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## Estrogen influences the differentiation, maturation and function of dendritic cells in rats with experimental autoimmune encephalomyelitis<sup>1</sup>

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**KEY WORDS** multiple sclerosis; experimental autoimmune encephalomyelitis; dendritic cells; estrogens; nitric oxide

### ABSTRACT

**AIM:** To examine if estrogen can affect the immune response at the dendritic cells (DCs) level in rats with experimental autoimmune encephalomyelitis (EAE). **METHODS:** Lewis rats were immunized with inoculum containing MBP<sub>68-86</sub>. DCs were derived from spleen monocytes of EAE rats with IL-4 and GM-CSF in presence of 17 $\beta$ -estradiol (E2). Nitric oxide (NO) was detected by Griess reagent. The surface markers and cytokines production of DCs were shown by flow cytometry. DCs were cocultured with MBP-specific T cells, [<sup>3</sup>H]-TdR incorporation was used to reveal the antigen presentability, the supernatant of the coculture were collected to examine the cytokines secretion by ELISA. **RESULTS:** E2 activated DCs by accelerating the maturation process characterized by upregulation of MHC II and costimulating molecule B7-1, B7-2, drastic high expression of CD40. IFN- $\gamma$ -producing DCs were also elevated without any alteration of IL-10. Estradiol-treated DCs (E2-DCs) secreted more NO in the culture supernatant. By contrast, E2-DCs showed decreased antigen presentation ability with reduced secretion of IFN- $\gamma$  but no alteration of IL-10 in the coculture with T cells. **CONCLUSION:** Estrogen can affect the differentiation, maturation and function of DCs from EAE rats, which may be attributed to its protection against EAE and the remission of multiple sclerosis patients in pregnancy.

### INTRODUCTION

Autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and Grave's disease preferentially affect women and likely involve Th1 cells directed at tissue-specific antigens. By contrast, in-

creased levels of sex hormones produced during pregnancy have been associated with clinical remissions in MS and RA patients. The underlying mechanisms are still not well known.

It has been demonstrated that combining T-cell receptor vaccination with supplemental estrus doses of estrogen potentiated IL-10 and TGF $\beta$  production in antigen-specific T cells, resulting in full protection against experimental autoimmune encephalomyelitis (EAE)<sup>[1]</sup>, and a moderate shift to Th2 cytokines production of T cells in pregnancy or estrogen treatment. All these can not account for all the protective mechanisms of estro-

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gen on EAE.

As dendritic cells (DCs) are professional antigen presenting cells, they have a central role in the initiation and regulation of immune responses. DCs exhibit considerable plasticity in their ability to skew Th responses, and DCs that normally induce Th1 profiles can be converted to Th2-skewing cells when treated with IL-10 and TGF $\beta$  or with steroids or prostaglandin E2<sup>[2]</sup>. DCs plasticity are also reflected in their differentiation, a process influenced by many factors<sup>[3]</sup>. We wonder if the protective effect of estrogen may happen at DCs level by affecting their functional differentiation so as to result in the remission of EAE.

## MATERIALS AND METHODS

**Animals and reagents** Male Lewis rats, weighing 150-180 g, were obtained from Zentralinstitut für Versuchstierzucht (Hannover, Germany). Guinea pig MBP<sub>68-86</sub> (YGSLPQKSQRS QDENPV) was produced in an automatic Tecan-Syro Synthesizer (Multisytech, Bochum, Germany).

Monoclonal PE-conjugated anti-rat MHC class II, antibodies were obtained from SeroTec (Oxford, UK). Monoclonal PE-anti-rat B7-1 (CD80), B7-2 (CD86), CD40 were from PharMingen (San Diego, CA). PE-conjugated anti-rat IFN- $\gamma$  and FITC-conjugated anti-rat IL-10 mAb were from SeroTec. Water-soluble estradiol-3-benzoate (E2) was from Sigma (USA).

**Generation of DCs from spleen mononuclear cells** Splens were collected under aseptic conditions from EAE rats on d 14 post immunization (pi). Mononuclear cells (MNC) were prepared by grinding through a wire mesh, depleted of erythrocytes with osmotic lysis, and suspended in serum-free DMEM (Gibco, UK), and were seeded in flasks (Becton). Two hours later, non-adherent cells were gently removed by swirling the plates and aspirating the medium, and plates were washed 5 times with serum-free medium. New medium containing 10 % fetal calf serum (FCS; Gibco), 10 g/L minimal essential medium (MEM) amino acids (Gibco), 2 mol/L glutamine (Flow Labs, UK), 50 000 U/L penicillin and 50 ng/L streptomycin (Gibco) and 10 mmol/L HEPES (Sigma) supplemented with rat granulocyte-macrophage colony-stimulating factor (GM-CSF, 10  $\mu$ g/L; R&D, Germany) and rat IL-4 (10  $\mu$ g/L, R&D) with E2 (E2-DC) or without E2 (ctr-DC). We set low dose (L, 2  $\mu$ g/L), mild dose (M, 10  $\mu$ g/L), and high dose (H, 50  $\mu$ g/L) of E2, representing the physiologic

concentrations found in the peripheral circulation during pregnancy (up to 35  $\mu$ g/L)<sup>[2]</sup>. The medium were changed for half fresh medium after 4 d. Floating DCs were used in the study 7 d later.

**Induction of EAE** Every rat was immunized in both hind footpads via sc with 200  $\mu$ L of inoculum containing 25  $\mu$ g MBP<sub>68-86</sub>, 2 mg *Mycobacterium tuberculosis* (strain H37RA; Difco), 100  $\mu$ L saline and 100  $\mu$ L Freund's incomplete adjuvant (FIA; Difco). Rats injected with PBS were set as normal control.

**FACS analysis** DCs were incubated with PE-conjugated monoclonal antibodies or with nonconjugated mAbs for 30 min on ice, followed by incubation with isotype-specific secondary mAb. Before and after each incubation, DCs were washed twice with 10 g/L BSA in PBS. DCs were analyzed with flow cytometer (Becton Dickinson) using CellQuest software. The data were expressed as mean fluorescence intensity, in exception for CD40, the data was shown in dot-plot.

Two-colour intracellular cytokines staining for IL-10 and IFN- $\gamma$  was also performed. DCs were fixed with 20 g/L paraformaldehyde in PBS, permeabilized with 5 g/L saponin, stained with FITC-conjugated mouse anti-IFN- $\gamma$  and PE-conjugated mouse anti-IL-10 at room temperature for 30 min.

**ELISA** Measurement of IL-10 and IFN- $\gamma$  in the supernatant of coculture of DC with T cells was performed by using OptEIA kits from BD PharMingen, as the manufacturer's protocol. Determinations were performed in duplicate and results were expressed as ng/L.

**DC-T cell cocultures** DCs were extensively washed, irradiated (3000 rad), and the number was calculated using a Hemacytometer. DCs were then added in graded doses to  $1 \times 10^5$  cells/well purified allogeneic responder T cells in complete medium in the presence of MBP<sub>68-86</sub> (10 mg/L) in 96-well round-bottomed plates (Nunc) for 3 d. Coculture were pulsed for the last 16 h with  $37 \times 10^3$  Bq/well <sup>3</sup>[H]thymidine. Results were given as mean cpm $\pm$ SD of 6 well cultures.

**Preparation of T cells from lymph nodes** On 14 d pi, lymph node MNC suspension from EAE rats were prepared by grinding through a wire mesh. For isolation of T cells, MNC suspensions were incubated in flasks in serum-free medium at 37 °C for 1.5 h. The non-adherent fraction was collected and incubated over a 20-mL nylon wool column. T cells were obtained by depletion of nylon wool-adherent cells.

**Assay of nitrite** The level of nitrite as a reflection of NO production in culture supernatants was mea-

sured using modified Griess reagent (Sigma). In brief, 100  $\mu$ L of the culture medium of DCs were mixed with equal volume of Griess reagent at room temperature for 10 min, then the absorbance at 560 nm was measured using an automated plate reader.

**Statistical analysis** Statistical analysis was performed using one-way ANOVA and Tukey-Kramer multiple comparisons test when ANOVA showed significant differences.

**RESULTS**

**Effect of estrogen on the functional differentiation of monocyte-derived DCs** Adherent spleen monocytes were cultured in the presence of IL-4 and GM-CSF, which resulted in a typical phenotype of immature DCs after a 7-d culture. The typical surface markers of DCs such as MHCII, CD40 and costimulating molecules B7-1 and B7-2 were upregulated in the differentiation process of DCs in the presence of consecutive doses of E2, suggesting the accelerated maturation process (Fig 1).

**DCs number, NO production and cytokines expression by DCs derived in various doses of estrogen**

In the presence of E2, the amount of harvested DCs from the culture flasks were reduced dose-dependently (Fig 2a). NO secretion in the culture supernatant was increased in E2-DCs (Fig 2b), indicating that E2 can induce NO production in the differentiation process of DCs. Intracellular staining showed augmented IFN- $\gamma$  positive DCs in E2-DCs, a small increase of IL-10 was also found (Fig 2c).

The above data taken into account, DCs derived in presence of estrogen demonstrated increased nitric oxide production and augmented IFN- $\gamma$  positive cells even the differentiated DC number was reduced.

**Inhibition of Ag-induced T cell proliferation by estrogen-treated DCs** The more mature stage of E2-DCs, characterized by upregulation of the surface markers, was supposed to be more potent to stimulate T cell proliferation, in other words, higher antigen presentability.

To our surprise, T cells response was significantly inhibited in E2-DCs (Fig 3). The results indicate that E2-DCs are able to suppress Ag-specific T cell response even with higher expression of costimulators. So may exists a different mechanism underlying the Ag present-

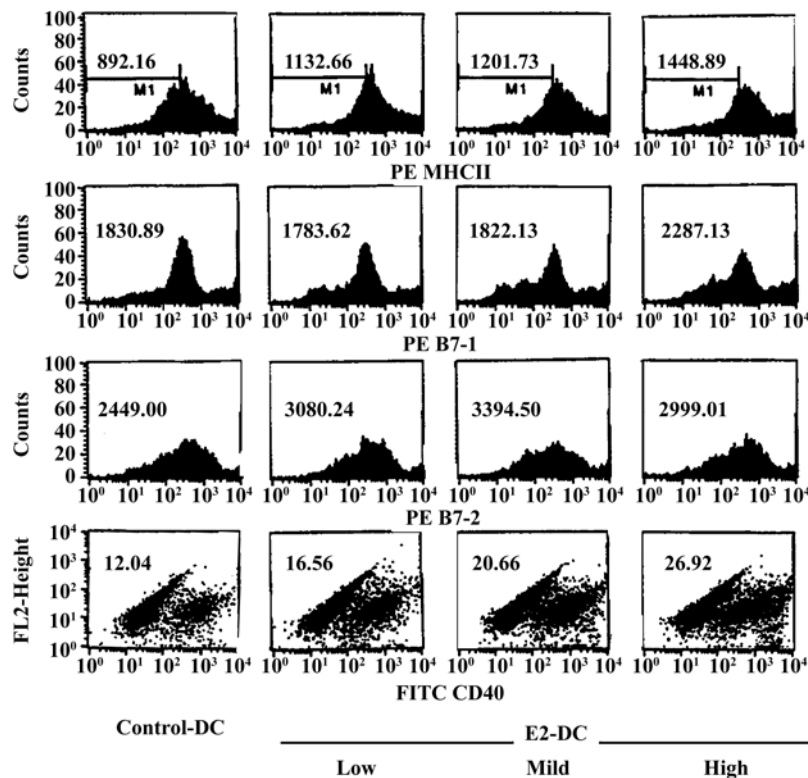


Fig 1. E2 affected the phenotype of monocyte-derived DCs from EAE rats. Representative histograms of 3 individual experiments are shown.

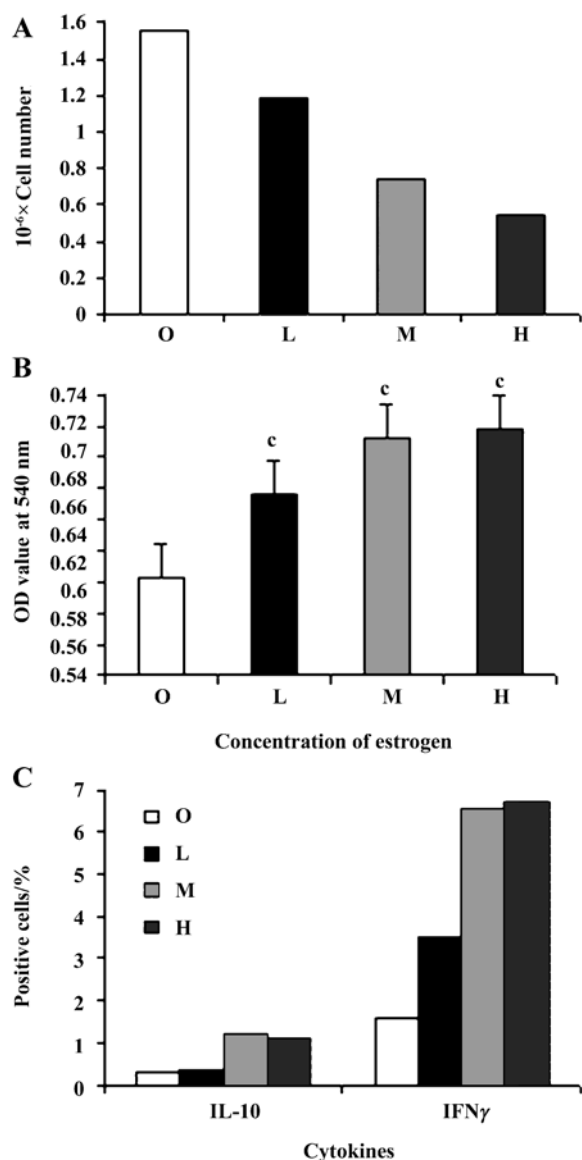


Fig 2. Total cell number (A), NO production (B), and cytokines expression (C) of DCs derived under various concentration of E2 for 7 d. <sup>c</sup>*P*<0.01 vs control.

tation procedure of E2-DCs.

**Cytokines shift of T cells by E2-DCs** We detected the supernatant of coculture of DCs with autogenous T cells. T cells primed with E2-DCs released less Th1 cytokine IFN- $\gamma$ , but without much change of Th2 cytokine IL-10 (Fig 4), indicating an incline to Th2 response.

**DISCUSSION**

Dendritic cells are professional APC with a unique capacity to initiate and regulate immune responses. They provide not only an array of antigenic peptides needed

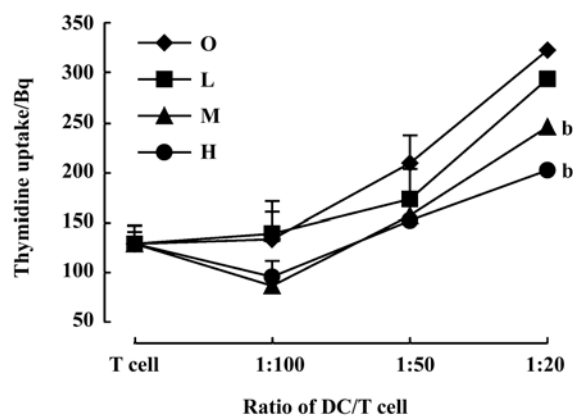


Fig 3. DCs derived in different doses of E2 exert weaker antigen presentability than that without estrogen (O,  $\blacklozenge$ ). The significance were revealed in DCs treated with middle (M,  $\blacktriangle$ ) or high dose of E2 (H,  $\bullet$ ). <sup>b</sup>*P*<0.05 vs control (O,  $\blacklozenge$ ).

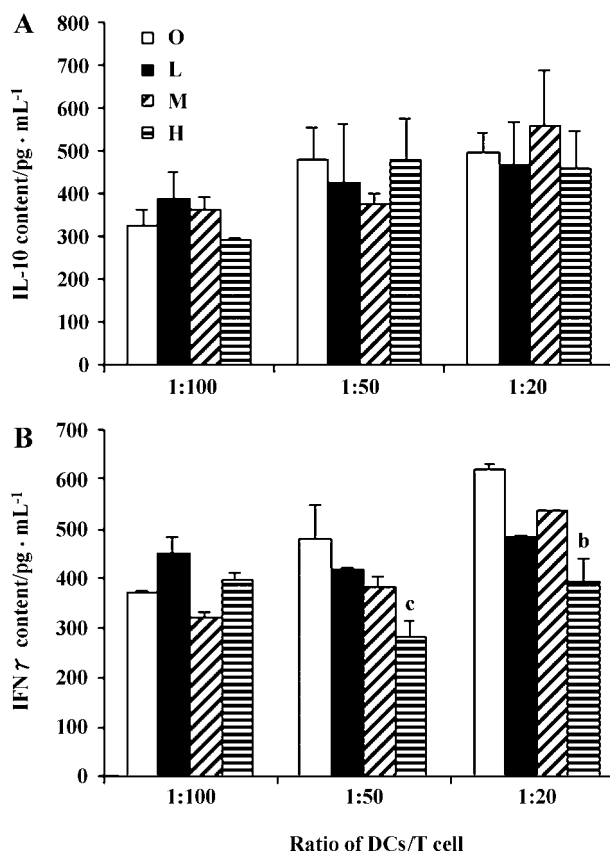


Fig 4. Profile of IL-10 and IFN- $\gamma$  secretion by T cells restimulated with MBP<sub>68-86</sub> (10 mg/L) following primed by E2-DCs or ctr-DCs. No difference in IL-10 secretion was seen in E2-DCs primed T cells, but reduction of IFN- $\gamma$  occurred if the ratio of DCs/T cell was as high as 1:50. <sup>c</sup>*P*<0.01, <sup>b</sup>*P*<0.05 vs control.

to activate the appropriate antigen-specific T cells, but also produce potent costimulatory signals that drive

quiescent T cells into the cell cycle<sup>[4]</sup>. Accumulating evidence has demonstrated that DCs bear much more plasticity, different functional types of DCs can be generated from the same precursor<sup>[5]</sup>.

The sex hormones, especially estrogen, which is remarkably increased in pregnancy, have been reported to be beneficial to EAE even at low doses<sup>[6]</sup>. Furthermore, estrogen was described as dose-dependent enhancement of Ag-stimulated IL-10 and IFN- $\gamma$  secretion<sup>[7]</sup>. Most importantly, estrogen can interact with the transcription factor NF- $\kappa$ B, which is a key regulator of inflammatory responses, suggesting a crucial role in autoimmunity<sup>[8]</sup>.

Estrogen receptor (ER) $\alpha$  and ER $\beta$  are expressed at all stages of DCs differentiation in human peripheral mononuclear cell (PMNC)<sup>[9]</sup>, providing strong evidence that estrogen can bind to and regulate DCs, thus allowing the possibility that estrogen might dampen inflammation by directly affecting the function of DCs.

In the present study, we provide phenotypical and functional evidences that estrogen is able to activate DCs derived from monocytes of EAE rats with increased IFN- $\gamma$  and NO production. Unexpectedly, E2-DCs were less potent than ctr-DCs in inducing allogeneic T cell proliferation and were clearly impaired in producing the biologically IFN- $\gamma$  secretion. This suggests that by promoting the maturation, especially by inducing NO production of DCs, estrogen may inhibit the development of inflammatory Th1 responses.

So far only one report studied the effect of estrogen on DCs differentiation from human PMNC, but ended with negative results. Instead, nonsteroidal anti-estrogens such as toremifene and tamoxifen could inhibit the functional differentiation of DCs<sup>[9]</sup>. The incompatibility with our results may be due to the different sources of monocytes. We collected the monocytes from EAE splenocytes while they selected healthy human PMNC in their study. Activated spleen monocytes may obtain quite different characteristics from those separated from healthy human PMNC.

In autoimmunity, Th1/Th2 balance and the activation of effector T cell subset are often critical for the progression or the remission of disease<sup>[10]</sup>. IFN- $\gamma$  plays an important role as a regulator of self-reactive lymphocytes. The disease-preventing effect of IFN- $\gamma$  invokes an "immunosuppressive" pathway. One possible mechanism may be the over induction of NO<sup>[11]</sup>.

We demonstrate in this study that E2-DC with elevated IFN- $\gamma$  expression can produce higher levels of

NO. The role of NO in autoimmune diseases comprises both regulatory and effector functions<sup>[12]</sup>. It is found that NO suppress lymphocyte proliferation<sup>[13,14]</sup> and induce apoptosis of the most potent source of alloantigenic stimulation<sup>[15]</sup>. Thus, although DCs function initially as the most potent APC for T cell activation, they may inhibit T cell proliferation if induced to produce NO by estrogen. An additional functional consequence of NO production is the induction of apoptosis of DCs itself. We harvested fewer amount of DCs from that derived in presence of estrogen, which implying apoptosis of DCs itself.

We propose that splenic DCs from EAE rats, while differentiated in presence of estrogen could produce high amount of NO, which directly down-regulated autoreactive T cell responses and IFN- $\gamma$  secretion. Taken together, we conclude that estrogen can change the plasticity of DCs derived from EAE monocytes leading the immune response to the protection against diseases.

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