphocyte proliferation was not altered when these were added after the 4-day washout period (data not shown). In contrast to the DC phenotype and cytokine production, lymphocyte proliferation was reduced when otherwise equally treated DC had not been pulsed with allergen (data not shown).

Combined MALP-2 and IFN- γ pretreatment shifts a Th2-skewed immune response to Th1

To analyze the effect of MALP-2 and IFN- γ pretreatment on the allergen-induced Th2-skewed immune response, the cytokines IFN- γ and IL-4 were measured on day 10, the third day of the coculture, in the supernatant of a coculture of DC from allergic donors with autologous lymphocytes. For this experiment, an optimal titration ratio of 1:3 (DC:lymphocytes) was used. Fig. 6 shows the cytokine concentration and the ratio of IFN- γ to IL-4 in

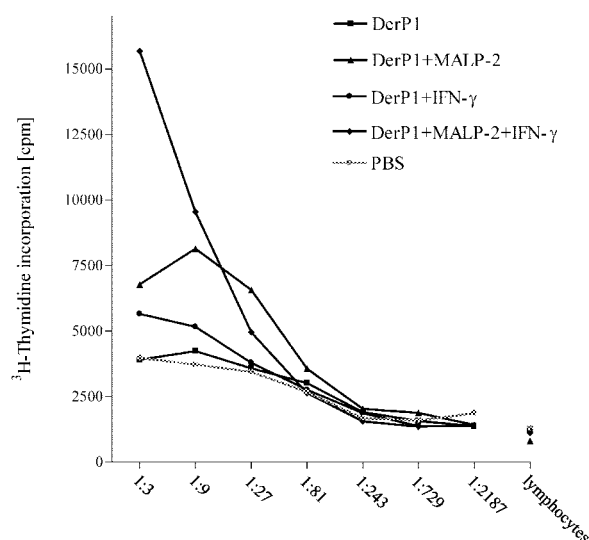
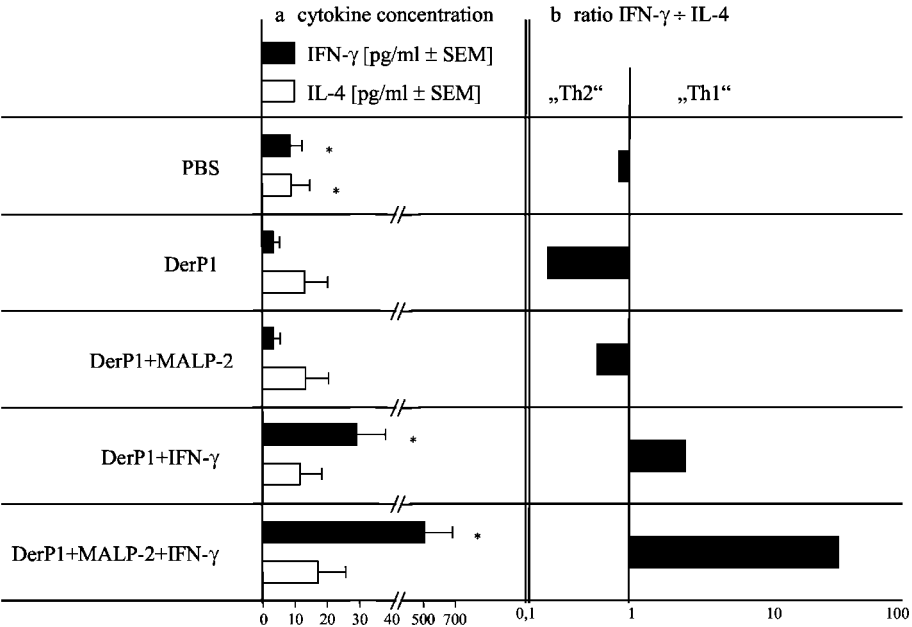


FIGURE 5. Proliferative response of autologous lymphocytes cocultured with DC pretreated with PBS, Der p 1, Der p 1 + MALP-2, Der p 1 + IFN- γ , or Der p 1 + MALP-2 + IFN- γ . Cells were generated from subjects allergic to Der p 1. Each curve represents the mean of six individual experiments. Decreasing numbers of DC were cocultured with 1×10^5 lymphocytes, giving the ratio of DC to lymphocytes x-axis. The last point (lymphocytes) shows lymphocytes without DC.

FIGURE 6. Production of IFN- γ and IL-4 in a coculture of autologous lymphocytes with DC pretreated with PBS, Der p 1, Der p 1 + MALP-2, Der p 1 + IFN- γ , or Der p 1 + MALP-2 + IFN- γ . Cells were generated from subjects allergic to Der p 1. *a*, Cytokine concentrations $\pm$ SEM; *, indicates a significant difference ($p \leq 0.05$) compared with the group treated with allergen alone (Der p 1). *b*, Ratios of IFN- γ to IL-4 are shown on a logarithmic scale.



the coculture supernatant. Pulsing with Der p 1 induced an increase in IL-4 and a reduction of IFN- γ production. Additional MALP-2 pretreatment showed no effect. IFN- γ pretreatment had little effect on IL-4, but augmented IFN- γ production. This effect was boosted by simultaneous preincubation of DC with MALP-2 and IFN- γ . Although IL-4 was not raised significantly, IFN- γ production increased >50-fold compared with cocultures with allergen alone, indicating a Th1 immune response. The massive increase of IFN- γ production in the supernatant of the coculture was correlated with a rise in the number of IFN- γ -producing lymphocytes detected by ELISPOT. Although 27 ± 3 spots were detected in the group treated with allergen alone, this was tripled (89 ± 5) following simultaneous stimulation with MALP-2 and IFN- γ . Neither the amount of IL-4-producing cells nor the cytokine content in the supernatant was affected (21 ± 6 (Der p 1-pretreated DC) vs 18 ± 8 (Der p 1 + MALP-2 + IFN- γ -pretreated DC); note the 3-fold higher cell concentration necessary in this experiment compared with the IFN- γ ELISPOT (Fig. 7)). Neither IFN- γ - nor IL-4-positive spots were detected in the controls after stimulation of DC with allergen alone nor after treatment with allergen plus MALP-2 and IFN- γ without lymphocytes, nor by culturing lymphocytes alone.

Culture Conditions		IFN- γ Spot-		IL-4 Spot-	
Cells	Conditions	-Photo	-No.	-Photo	-No.
DC	DerP1		0		0
DC	DerP1+MALP-2+IFN- γ		0		0
Ly			0		0
DC+Ly	DerP1		27 ± 3		21 ± 6
DC+Ly	DerP1+MALP-2+IFN- γ		89 ± 5		18 ± 8

FIGURE 7. Frequency of IFN- γ - or IL-4-producing cells as determined by ELISPOT. Lymphocytes from allergics to Der p 1 were cocultured with autologous DC pulsed with Der p 1 or additionally stimulated with MALP-2 and IFN- γ . Either cell type alone is given as control. Spot numbers are given as means with SEM from duplicate experiments.

The Th1-inducing effects depend on IL-12p70

IL-12p70 is known to stimulate Th1 responses. To analyze the mechanism of MALP-2- and IFN- γ -stimulated DC to induce such a pronounced Th1 response, IL-12p70, which is produced only by DC treated accordingly (Fig. 4b), was blocked with an appropriate Ab in the coculture. Such treatment indeed resulted in a significantly diminished production of IFN- γ ($443 \text{ pg/ml} \pm 73$) compared with the control group treated with an unspecific isotype control Ab ($664 \text{ pg/ml} \pm 28$) (Fig. 8a). IL-4 production was not affected. Lymphocyte proliferation was consequently reduced to two-thirds by the specific Ab compared with the control Ab (Fig. 8b).

Discussion

Several methods were published that describe the generation and modulation of DC for clinical use (reviewed in Refs. 16–18). These studies are mostly concerned with the exploitation of DC for use in tumor therapy. Our interest lies in the generation of DC that might be used to alleviate or abolish serious allergic symptoms caused by a misdirected reaction to ubiquitous, normally harmless allergens. A hypothesis has been formed that a conversion of a Th2 reaction against such Ags into a Th1-mediated reaction might be a feasible approach to a causative treatment of allergies (19–21). As a first step, it was the aim of this in vitro study to explore the ability of DC subjected to various stimuli to modulate a pre-existing Th2 response and to revert it to a Th1-type response.

Our experimental system using DC and autologous lymphocytes from allergics, in which allergen stimulation induces a Th2 response, has been well described (22–24). We used the Der p 1 Ag from the house dust mite as a relevant Ag. Some studies report that Der p 1 is capable of modulating DC to induce a Th2 response because of an intrinsic enzymatic activity of the molecule (25–27). To exclude such interference with our experiments, purified Der p 1 without protection of the reactive thiol group was used. This leads to inactivation of the enzyme activity, resulting in a Der p 1 variant that is unable to induce Th2 per se, but is still antigenic. Indeed, no effect on isolated DC was observed (see Table I and Fig. 4). The antigenicity of the Der p 1 variant was shown by its capacity to cause cytokine production, as was observed in the coculture of DC and lymphocytes from allergic subjects (see Fig. 6). This suggests that the DC take up, process, and successfully

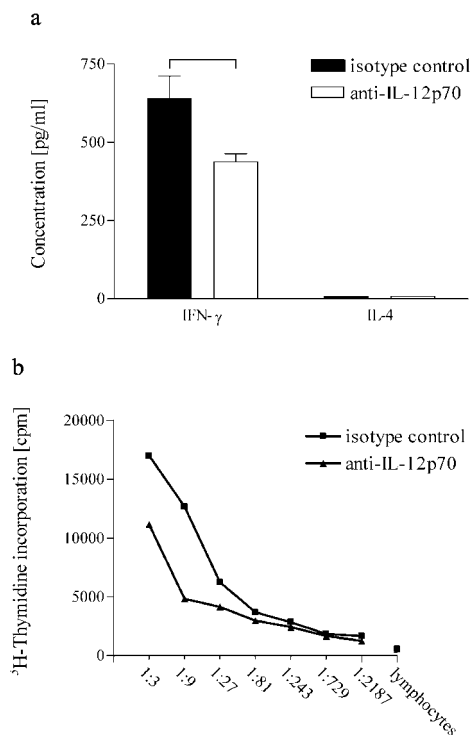


FIGURE 8. Effect of a blocking anti-IL-12p70 Ab on cytokine production and lymphocyte proliferation. Lymphocytes from allergics to Der p 1 were cocultured with allergen-pulsed autologous DC prestimulated with MALP-2 and IFN- γ in the presence of the Ab: *a*, cytokines measured by ELISA; *b*, proliferation measured by [^3H]thymidine incorporation. Control cultures were treated with an unspecific isotype control Ab.

present the enzymatically inactivated allergen to the in vivo Der p 1-primed Th2 cells.

Using cells from healthy donors, we had previously shown that MALP-2, although inducing DC maturation and DC-mediated lymphocyte proliferation, has no influence on the polarization of the lymphocyte response (10). As shown in the present study, the same was true for cells from allergic subjects. Only pretreatment of DC with a combination of MALP-2 and IFN- γ led to maturation of DC, associated with a high IL-12 production (see Table I and Fig. 4). The thus modulated DC reversed the prevailing Th2 reaction to a Th1-like response characterized by a pronounced enhancement of lymphocyte-derived IFN- γ (see Figs. 6 and 7). The frequency of IL-4-producing cells and the IL-4 concentration in the supernatant were not affected by MALP-2 + IFN- γ stimulation. We conclude thereof that the existing Th2 cells are not modulated, but that the Th1 shift is due to the augmentation of Th0 cells or of existing Th1 clones. In this regard, it will be of major interest to evaluate the in vivo situation after application of MALP-2 + IFN- γ and of accordingly treated DC. Such experiments will be conducted first of all in an animal model of allergic disease.

As it was produced by DC only after a combined stimulation with both substances (see Fig. 4b), IL-12p70 is the most probable factor that explains the observed Th1 shift. The role of IL-12p70 for the shift is further substantiated by the blocking experiments, which resulted in a diminished proliferation and IFN- γ production (Fig. 8). A comparable effect of blocking IL-12p70 was described in the allogenic system (28, 29). The involvement of IL-12 in Th1 reactions is well documented (30–33). Our study is consistent with the paradigm of a two-signal model for IL-12 induction, in our case MALP-2 and IFN- γ , as brought forward by Snijders et al. (34).

The modulating influence of IFN- γ alone on monocyte-derived DC has been discussed controversially: although some studies demonstrate an enhancing effect of IFN- γ on DC maturation and function (35, 36), others describe a down-regulation of immunostimulatory capacity in the human (37) and in the murine (38) system. In our study, DC pretreated with IFN- γ caused only a marginal enhancement of lymphocyte proliferation and production of IFN- γ (see Figs. 4 and 5).

A recent study of Dalpke et al. (39) showed the cross talk between the signal transduction pathways of TLR and IFN- γ receptors for the first time. The authors describe an amplification of IFN- γ signaling by a combined stimulation with TLR agonists such as CpG-oligodeoxynucleotide, LPS or lipoteichoic acid, and IFN- γ . The effect is reported to be due to the phosphorylation of STAT1, an event of importance in the IFN- γ signal transduction, by TLR-dependent stimuli. These findings may suggest a possible molecular mechanism for the observed synergism of MALP-2 and IFN- γ in modulating the DC.

This in vitro study shows the potential of appropriately treated DC to reverse a pre-existing Th2 response to a Th1-like reaction. As DC treated with MALP-2 and IFN- γ remained stable for at least 4 days without modification of phenotype and function, they can be termed terminally mature and are in this regard potentially suited for the evaluation of in vivo efficacy. Thus generated DC should have a constant phenotype and should not redifferentiate (13, 14). The step from cell culture work to an effective treatment of patients needs further studies. In this context, it may be of interest that MALP-2 can be effectively administered by the intranasal and intratracheal route (40–42).

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