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Long-Term Effects of IFN-γ, IL-10, and TGF-β-Modulated Dendritic Cells on Immune Response in Lewis Rats

RUI-SHENG DUAN,[1,3,5] HANS LINK,[1] and BAO-GUO XIAO[1,2,4]

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Increasing data have shown that IFN-γ, IL-10, and TGF-β-modulated dendritic cells (DC) provide a promising strategy in treatment of experimental allergic encephalomyelitis and experimental autoimmune myasthenia gravis through different manner. To explore the immune response status after long-term application of these cytokine-modulated-DC, Lewis rats were injected subcutaneously into naive DC and IFN-γ, IL-10, and TGF-β-modulated DC (i.e., IFN-γ-DC, IL-10-DC, and TGF-β-DC) at does of 1×10^6 cells/rat every month for continuous 18 months, respectively. No rats suffered from decreased vigor and activity as well as cachectic condition during 18-month observation, and no rats had body-weight loss after 18-month treatment. Exploratory laparectomy did not find any tumor in all rats. IL-10-DC and TGF-β-DC resulted in lower nonspecific (Con A-induced) and antigen specific (ovalbumin-stimulated) spleen mononuclear cells proliferation, accompanied by lower levels of IFN-γ, IL-10, and TNF-α. On the contrary, IFN-γ-DC did not suppress cell proliferation and IFN-γ and IL-10 production except only slightly decreased TNF-α levels. These results suggest that IFN-γ-DC seems to be a more ideal candidate in the treatment of autoimmune diseases without suppressing immune response.

KEY WORDS: Autoimmune disease; cytokine; dendritic cell; proliferation.

INTRODUCTION

Dendritic cells (DC) are the most potent antigen presenting cells (APC) within the immune system and are involved in the induction of both immunity and tolerance (1, 2). DC are characteristic of heterogeneity and plasticity, which means that different DC subsets, mature stage, and activation singles from the pathogens and cytokines may induce DC to exert either immunity or tolerance (2–4). In the past decade, many studies have been focused on the DC-based cancer immunotherapy to prime specific antitumor immunity (5). On the other hand, increasing evidence has shown that DC vaccine eliciting peripheral tolerance might also be a promising therapy for some autoimmune disease. Up to now, three different strategies have been used to prepare the tolerogenic DC: (i) isolation of tolerogenic DC subsets; (ii) utilization of immature DC; and (iii) modification of DC to a tolerogenic status (2). Previously, we found that DC from health rats, modulated *in vitro* by exposure to IFN-γ and TGF-β1, effectively suppress the ongoing experimental allergic encephalomyelitis and experimental autoimmune myasthenia gravis (EAMG) when given by the subcutaneous route (6–8). DC *in vitro* treated with IFN-γ also suppressed autoimmune diabetes in NOD mice (9). Furthermore, DC from the severe EAMG rats, modulated *in vitro* with IL-10, also inhibited the ongoing EAMG when given intraperitoneally (10). The evidence indicates that autologous DC, after *in vitro* modulation, might be a useful strategy to treat human myasthenia gravis and multiple sclerosis. Therefore, before clinical trial in human as a novel therapeutic strategy, it is essential to understand the effects of long-term application of cytokine-modulated-DC on general status and immune system of organism.

[1]Division of Neuroimmunology, Neurotec, Karolinska Institute, Stockholm, Sweden.
[2]Institute of Neurology, Fudan University, Shanghai, China.
[3]Division of Neurology, Shandong Qianfoshan Hospital, Shandong, China.
[4]To whom correspondence should be addressed at Division of Neuroimmunology, Neurotec, Karolinska Institute, Alfred Nobels Alle 10, SE-141 83 Stockholm Sweden; e-mail: bao-guo.xiao@neurotec.ki.se.
[5]Dr. Rui-Sheng Duan is a guest researcher from Division of Neurology, Shandong Qianfoshan Hospital, Shandong, China.

Abbreviations used: DC, dendritic cells; MNC, mononuclear cells; OVA, ovalbumin.

MATERIALS AND METHODS

Animals

Male Lewis rats (6–8 weeks old and body weight 160–180 g, from Zentralinstitut fur Versuchstierzucht Hannover, Germany) were used in this study.

Generation, Modulation and Injection of DC

Spleen mononuclear cell (MNC) suspensions from health rats were prepared by grinding spleen through a cell strainer (Beckton-Dickinson, Franklin Lakes, NJ), depleted of red cells with osmotic lysis, and suspended in serum-free Dulbecco's modication of Eagle's medium (Gibco, Paisley, UK). Cells (5×10^6 cells/mL) were incubated in 75 cm^2 Falcon culture flasks (Beckton-Dickinson). After 2 h, nonadherent cells were gently removed by swirling flasks and aspirating medium, and flasks were washed 3 times with serum-free medium to remove the rest of nonadherent cells. New complete medium supplemented with 1% (v/v) MEM (Gibco), 2 mM glutamine (Flow Lab., Irvine, UK), 50 IU/mL penicillin, 50 μg/mL streptomycin (Gibco), and 10% (v/v) fetal calf serum (FCS) (Gibco) were added into flasks. After 18 h, refloating cells were harvested as a DC-enriched fraction by shaking for 20 min at 200 rpm, while adherent cells mostly consisted of macrophages. The DC-enriched population contained 80–85% DC by staining with OX62 mAb, which recognizes the αE2 subunit of an intergrin specifically expressed on rat DC. Anti-rat CD3 (T cells), CD45RA (B cells), and CD161 (NK cells) were used to detect contamination by other cells, revealing that contamination of T cells was about 2.3%, of B cells 1.9%, and $\leq$1% of NK cells.

DC were exposed to 100 U/mL rat recombinant IFN-γ(Innogenetics, Ghent, Belgium) (IFN-γ-DC), 200 ng/mL recombinant human IL-10 (82% homology to rat IL-10, Bchering Plough Research Institute, NJ) (IL-10-DC), and 50 ng/mL recombinant human TGF-β1 (99% homology to rat TGF-β1, Genentech, San Fransisco, CA) (TGF-β-DC) and medium alone (naive DC) for 48 h, respectively. IFN-γ, IL-10 and TGF-β-DC, and naive DC were washed with serum-free medium, resuspended in serum-free medium.

Part of DC was used in *in vitro* experiments and the remaining DC were transferred subcutaneously (s.c.) into Lewis rats at dose of 1×10^6 cells/rat every month for continuous 18 months. Control rats only received the same volume medium ($n = 8$/group).

Phenotyping

DC were stained with anti-rat PE-MHC class II, PE-CD80, and PE-CD86 monoclonal antibodies (BD Biosciences, San Diego, CA) for 30 min at 4°C, and analyzed with an FACScan (Becton Dicknson). The intensity of these molecules on DC was expressed by mean fluorescence intensity (MFI).

Preparation of T Cells

Mononuclear cells from spleen were prepared by grinding spleen through a cell strainer, and T cells were separated using positive selection of anti-rat CD3 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). After cell fractionation, the purity of T cells was made up of $>$98% $CD3^+$ cells.

Mixed Lymphocyte Reactions

For coculture of DC with T cells, DC were radiated (3,000 rad), and cocultured with T cells (ratio 1:20) for 60 h at 37°C. Cells were then labeled for an additional 12 h with 10 μL aliquots containing 1 μCi of [^{3}H] methylthymidine (Amersham, Little Chalfont, UK) before harvesting. The amount of radioactivity incorporated into DNA was measured in a β-scintillation counter. Cultures were run in triplicate and the results were expressed as cpm.

Clinical Observation and Exploratory Laparectomy

Activities of all rats were observed during the period of treatment. After 18-month treatment, the body weight was measured. Exploratory laparectomies were performed on all rats when they were sacrificed for experiments. Organs of lung, liver, spleen, stomach, and kidney were observed with naked eyes and under the microscopes.

Immunization with Ovalbumin

After 18-month treatment with DC (1 month after last DC injection), 4 rats in each group were immunized s.c. with 200 μL inoculum including 50 μg ovalbumin (OVA; Sigma, St. Louis, MO), 2 mg Mycobacterium tuberculosis (strain H37RA, Difco, Detroit, MI), 100 μL saline, and 100 μL Freund's incomplete adjuvant (Difco) at the base of tail.

Preparation of Spleen Mononuclear Cells

Mononuclear cells were obtained from 4 rats without immunization after 1 month of last DC injection and 4 rats immunized with OVA on day 9 postimmunization (p.i.) in

each group. Spleen MNC suspensions were prepared by grinding spleen through a cell strainer. Cells were washed 3 times with serum-free medium, and re-diluted to a cell concentration of 2×10^6/mL in complete medium for all experiments.

Cell Proliferation

Triplicate aliquots (200 μL) of MNC suspensions containing 4×10^5 cells were applied into 96 well round-bottomed microtiter plates (Nunc, Copenhagen, Denmark). Aliquots of Con A (10 μL; final concentration 5 μg/mL, Sigma) and OVA (final concentration 10 μg/mL, Sigma) were added into appropriate wells containing MNC from unimmunized and OVA-immunized rats, respectively. PBS (10 μL) were added into MNC from OVA-immunized rats as a spontaneous proliferation level. After 48 h, cells were pulsed for 18 h with 10 μL aliquots containing 1 μCi of ^{3}H-methylthymidine. Cells were harvested onto glass fiber filters and thymidine incorporation was measured. The results were expressed as cpm and stimulation index (SI):

$$SI = cpm_{(OVA)}/cpm_{(No\ antigen)}$$

Determination of Cytokines by ELISA

Mononuclear cells (4×10^5 cells/well) from unimmunized and OVA-immunized rats were incubated for 48 h at 37°C in the presence of Con A (5 μg/mL) and OVA (10 μg/mL), respectively. Supernatants were harvested and stored at -20°C for cytokine determination. Levels of IFN-γ, IL-10, and TNF-α in the supernatants were measured by quantitative ELISA as described in the protocol supplied by the manufacturer (PharMingen, San Diego, CA).

RESULTS

Phenotype and Function of Dendritic Cells

Phenotypic analysis of DC showed that IFN-γ, IL-10, or TGF-β did not alter the expression of MHC class II on DC (Fig. 1). However, IL-10 effectively declined the expression of CD80 (MFI = 53) and CD86 (MFI = 50) as compared with naive DC (MFI = 158 and MFI = 375, respectively). Additionally, the expression of CD80 (MFI = 80) and CD86 (MFI = 91) on TGF-β-DC was moderately inhibited as compared with IL-10-DC

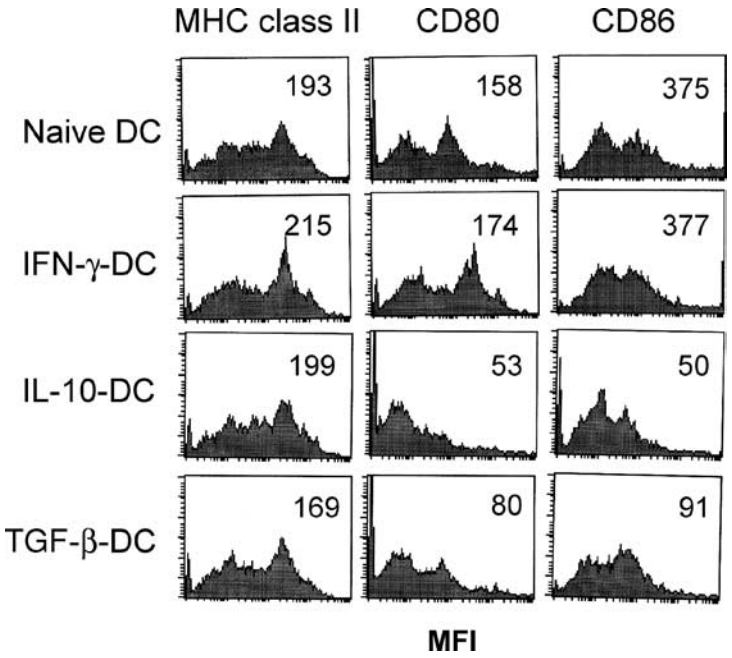

Fig. 1. Expression of MHC class II, CD80, and CD86 on DC. DC were exposed to IFN-γ (100 U/mL), or IL-10 (200 ng/mL), or TGF-β (50 ng/mL) for 48 h at 37°C. DC were then washed with 1% BSA–PBS, and were stained with labeled mAbs or appropriate isotypic controls. A minimum of 10^4 cells was analyzed using CellQuest software. Results are representative of three independent experiments.

(Fig. 1). No differences in the expression of co-stimulatory molecules were observed between IFN-γ-DC and naive DC.

As T-cell stimulation via TCR in the absence of a second signal by co-stimulatory molecules induces a state of anergy, we further tested T-cell proliferation cocultured with DC. Naive DC and IFN-γ-DC stimulated a slight T-cell proliferation ($p \cong 0.05$, respectively) (Fig. 2). In contrast, IL-10-DC induced a markedly impaired proliferation of T cells (mean cpm = 1349) as compared with T cells alone (mean cpm = 2352). No differences were observed between T cells alone and TGF-β-DC, indicating that TGF-β-DC did not effectively stimulate T-cell proliferation (Fig. 2). In preliminary experiments, the production of cytokines was very low in radiated DC alone.

Clinical Observation and Exploratory Laparectomy

There were no symptoms of decreased vigor and activity, cachectic condition and other abnormality for all rats in each group during the 18-month observation. There was also no significant difference of body weight among these groups after 18-month treatment. Exploratory laparectomies showed that there were no tumors in lungs, livers, spleens, gastr-intestine, and kidneys by microvivisection. All these organs appeared the normal size and color.

Cell Proliferation

After 18-month treatment with different DC, MNC were obtained from unimmunized rats. There were no significant differences of Con A-induced MNC proliferation between control rats and rats injected with naive-DC. However, there was lower Con A-induced MNC proliferation in rats injected with IL-10-DC ($p < 0.001$) or TGF-β-DC ($p < 0.001$), whereas there was increased Con A-induced MNC proliferation in rats injected with IFN-γ-DC ($p < 0.01$), as compared with control rats (Fig. 3).

To explore the specific immune response after the 18-month treatment with different DC, 4 rats in each group were immunized with OVA as described in "Materials and Methods." Spleen MNC were obtained on day 9 p.i. Increased proliferation in rats injected with IFN-γ-DC ($p < 0.05$) and decreased proliferation in rats injected with IL-10-DC ($p < 0.05$) were evidence compared with control rats when MNC were cultured with no antigen. Meanwhile, there were lower OVA-induced specific MNC proliferation in rats injected with IL-10-DC ($p < 0.001$) or TGF-β-DC ($p < 0.001$). Stimulation index analysis indicated that suppression of MNC proliferation mediated by IL-10-DC ($p < 0.01$) or TGF-β-DC ($p < 0.05$) was antigen specific (Fig. 4).

Cytokine Production

After 18-month treatment with DC, spleen MNC from unimmunized rats were obtained from each group and cultured in the presence of Con A. There were no difference for production of IFN-γ and IL-10 in MNC supernatants between control rats and rats injected, respectively, with either naive DC or IFN-γ-DC. There was slightly decreased TNF-α in supernatants from rats injected with naive DC or IFN-γ-DC as compared with control rats

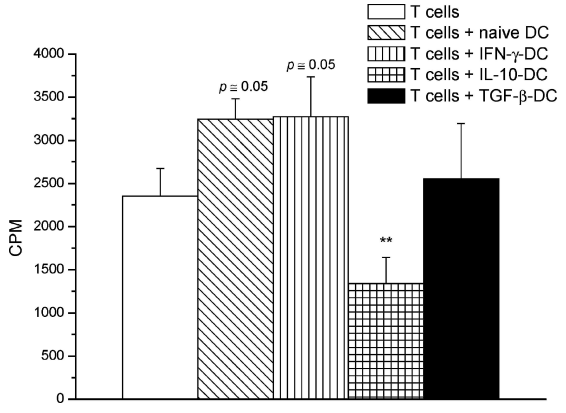

Fig. 2. Mixed lymphocyte reaction. DC were exposed to IFN-γ (100 U/mL), or IL-10 (200 ng/mL), or TGF-β (50 ng/mL) for 48 h at 37°C. After washing with medium, DC were radiated (3000 rad), and cocultured with T cells (ratio 1:20) for 60 h at 37°C. Cells were then labeled for an additional 12 h with 10 μL aliquots containing 1 μCi of [^{3}H]. The results are expressed as the mean valves ±SD. Differences between groups were evaluated by ANOVA. ** $p < 0.01$.

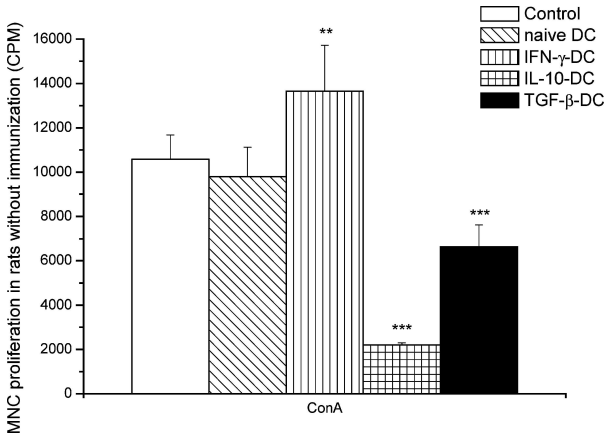

Fig. 3. Spleen MNC from unimmunized rats after 18-month treatment with different DC ($n = 4$/group) on day 9 p.i. were cultured with Con A. The results are expressed as the mean valves ±SD. Differences between groups were evaluated by ANOVA. ** $p < 0.01$ and *** $p < 0.001$.

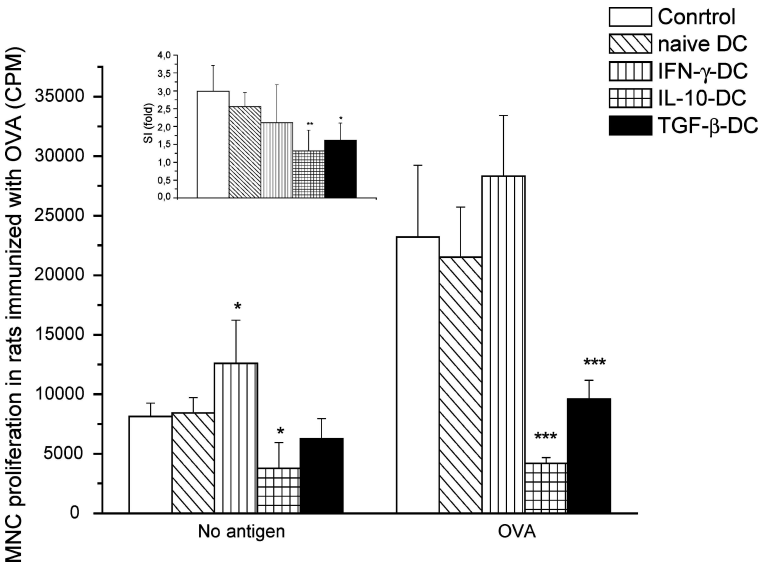

Fig. 4. Spleen MNC from OVA-immunized rats after 18-month treatment with different DC ($n = 4$/group) on day 9 p.i. were cultured in presence or absence of OVA. The results are expressed as the mean valves ±SD and SI. Differences between groups were evaluated by ANOVA. $^{**}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

($p < 0.001$ for both comparison) (Fig. 5). The levels of both IFN-γ and TNF-α were significantly decreased in rats injected with IL-10-DC or TGF-β-DC as compared with control rats ($p < 0.01$ for both comparison of IFN-γ; $p < 0.001$ for both comparison of TNF-α) (Fig. 5). IL-10 level of supernatants was lower only in rats injected with TGF-β-DC as compared with control rats ($p < 0.05$) (Fig. 5).

Spleen MNC from OVA-immunized rats were obtained from each group on day 9 p.i. and cultured in the presence of specific antigen OVA. With exception of slightly decreased TNF-α level in MNC supernatants in rats injected with IFN-γ-DC ($p < 0.001$), there were no differences on levels of IFN-γ, IL-10, and TNF-α production among rats injected with naive DC or IFN-γ-DC and control rats (Fig. 5). However, both IL-10 and TNF-α levels in supernatants from rats injected with IL-10-DC or TGF-β-DC, as well as IFN-γ level from IL-10-DC-injected rats, were significantly decreased as compared with control rats ($p < 0.001$ for all comparison) (Fig. 5).

DISSCUSSION

In this study, we demonstrate that the application of IFN-γ-DC, IL-10-DC, and TGF-β-DC at dose of 1×10^6 cells/rat every month for 18 months did not induce any tumor and body-weight loss in rats. However, IL-10-DC and TGF-β-DC resulted in lower Con A-induced and antigen (OVA)-specific cell proliferation, accompanied by lower levels of Th1 and Th2 cytokines in corresponding supernatants. On the contrary, IFN-γ-DC did not decrease cell proliferation and levels of IFN-γ, IL-10 production, with the exception of slightly decreased TNF-α, in Con A- and OVA-stimulated supernatants.

Immune tolerance can be operationally mediated by different mechanisms, including lack of antigen accessibility (ignorance), absence of T cells (deletion), lack of sufficient activation signals (unresponsiveness), or regulatory T cells. Co-stimulatory molecules, such as CD80 and CD86, are considered as phenotypic markers of DC maturation. In the present study, both IL-10 and TGF-β declined the expression of CD80 and CD86 as compared with naive DC. These immature DC, which lack co-stimulatory molecules, present the tissue antigens to autoreactive T cells, which in the absence of co-stimulation, enter into a state of anergy. If T cells recognize only low levels of MHC/peptide, have a low affinity for their cognate ligand, or receive no co-stimulation from DC, they become anergic or undergo apoptosis. The effect of IL-10- or TGF-β-DC may be directly due to the inhibitory influence of the co-stimulatory molecules of DC, because preincubation of T cells with IL-10 did not result in an inhibited proliferative response (11). An induction of alloantigen-specific anergy in $CD4^+$ T cells and an antigen-specific state of anergy of the influenza hemogglutinin-specific T cell clone HA1.7 was observed after treatment of DC with IL-10, supporting the idea that co-stimulatory molecules are targets for IL-10 action

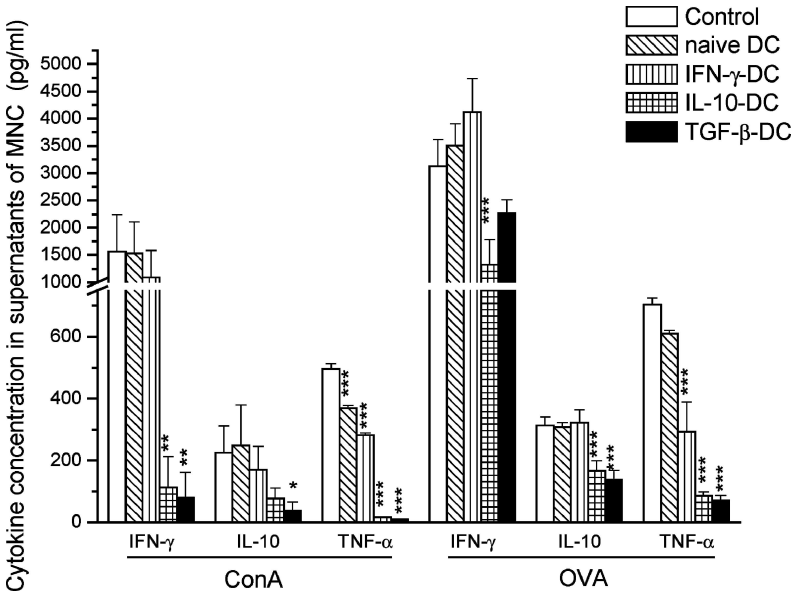

Fig. 5. Cytokine production. Spleen MNC from unimmunized rats after 18-month treatment with different DC ($n = 4$/group) and from OVA-immunized rats on day 9 p.i. ($n = 4$/group) were cultured with Con A and OVA for 48 h, respectively. The supernatants were collected and cytokines were measured by ELISA. The results are expressed as the mean valves ±SD. Differences between groups were evaluated by ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

on DC (11). The state of anergy was characterized by an inhibited T-cell proliferation and reduced production of IL-2 and IFN-γ. Here, our present data demonstrate that long-term application of TGF-β-DC and IL-10-DC resulted in decreased cell proliferation, accompanied by lower levels of both Th1 and Th2 cytokine *in vivo*. Once generated, anergic T cells can suppress development of an immune response by directly suppressing the expression of MHC class II, CD80, and CD86 on DC in culture (12).

Other potential mechanism of tolerance involves DC production of tryptophan metabolites through the action of indoleamine 2,3-dioxygenase (IDO). IDO activity can be induced via IFN-γ receptors, and this leads to T-cell apoptosis, especially activated T cells *in vivo*. Maturation signals of DC include proinflammatory cytokines (GM-CSF, IL-1β, TNF-α, and IFN-α), bacterial or viral components (LPS, unmethylated cytosine poly-guanine or CpG, double-stranded DNA). IFN-γ did not promote the maturation of DC and the down-regulation of CD80 and CD86 by DC, but can induce the expression of IDO that is more sensitive to activated T cells. IFN-γ was found to enhance the regulatory activity of CD8α^{+} DC through enhanced functional activity of IDO and tryptophan degradation, leading to apoptosis of T cells *in vivo* (13). The evidence that IFN-γ induces IDO production provides support for the immunoregulatory role of IFN-γ. Therefore, it seems that IFN-γ-DC might be more ideal candidate in treatment of autoimmune diseases without suppressing immune response. It may be very important for long-term application of cytokine-modified-DC to treat human autoimmune diseases in that long-term immune suppression could increase the risk of infection and tumour.

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