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Modulation of dendritic cell function by cowshed dust extract

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We have shown previously that inhalation of cowshed dust extract (CDE) resulted in decreased airway reactivity, eosinophilic inflammation and sensitization in a mouse model of allergic asthma. Our data suggested down-regulation of allergic immune response rather than activation of a Th1 response towards the model allergen. However, the precise mechanism of allergy protection is not yet understood in detail. To gain deeper insight into CDE-induced immune modulation, we have analysed the effects of CDE on dendritic cell biology. Dendritic cells were generated from murine bone marrow cells (BMDC). Cells were stimulated with CDE and subsequently used to sensitize mice via the airways. Our results showed that cells were not able to prime mice for allergic immune response when they were treated with CDE 2 days before pulsing with allergen, whereas cells that were stimulated with CDE simultaneously to OVA pulsing induced a fully developed allergic immune response. Surprisingly, CDE-treated cells that were not able to prime mice for allergic immune response exhibit an activated phenotype with high expression of the co-stimulatory surface molecule CD86. Moreover, CDE-treated cells transiently produced high amounts of cytokines such as IL-10, IL-12p70 and TNF- α . Interestingly, blocking of autocrine-produced IL-10 *in vitro* partially restored the allergy-inducing capacity of CDE-exposed cells. Thus, we conclude that prolonged exposure to CDE reduces the allergy-inducing capacity of dendritic cells. Furthermore, we present evidence that an autocrine IL-10 dependent mechanism seems to be involved in down-regulation of dendritic cell function due to stimulation with CDE.

Keywords: Allergy, dendritic cells, tolerance

INTRODUCTION

Recently, we have shown that inhalation of cowshed dust extract (CDE) protected from allergic immune response in a mouse model of asthma.¹ This was the first experimental evidence linking the epidemiological observation that growing up on a traditional farm protects from allergic disease to inhalation of environmental dust. The precise mechanism of CDE-induced immune modulation is not understood in detail; however, in contrast to studies in which high doses of lipopolysaccharides (LPS) were applied, CDE down-regulates the immune response

towards the allergen rather than inducing a Th1 response.^{2,3} Moreover, our published data revealed that human monocyte derived dendritic cells that were treated with CDE exhibited reduced immune stimulatory properties. Since lung bronchi contain dendritic cells that are specialized to encounter inhaled substances, it is tempting to speculate that the immune stimulatory properties of lung dendritic cells are reduced in a similar manner after inhalation of CDE.⁴

From murine asthma models, it is well known that dendritic cells are of particular importance for the induction phase of the allergic immune response. Furthermore, it was demonstrated recently that these cells are also

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involved in the on-going allergic immune response.⁵ It is not understood in detail how the disease is induced by dendritic cells; however, data from animal models indicated that co-stimulation during the sensitization phase is of importance for the decision whether Th1, Th2 or a regulatory immune response is induced.^{3,6,7} Mice that were sensitized with allergen in the presence of a high dose of TLR4 ligands like LPS tended to develop a Th1 response, whereas mice that were sensitized and challenged with allergen without adjuvant or in the context of TLR2 binding substances developed a Th2 response. Interestingly, experiments from Kuipers and colleagues⁸ revealed that immune modulatory effects mediated by TLR4 binding substances take place on the dendritic cell level. For this purpose, they utilized an airway sensitization model with allergen-pulsed dendritic cells as sensitizing agent. They demonstrated that dendritic cells that were treated with LPS during allergen exposure were subsequently not able to prime mice for allergic immune response due to induction of a protective Th1 response towards the allergen.⁸

To gain deeper insight into the immune modulatory activity of CDE, we have tested here whether treatment with CDE modulates the biology of dendritic cells thus influencing their ability to induce an allergic immune response in a mouse model of allergic airway inflammation.

MATERIALS AND METHODS

Extraction of cowshed dust

Cowshed dust was collected and extracted with isotonic sodium chloride solution as previously described.¹ Cowshed dust extract (CDE) was stored lyophilized. After resolubilization in isotonic NaCl solution, extracts were sterile filtered (0.22 µm) before use.

Animals

Female BALB/c mice (Charles River, Sulzfeld, Germany) aged 7–8 weeks were purchased and then adapted to the animal facility for 14 days prior to experiments. Animals had access to food and water *ad libitum*. All experimental procedures were approved by the animal ethics committee at Bezirksregierung Arnsberg, Germany.

Generation of BMDC and cultivation with CDE

Femora and tibia of untreated mice were flushed with phosphate-buffered saline (PBS) to harvest bone marrow dendritic cells (BMDC). Subsequently, erythrocytes were lysed by hypotonic shock. Bone marrow cells were

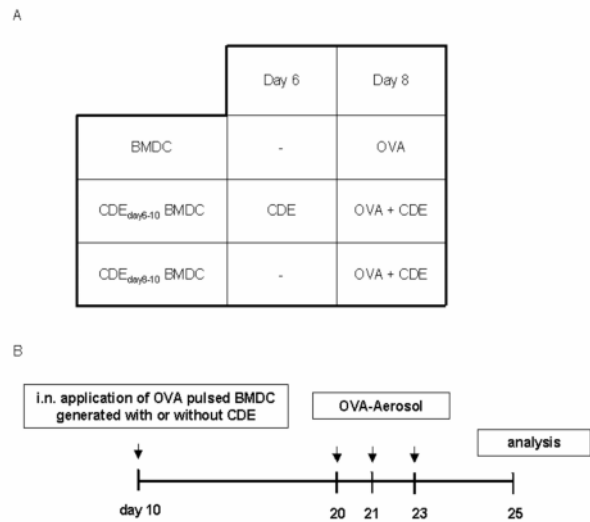


Fig. 1. Design of *in vitro* (A) and *in vivo* (B) experiments. (A) On day 0, bone marrow cells were harvested and cultured with GMCSF to generate BMDC. Cells were stimulated with CDE either on day 6 (CDE_{day6-10} BMDC) or on day 8 (CDE_{day8-10} BMDC). BMDC were pulsed for 2 days with 100 µg/ml ovalbumin (OVA) on day 8. (B) Naïve mice were sensitized by intranasal application of BMDC on day 10. OVA-aerosol, indicates challenges with 1% OVA aerosol via the airways. Analysis denotes harvest of BAL cells, serum and splenocytes for analysis.

cultured with 20 ng/ml rmuGMCSF (Immunotools, Friesoythe, Germany) for 6 days with one exchange of supplemented RPMI 1640 medium (RPMI 1640 with 5% fetal calf serum [FCS], 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin; all from Biochrom, Berlin, Germany) after 3 days. On day 6, low-adherent dendritic cells were collected and subsequently cultured with 100 µg/ml of CDE for an additional 4 days (from days 6–10) or 2 days (from days 8–10), respectively (Fig. 1A). On day 8, medium was exchanged and cells were pulsed with 100 µg/ml chromatographically purified ovalbumin (OVA) for 2 days (Worthington Biochemical Corporation, New York, USA). In some cultures, 4 µg/ml of an IL-10 neutralizing antibody (clone JES5-2A5; BD Bioscience, Heidelberg, Germany) was included for 4 days at the same time as stimulation with CDE. It was checked by ELISA that this concentration is sufficient to block endogenously produced IL-10 completely. In other cultures, BMDC were treated for 4 days with 1 ng recombinant murine IL-10 (Peprotech, London, UK) instead of stimulation with CDE.

Sensitization of mice with OVA-pulsed BMDC and airway challenge

For sensitization of mice with dendritic cells via the airways, they were anesthetized with a mixture of Ketamin and Rompun. OVA-pulsed BMDC (10⁶ cells)

were administered intranasally in 50 μ l PBS (Fig. 1B). Different treatment groups were primed with OVA-pulsed BMDC that were either cultivated for 2 or 4 days with CDE. Subsequently, for induction of airway inflammation and antibody response, mice were challenged on days 11, 12 and 13 with 1% OVA aerosol for 30 min using a PARI-Boy aerosol generator (PARI, Starnberg, Germany). For analysis of ovalbumin-specific antibody production, mice were challenged twice a week for a total of 9 weeks. Controls were treated with OVA aerosol alone.

Measurement of OVA-specific IgG1, IgG2a, IgE and IgA

OVA-specific antibodies in serum of mice were measured as described previously.¹ Levels of OVA-specific IgG1 and IgE was related to combined standards generated in our laboratory and expressed as arbitrary units per millilitre. Levels of OVA-specific IgG2a was expressed as optical density measured at 405 nm. OVA-specific IgA was measured in broncho-alveolar lavage (BAL) fluid of mice diluted 1/500. An alkaline phosphatase conjugated, isotype-specific antibody was used for detection (goat anti-mouse IgA; Sigma-Aldrich, Germany). Titer of IgA was expressed as optical density measured at 405 nm.

In vitro cytokine production of mouse lymphocytes

Spleens and lung lymph nodes were harvested 2 days after the third aerosol challenge. Single-cell suspensions were prepared by mechanical disruption and erythrocytes were lysed. Lymphocytes were cultured at a concentration of 5×10^6 /ml in complete tissue culture medium (RPMI 1640 with 10% fetal calf serum [FCS], 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin; Biochrom). After 48 h of culture, supernatants were taken and stored at -80°C until analysis.

Measurement of cytokines in cell culture supernatants

Levels of IL-5, IL-10, and IFN- γ were assessed using optEIA kits (BD Biosciences), IL-12p70 and TNF- α was measured with an ELISA set (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Broncho-alveolar lavage (BAL)

Two days after the last ovalbumin challenge, lungs were lavaged via a tracheal tube with 2×1 ml PBS, and leukocytes in the lavage fluid were counted. After

centrifugation, BAL fluid was frozen for further analysis. Cytospin slides of BAL cells were stained with a fast-staining procedure (HAEME-Schnellfärbung, Labor+ Technik Eberhard Lehmann, Berlin, Germany), according to the manufacturer's instructions. The percentages of eosinophils, lymphocytes and macrophages in BAL samples were determined by light microscopy. At least 300 cells per sample were differentiated by a blinded investigator.

Endocytosis

Endocytotic activity of BMDC was determined by flow cytometry. For this purpose, cells were cultured for 2 days with 100 μ g/ml fluorescein-labeled dextran (FITC-Dextran; Sigma-Aldrich, Germany). Dead cells were counterstained with propidium iodide.

Homing of BMDC to regional lymph nodes

For analysis of traffic of CDE-treated BMDC, cells were labeled with the cell marker carboxyfluorescein diacetate succinimidylester (CFSE). Briefly, cells were suspended in PBS and labeled with 5 μ M CFSE (Invitrogen, Karlsruhe, Germany) for 30 min. Subsequently, cells were centrifuged, resuspended in medium with 10% FCS and incubated at 37°C for an additional 30 min. Green fluorescent cells (2×10^6) were applied intranasally into mice. After 24 h, mice were killed and lavaged with PBS. Subsequently, lungs and mediastinal lymph nodes were removed and digested with collagenase IV (Sigma) as previously described. Single cell suspensions were analyzed for the presence of green fluorescent BMDC by flow cytometry.

Flow cytometric analysis

Flow cytometric analysis of dendritic cell-specific surface antigens was performed on a flow cytometer (Partec, Münster, Germany). The following fluorescein-isothiocyanate (FITC) or phycoerythrin (PE) conjugated rat anti-mouse monoclonal antibodies (mAbs) were used for direct labeling of the cells: antiCD11c-PE (clone N418) from BD Bioscience, antiCD86-FITC (clone GL1), and antiMHC II-Fitc (clone M5/114.15.2) from eBioscience. Isotype-matched mAbs of irrelevant specificity were used as controls.

Statistical analysis

All data were analyzed by the two-tailed Mann-Whitney test. Graph Pad Prism Software (v.3.03) was used for

analysis. Values of $P < 0.05$ were considered statistically significant. All results are presented as mean $\pm$ SD.

RESULTS

BMDC stimulated with CDE are not able to prime mice for allergic immune response

To analyse whether the ability of dendritic cells to induce an allergic immune response is modified by treatment with CDE, we generated dendritic cells from the bone marrow of mice. These BMDC were treated with CDE either from days 6–10 ($\text{CDE}_{\text{day6-10}}$ BMDC) or days 8–10 ($\text{CDE}_{\text{day8-10}}$ BMDC). Control dendritic cells were cultured in medium without CDE (BMDC). On day 8, two days before cells were transferred to mice, they were pulsed with OVA. After priming of mice with dendritic cells and subsequent OVA challenge, they were evaluated for eosinophilic airway inflammation and cytokine production (Fig. 2).

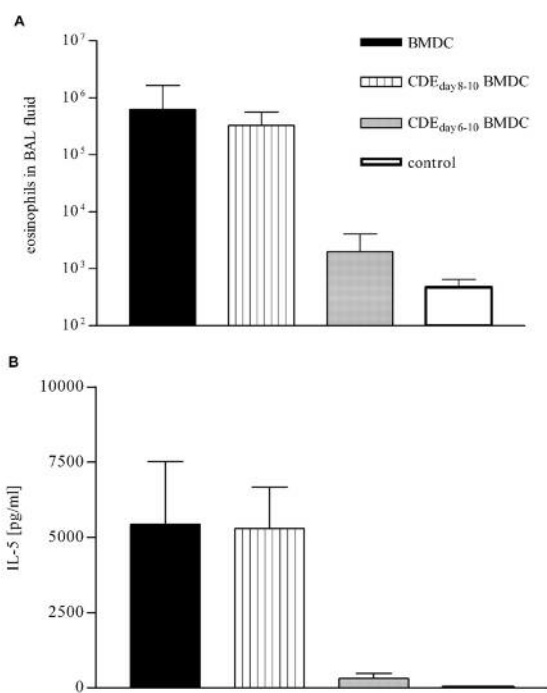


Fig. 2. Eosinophilic airway inflammation and IL-5 production of mice sensitized with BMDC. Total number of eosinophils in BAL fluid (A) and IL-5 production in cell culture supernatants of splenocytes (B) from mice that were sensitized with OVA-pulsed dendritic cells (BMDC) or OVA-pulsed dendritic cells that were treated with CDE from days 8 to 10 ($\text{CDE}_{\text{day8-10}}$ BMDC) or OVA-pulsed dendritic cells that were treated with CDE from days 6 to 10 ($\text{CDE}_{\text{day6-10}}$ BMDC) or challenged with OVA alone without prior sensitization (control). Results are expressed as mean with standard deviation from 4 mice per group. Data are representative for two independent experiments. Peribronchial inflammation of lung tissue was evaluated in H&E-stained lung slices from BMDC (C) and $\text{CDE}_{\text{day6-10}}$ BMDC (D) primed mice, respectively.

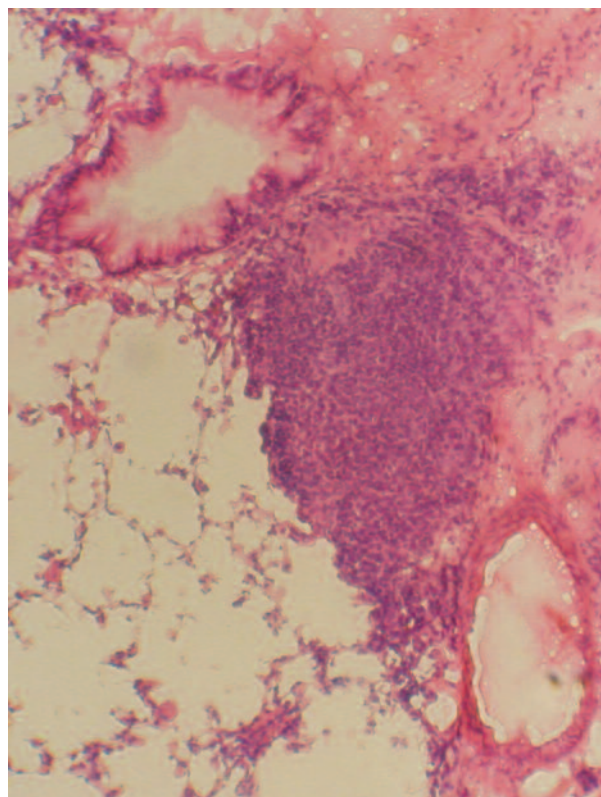


Fig. 2C.

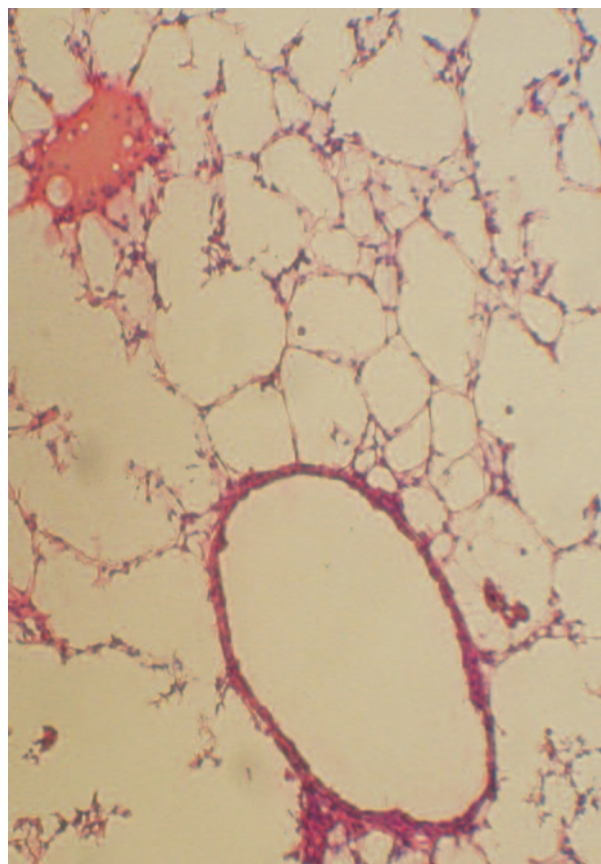


Fig. 2D.

As demonstrated in Figure 2A, mice that were primed with OVA-pulsed BMDC show massive eosinophilic infiltration in their airways ($63 \pm 52 \times 10^4$). Furthermore, as shown in Figure 2B, lymphocytes of these mice exhibit production of large amounts of IL-5 (spleen cells, $5.4 \pm 1 \times 10^3$ pg/ml; cells from bronchial lymph nodes, 4.07×10^3 pg/ml). However, mice that were primed with CDE_{day6-10} BMDC exhibit almost completely abolished eosinophilic infiltration in the airways ($0.2 \pm 0.2 \times 10^4$) and, furthermore, significantly reduced IL-5 production by lymphocytes (spleen cells, $0.3 \pm 0.2 \times 10^3$ pg/ml; cells from bronchial lymph nodes, not detectable). In contrast, mice that were primed with CDE_{day8-10} BMDC exhibit similar Th2 response compared to mice primed with BMDC that were not stimulated with CDE (eosinophils, $33 \pm 24 \times 10^4$; IL-5 from spleen cells, $5.3 \pm 1.4 \times 10^3$ pg/ml; IL-5 from cells from bronchial lymph nodes, 10.3×10^3 pg/ml). As demonstrated for IL-5, the production of IL-4, IL-10, IL-13 and IFN- γ was also down-regulated (data not shown) when mice were sensitized with CDE_{day6-10} BMDC indicating that CDE-treated dendritic cells show reduced capacity to activate T-lymphocytes.

These results were supported by histological examination of lung tissue. As demonstrated in Figure 2C, lung slices of mice that were primed with OVA-pulsed BMDC showed massive infiltration of leukocytes into peribronchial tissue compared to mice primed with CDE_{day6-10} BMDC where infiltration is almost absent. Thus, it can be concluded that treatment with CDE only leads to reduced allergy-inducing properties of dendritic cells when cells were treated before pulsing with antigen.

Dendritic cells that were treated with CDE lose their capacity to sensitize mice for OVA-specific IgG1 and IgE production

Exposure of mice to ovalbumin aerosol normally leads to tolerance towards the allergen.⁹ To test whether priming of mice with dendritic cells via the airways sensitizes them for a Th2 response and subsequently leading to OVA-specific antibody response, we applied 10^6 OVA-pulsed BMDC intranasally and subsequently challenged mice with an OVA aerosol over a period of 9 weeks.

As expected, mice that were challenged with OVA without prior sensitization exhibited a minor antibody response. In contrast, mice that were sensitized with OVA-loaded dendritic cells showed massive production of OVA specific IgE (Fig. 3A) and IgG1 (Fig. 3B) and of IgG2a, although to a lesser extent (Fig. 3C). Thus, sensitization of mice with dendritic cells seems to be sufficient to prime mice for Th2 response and subsequent IgE and IgG1 production. However, as already observed for

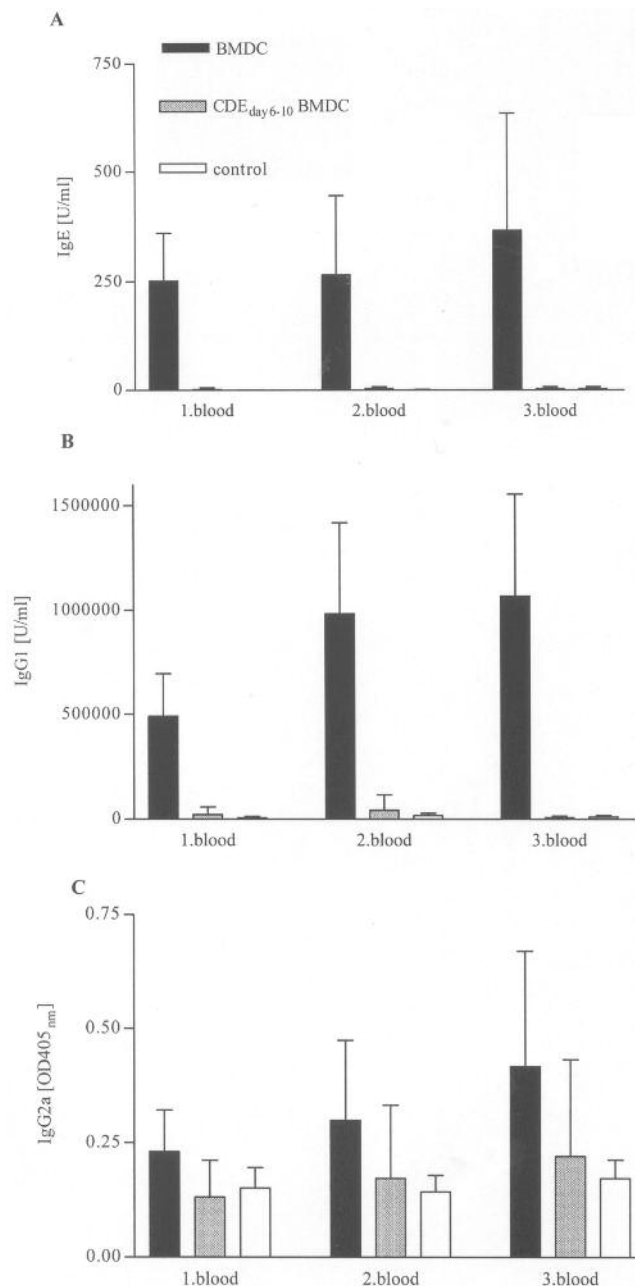


Fig. 3. OVA-specific antibody production in mice sensitized with BMDC and challenged with OVA for 6 weeks. Mice that were sensitized with OVA-pulsed dendritic cells (BMDC) or OVA-pulsed dendritic cells that were treated with CDE from days 6 to 10 (CDE_{day6-10} BMDC) were bled three times during a 9-week challenge protocol. Control mice were subjected to the challenge protocol without prior sensitization. Concentrations of OVA-specific antibody from the IgE (A), IgG1 (B) and IgG2a (C) isotypes were measured by isotype-specific ELISA and expressed as arbitrary units. Results are shown as mean with standard deviation from 5 mice per group.

allergic airway inflammation, CDE_{day6-10} BMDC were not able to prime mice for IgE, IgG1 and IgG2a production. These mice had low antibody titres comparable to

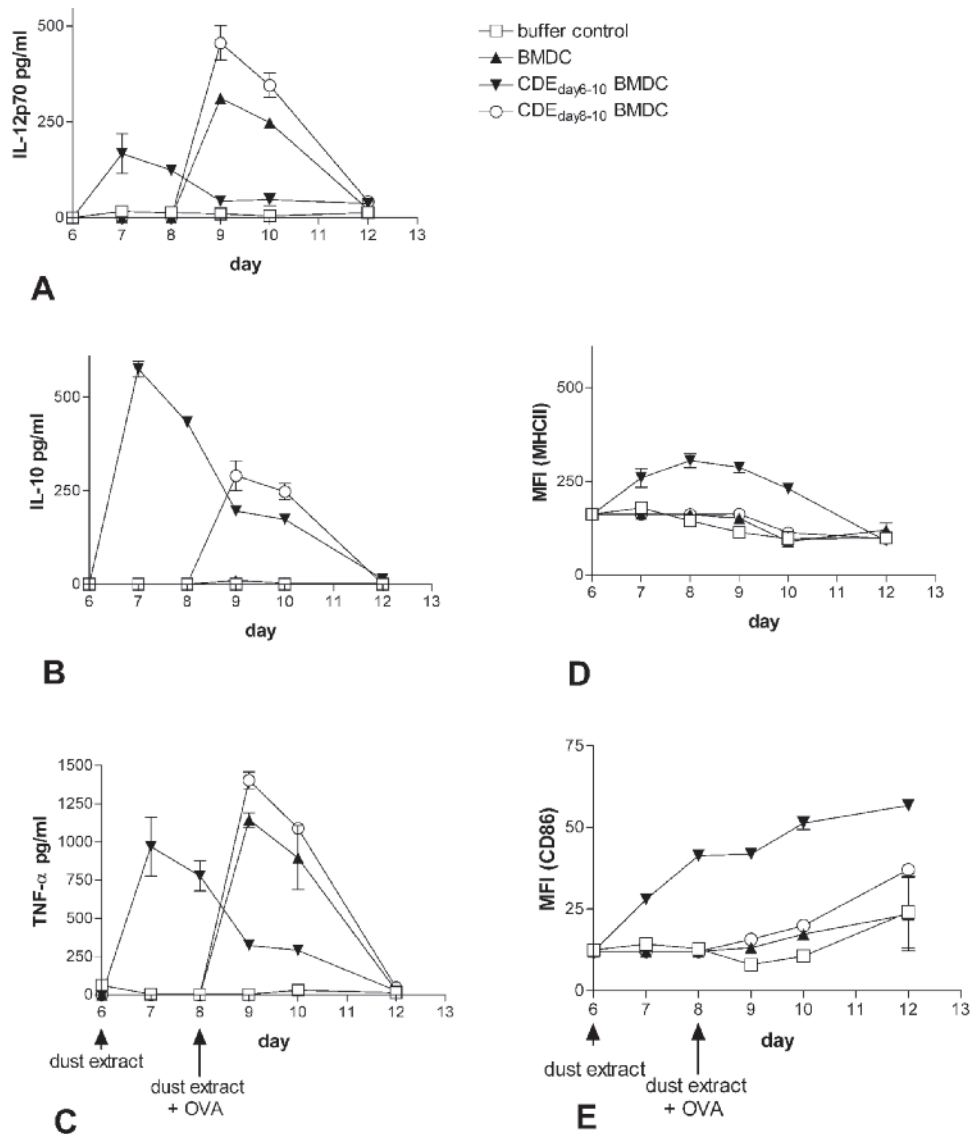


Fig. 4. Cytokine production and surface molecule expression of BMDC that were treated with CDE. BMDC were either left unstimulated (buffer control) or were pulsed with OVA on day 8 (OVA) or treated with CDE from either days 6 to 10 (OVA + CDE_{day6-10}) or days 8 to 10 (OVA + CDE_{day8-10}) simultaneously with OVA pulsing. At indicated time points, supernatants were harvested for measurement of cytokines IL-12 (A), IL-10 (B) and TNF- α (C). Cytokines were measured by specific ELISA and expressed as pg/ml cell culture supernatant. Furthermore, dendritic cells were collected for evaluation of surface molecule expression MHC II (D) and CD86 (E). Surface molecule expression was determined with a flow cytometer and expressed as mean fluorescence intensity (MFI). Data are representative for three independent experiments.

titres of mice challenged with OVA only. This demonstrates that treatment of BMDC with CDE not only affected the cellular, but also the humoral, immune response towards allergens. Interestingly, protected mice did not develop an OVA-specific IgG2a response indicating that CDE-treated BMDC did not induce a Th1 response towards the allergen. Since allergen-specific IgA production is discussed as a protective mechanism in allergic disease, we measured OVA-specific IgA production in BAL fluid. However, production of IgA in mice sensitized with CDE_{day6-10} BMDC ($OD_{405} = 0.048 \pm$

0.069) was also reduced compared to mice sensitized with BMDC not treated with CDE ($OD_{405} = 0.618 \pm 0.331$) showing that IgA antibodies are probably not involved in allergy protection in our model.

Treatment of BMDC with CDE leads to an activated phenotype and production of cytokines

T-cell stimulatory capacity of dendritic cells depends on expression of surface molecules and production of

cytokines. Therefore, we analysed the expression of MHC II and the co-stimulatory molecule CD86 on these cells. Furthermore, we measured production of TNF- α , IL-12 and IL-10 as important cytokines for either immune stimulation or immune suppression, respectively. As shown in Figure 4A–C, treatment with CDE induced strong production of all cytokines measured. Release of cytokines reached maximum 1 day after stimulation of cells with extract (CDE_{day6–10} – IL-10, 575 ± 30 pg/ml; IL-12, 167 ± 73 pg/ml; TNF- α , 969 ± 273 pg/ml; CDE_{day8–10} – IL-10, 290 ± 56 pg/ml; IL-12, 456 ± 64 pg/ml; TNF- α , 1404 ± 79 pg/ml) and then continuously decreased. Remarkably, CDE_{day6–10} BMDC produced significantly higher amounts of IL-10 than CDE_{day8–10} BMDC. Even more importantly, on day 10, the time point when cells were used for sensitization of mice, CDE_{day8–10} BMDC showed high IL-12 and TNF- α production comparable to cells that were pulsed with OVA alone, whereas CDE_{day6–10} BMDC exhibited low production of all cytokines measured.

The expression of co-stimulatory surface molecules

showed different characteristics (Fig. 4D–E). CD86 expression continuously increased after stimulation of cells with CDE. Interestingly, increased expression of this co-stimulatory surface molecule was significantly more pronounced when CDE was present in culture starting on day 6. At day 10, the expression of CD86 was higher on cells that were treated with CDE than on cells that were either stimulated with OVA only or left untreated. In contrast, enhanced MHC II expression was only observed when cells were stimulated with CDE from day 6.

Since our previously published experiments revealed that treatment of human monocytes with CDE affected generation of monocyte-derived dendritic cells, we have analysed CD11c expression as a marker for murine dendritic cells. However, we found no difference between CDE treated and untreated BMDC regarding the percentage of CD11c expressing cells throughout the time points studied (data not shown). This strongly suggests that generation of BMDC is not influenced by stimulation of cells with CDE starting from day 6.

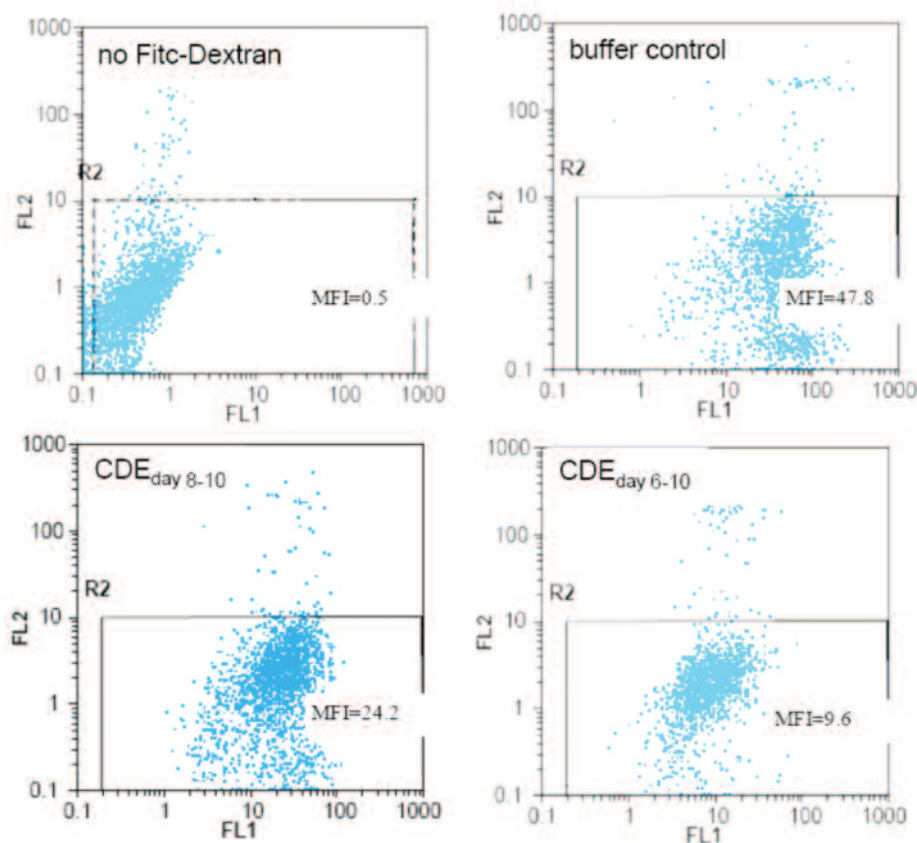


Fig. 5. Endocytotic capacity of CDE-treated BMDC. BMDC were either left untreated (upper right) or were treated with CDE from days 6 to 10 (lower right) or days 8 to 10 (lower left), respectively. On day 8, cells were pulsed for 2 days with fluorescein-labeled dextran (x-axis) except for cells that were shown in the upper left diagram. On day 10, dead cells were counterstained with propidium iodide (y-axis) and fluorescence was measured by flow cytometry. Average FITC-dextran uptake of living cells is expressed as mean fluorescence intensity (MFI). Data are representative for three independent experiments.

Endocytotic capacity of CDE treated BMDC and transport of antigen to the lymph node

To assess whether the reduced immune stimulatory activity of BMDC was due to inhibition of antigen uptake, we examined the endocytotic capacity of CDE-treated cells. Since the model allergen ovalbumin is believed to be taken up mainly via the mannose receptor, we used fluorescein-labelled dextran (FITC-dextran) to account for glycoreceptor-mediated endocytosis.¹⁰ After 1-h incubation with FITC-dextran, we found phagocytic activity of BMDC no matter whether they were treated with CDE or not. However, we found no difference in antigen uptake between CDE_{day8-10} BMDC, CDE_{day6-10} BMDC and untreated BMDC in this short-time incubation (data not shown). In contrast, after a prolonged incubation period with fluorescein-labelled dextran for 48 h, cells that were treated with CDE either on day 6 or on day 8 showed reduced fluorescence intensity compared to untreated cells indicating that glycoreceptor-mediated antigen uptake was reduced due to stimulation with CDE (Fig. 5).

To test whether CDE-treated dendritic cells home to lung draining mediastinal lymph nodes, we cultured BMDC with CDE 2 days before OVA pulsing and subsequently labelled cells with fluorescein (CFSE). The fluorescent BMDC were applied intranasally to mice and the distribution of cells was compared to BMDC that were not treated with CDE. As shown by FACS analysis (Fig. 6), fluorescein-labelled BMDC traffic from BAL fluid to lung tissue and subsequently homed to draining lymph nodes no matter whether they were treated with CDE or left untreated. Thus, it could be concluded that traffic of dendritic cells to lung tissue and subsequent homing in to bronchus-draining mediastinal lymph nodes was not impaired due to treatment of cells with CDE.

Blocking the biological activity of autocrine IL-10 partly restores Th2-inducing capacity of BMDC

As demonstrated above, we found significant IL-10 production by BMDC after stimulation with CDE. Furthermore, CDE_{day6-10} BMDC which were not able to prime mice for allergic immune response exhibited higher production of IL-10 than CDE_{day8-10} BMDC or cells that were pulsed with OVA only. Therefore, we hypothesized that reduced immune stimulatory properties of CDE-treated cells is probably due to autocrine production of this anti-inflammatory cytokine. To test this hypothesis, we compared mice sensitized with CDE-stimulated cells with mice sensitized with cells that were treated with IL-10. Indeed, addition of 1 ng recombinant murine IL-10 to culture medium, an amount comparable to autocrine produced IL-10 in CDE-stimulated BMDC cultures, was sufficient to block the allergy-inducing properties of BMDC (Fig. 7). To address the importance of autocrine IL-10 production more directly, we blocked IL-10 activity in cell cultures of BMDC stimulated with CDE by a neutralizing monoclonal antibody and compared the sensitizing capacity of these cells with BMDC stimulated with dust but without antibody. As shown in Figure 7, blocking the biological activity of the anti-inflammatory cytokine partly restored the Th2-inducing capacity of BMDC. Infiltration of eosinophils and lymphocytes into the airways was significantly enhanced ($P < 0.01$) in mice that were sensitized with BMDC stimulated with CDE in the presence of IL-10 neutralizing antibody. However, there was still significantly reduced eosinophilic infiltration in these mice compared with mice sensitized with OVA-pulsed BMDC that were not treated with extract, suggesting that additional factors were involved in the suppression of dendritic cell activity.

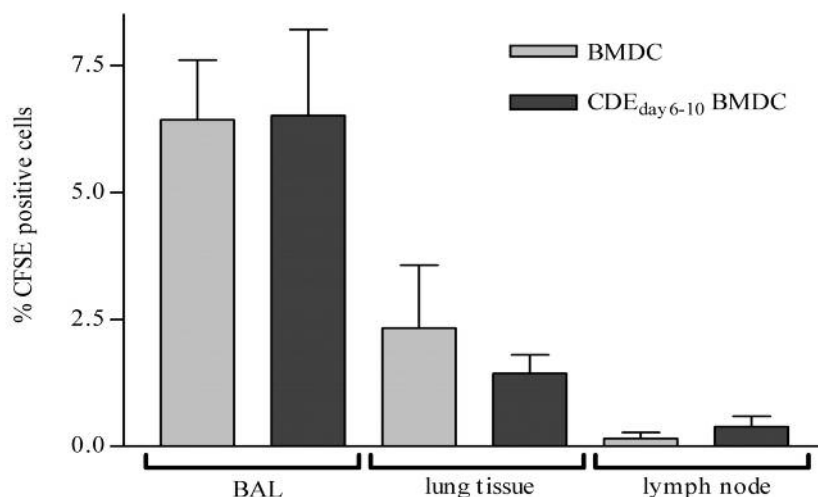


Fig. 6. Migration characteristics of CDE-treated BMDC. BMDC were either pulsed with OVA alone (BMDC) or additionally treated with CDE from days 6 to 10 (CDE_{day6-10} BMDC). On day 10, cells were labeled with CFSE and intranasally applied to mice. After 24 h, fluorescent cells were detected in BAL fluid, lung tissue and mediastinal lymph nodes by means of flow cytometry.

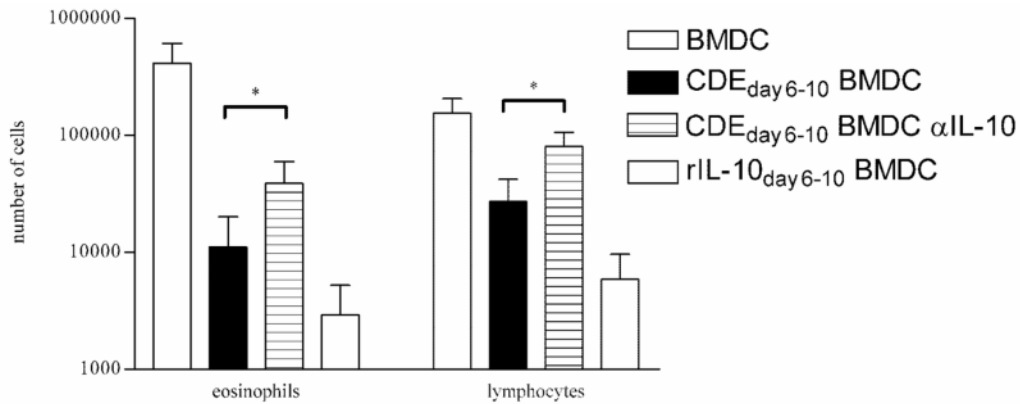


Fig. 7 Eosinophilic and lymphocytic infiltration in airways of mice sensitized with OVA-pulsed BMDC treated with CDE in the presence or absence of an IL-10 neutralizing antibody. Mice were sensitized with OVA-pulsed BMDC that were treated with either a buffer control (white bar) or CDE from days 6 to 10 (black bar) or CDE from days 6 to 10 in the presence of an IL-10 neutralizing antibody (dashed bar) or recombinant murine IL-10 (grey bar). Results are expressed as mean with standard deviation from up to ten mice per group.

DISCUSSION

Recently, we have presented data showing that treatment with cowshed dust extract led to protection against allergic disease in a mouse model of allergic asthma.¹ Our experiments indicated that CDE down-regulates the immune response towards the allergen rather than inducing a Th1 response. Surprisingly, in further studies, we found that treatment of mice with CDE did not induce FoxP3⁺ T-lymphocytes and that allergy-protection was not transferable by lymphocytes from protected to naïve mice (unpublished results). Therefore, we suggest that, in our model, treatment of mice with CDE did not lead to induction of regulatory T-cell response but rather affects cells of the innate immune system. To analyse the mechanism of CDE-induced allergy protection in more detail, we examined, in the present study, the effects of CDE on dendritic cell biology. Our experiments demonstrate for the first time that the allergy-inducing capacity of BMDC is impaired as a consequence of contact with CDE.

BMDC that were treated with CDE 2 days before OVA pulsing (CDE_{day6-10} BMDC) lose their property to prime mice for allergic immune response. Mice that were sensitized with these cells exhibit reduced Th2 cytokine production, eosinophilic lung inflammation and also decreased allergen-specific antibody production. Our first explanation for this observation was that cultivation of cells with extract leads to inhibition of antigen uptake or transport of antigen to the draining lymph nodes. Indeed, we found decreased, yet still significant, endocytotic capacity of BMDC that were treated with CDE. However, CDE_{day6-10} BMDC that were pulsed with antigen after stimulation with CDE and those that were pulsed simultaneously to stimulation

with CDE (CDE_{day8-10} BMDC) exhibited similar reduced antigen uptake, respectively. Since CDE_{day8-10} BMDC have significant Th2-inducing potential and induce pronounced eosinophilic airway inflammation, it was unlikely that reduced Th2-inducing properties of day-6 treated cells was due to decreased antigen uptake alone. Interestingly, we observed enhanced expression of MHC II and co-stimulatory molecule CD86 as a consequence of stimulation with dust extract. From the literature, it is well known that dendritic cells develop a mature phenotype due to microbial or cytokine stimulation and, consequently, exhibit reduced antigen uptake.¹¹ Albeit not exclusively responsible for impaired induction of Th2 response, we suggest that reduced uptake or processing of allergen contributed to the effects observed in our experiments. Although showing similar phagocytic activity, CDE_{day6-10} BMDC and CDE_{day8-10} BMDC behave completely differently *in vivo*. From this observation, the question arises whether the 4-day time span of treatment with CDE, the pre-treatment with CDE before OVA pulsing or the time point of contact with CDE during BMDC development is crucial for the different behaviour *in vivo*. However, since cells that were cultivated simultaneously with OVA and CDE for a total of 4 days show reduced immune stimulatory capacity comparable to CDE_{day6-10} BMDC, it is likely that the length of treatment is the most important factor (data not shown).

In contrast to antigen uptake, migration of BMDC to lung tissue and draining lymph nodes was not impaired after treatment of cells with CDE. Moreover, we found no relevant induction of apoptosis in CDE-treated and control cells, excluding that the reduced allergy-inducing capacity of BMDC was due to increased cell death (data not shown).

Beside enhanced expression of co-stimulatory surface molecules, we found significant TNF- α , IL-12 and, even more importantly, IL-10 production by BMDC. Furthermore, prolonged exposure to CDE led to reduced cytokine production by these cells suggesting that cell activity was down-regulated over time. Still, the cells developed an activated phenotype with high expression of CD86 and MHC II surface molecules despite accumulating concentrations of IL-10 in cell-culture medium. Since IL-10 had been described as a factor for negative regulation of cell activation, these results were somewhat surprising. However, our observations are in line with results previously reported by Commeren *et al.*,¹² where BMDC that were treated with IL-10 from an early time point of generation had increased surface expression of CD86, but lacked T-cell stimulatory capacity. More importantly, in this study, it was demonstrated, that the time point of IL-10 was crucial for the resulting phenotype; addition of IL-10 between days 0 and 6 of dendritic cell generation led to high expression of CD86 and low T-cell stimulatory capacity whereas late IL-10 (on day 8) resulted in low expression of CD86 and strong T-cell stimulation.¹² Whether increased CD86 expression as seen here resulted from direct stimulation of cells with substances from CDE or was a consequence of autocrine-produced IL-10 remains to be defined. However, it is probable that autocrine IL-10 is involved in modulation of dendritic cell function since neutralisation of this anti-inflammatory cytokine partially restored allergy-inducing capacity of BMDC. Therefore, it can be hypothesized that CDE acts as a stimulatory agent for autocrine IL-10 production which then down-regulates immune stimulatory activity of BMDC. Since *in vitro* neutralization of released IL-10 only partially restored the allergy-inducing capacity of BMDC and substitution of CDE-induced IL-10 production by recombinant IL-10 resulted in complete suppression of dendritic cells, it is likely that additional factors were involved. Fujita *et al.*¹³ reported that BMDC develop into a regulatory dendritic cell phenotype when generated in presence of IL-10 and TGF- β . This population of regulatory dendritic cells is able to suppress an allergic immune response due to induction of a regulatory T-cell response. Since we found autocrine TGF- β production by CDE-treated BMDC (data not shown), it is possible that this suppressive cytokine is involved in our study; however, we have no evidence for induction of regulatory T-cells.

Recently, autocrine production of IL-10 by BMDC was shown by Kajino *et al.*¹⁴ These authors demonstrated that stimulation with LPS or peptidoglycan led to IL-10 production which subsequently down-regulated production of inflammatory cytokines and co-stimulatory molecule expression in an autocrine manner. Although these results support our findings, on the contrary, we have observed that co-stimulatory molecule

expression continuously increased following stimulation with CDE. However, since CDE is composed of several immune-modulating factors, it is likely that a response was induced that differed from the effects observed after stimulation with a single PAMPs like peptidoglycan or LPS.

Other authors reported down-regulation of cytokine production after prolonged activation of dendritic cells. For example, Langenkamp *et al.*¹⁵ demonstrated that dendritic cells after short LPS stimulation were able to induce strong Th1 responses but became unresponsive to prolonged LPS stimulation with a marked reduction of cytokine production. Thus, LPS seems to have dual properties depending on duration and concentration of the stimulus. Data from animal experiments support this conclusion. Intranasal treatment of mice with low doses of LPS was described by Eisenbarth *et al.*² to act as a Th2 polarizing adjuvant *in vivo*, whereas high doses of LPS induced Th1 responses. Likewise, experiments by Kuipers *et al.*⁸ reveal that OVA-pulsed dendritic cells that were treated with LPS *in vitro* induce an OVA-specific Th1 response *in vivo*. In contrast to these observations, CDE induces a completely different response actually down-regulating both Th2- and Th1-inducing capacity of BMDC. In fact, the observed effects of down-regulation of dendritic cell function accompanied by high IL-10 and low IL-12 production were similar to observations of Chieppa *et al.*¹⁶ In this report, it was shown that cross-linking of mannose receptors on dendritic cells induced an anti-inflammatory cytokine profile resulting in reduced immune stimulatory function. Since CDE contains many different polysaccharides (unpublished results), it is tempting to speculate that BMDC function was modulated by a similar mechanism. However, these speculations have to be verified by further studies. These studies should address which receptors are involved in CDE-induced immune modulation by utilization of mannose receptor and Toll-like receptor knock-out mice.

Although exhibiting high expression of surface molecules like MHC II and CD86, CDE-treated BMDC were not able to induce an allergic immune response. A similar non-activating phenotype of dendritic cells is described in the literature as cells with high expression of co-stimulatory molecules and low-level cytokine production.¹⁷ This observation provides an interesting link to the ALEX study, in which the authors described that allergy protection of children that grew up in a farming environment correlated with reduced cytokine production of MNC suggesting down-regulation of the activity of the innate immune system.¹⁸ It is noteworthy that the mouse model seems to reflect the mechanisms also observed in humans.

Finally, our observations imply that the protective effects of CDE, as seen in our model, result mainly from

an early activation of the innate immune system and tolerization of dendritic cells rather than the induction of a Th1- or a T-regulatory response.

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