Interleukin-10 Gene-Transfected Mature Dendritic Cells Suppress Murine Experimental Autoimmune Optic Neuritis

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Purpose. We have reported that calcitonin gene-related peptide gene-transfected mature dendritic cells (mDC) suppress murine experimental autoimmune optic neuritis (EAON) and experimental autoimmune encephalitis (EAE) via interleukin-10 (IL-10) production. In our study, we examined whether IL-10–transfected mDC prevent development of EAON and EAE.

Methods. A plasmid expressing mouse IL-10 was constructed and used to transfect C57BL/6 mouse bone marrow–derived mDC by electroporation methods. C57BL/6 mice (with or without GFP expression) were immunized with myelo-oligodendrocyte glycoprotein$_{35-55}$ (MOG$_{35-55}$), and injected intravenously with IL-10–transfected mDC either in the induction or effector phase.

Results. When IL-10–transfected mDC were injected in the induction phase, EAE developed clinically in 60% of mice in the IL-10–transfected group compared to 100% in the mock-transfected group ($P < 0.05$), and mean pathologic score for EAON was 1.1 in the IL-10–transfected group compared to 2.1 in the mock-transfected group ($P < 0.05$). When IL-10–transfected mDC were injected in the effector phase, mean EAE clinical scores were not significantly different between the two groups (2.0 vs. 3.0), while the mean EAON pathologic score was lower in the IL-10–transfected group compared to the mock-transfected group (1.0 vs. 2.7, $P < 0.05$). Delayed hypersensitivity was suppressed significantly in the IL-10–transfected group. Interestingly, the proportions of CD80/86$^+$ and MHC class II$^+$ cells decreased significantly ($P < 0.05$), whereas Foxp3$^+$ cells increased significantly in the spleen and lymph node in the IL-10–transfected group by flow cytometry analysis. Immunohistochemical analysis demonstrated the localization of IL-10–transfected GFP-expressing mDC not only in the spleen and lymph nodes but also in the inflamed optic nerve.

Conclusions. Treatment with IL-10–expressing mDC was effective in suppressing the development of EAON and EAE. (*Invest Ophthalmol Vis Sci.* 2012;53:7235–7245) DOI: 10.1167/iovs.12-10587

Multiple sclerosis (MS) is considered to be a T-cell–mediated inflammatory autoimmune disease directed against myelin or oligodendrocytes in the central nervous system (CNS), and is one of the most common neurologic diseases of young adults in Asian countries, including Japan. MS often causes optic neuritis and myelitis, and this form of MS is called optic-spinal MS. In Europe and America, optic-spinal MS also is called Devic disease or neuromyelitis optica.[1] This condition is characterized clinically by severe optic neuritis and longitudinally extended transverse myelitis, and often is positive for serum aquaporin-4 antibody, but mostly negative for oligoclonal bands.[2]

The development of an optimal animal model is necessary to analyze the pathologic conditions of an important disease, and to develop new cure. Experimental autoimmune encephalomyelitis (EAE) is a well-studied, CD4$^+$ T-cell–mediated inflammatory demyelinating disease of the CNS and serves as the primary animal model for MS.[3] Much of the understanding of the pathogenesis of MS and many therapeutic advances are derived from studies using the EAE model. It generally is accepted that autoreactive, myelin-specific T-cells are responsible for disease initiation. In the EAE model, the antigens used for immunization are myelin basic protein (MBP), proteolipid protein, and myelo-oligodendrocyte glycoprotein (MOG).[4–7] In particular, mouse EAE induced by MOG antigen also manifests inflammation of the spinal cord, optic nerve, and optic chiasm, and resembles human disease.[4] Mice with EAE often suffer experimental autoimmune optic neuritis (EAON), which models acute optic neuritis in humans. The eye is an immune privileged site, in which the induction of conventional immunologic responses is inhibited. Aqueous humor that fills the anterior chamber contains numerous immunomodulatory cytokines and neuropeptides,[8–10] such as TGF-β, α-melanocyte-stimulating hormone; vasoactive intestinal peptide; and calcitonin gene-related peptide (CGRP). These peptides have been shown to have important roles as immunomodulatory factors.

Dendritic cells (DC) are professional antigen-presenting cells (APC) of the immune system, with the potential either to stimulate or inhibit immune response.[11] Normal mature DC (mDC) are potent APC that enhance T-cell activation, whereas normal immature DC (iDC) are engaged in the induction of

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peripheral T-cell tolerance under steady state conditions.[12–15] However, iDC may not be appropriate for clinical treatment of common inflammatory diseases.[15] Therefore, further development of DC with potent negative regulatory capability for T-cells is anticipated to facilitate their use for treatment or prevention of inflammatory diseases. Various laboratories have attempted to enhance the ability of DC to down-regulate T-cell response by pharmacologic methods or gene therapy, with the objective to generate regulatory DC for the treatment of T-cell–mediated autoimmune disorders.[16] Genetic modification of DC by introducing genes encoding immunoregulatory molecules is an attractive strategy for artificial generation of immunoregulatory DC, and this challenging approach could be used to develop new cellular therapies for graft rejection and autoimmunity diseases.[17]

Interleukin-10 (IL-10) is an anti-inflammatory cytokine associated most commonly with macrophages, both as a source of IL-10 and as the cell population most impacted by its action.[18] However, numerous other cells have been shown to secrete IL-10, including DC, T-cells, B-cells, neutrophils, eosinophils, and mast cells.[18] The action of IL-10 on target cells is mediated by the IL-10 receptor, a dimer consisting of an α and β subunit.[19,20] Engagement of the IL-10 receptor results in the activation of the Jak1 and Tyk2 tyrosine kinases as well as DNA binding of signal transducer and activator of transcription 3 (STAT3), leading to downstream alteration in biologic function of the target cell. The action of IL-10 results in down-regulation of MHC class II proteins as well as costimulatory molecules, such as CD80 and CD86, on the surface of target macrophages.[21,22] IL-10 is produced by macrophages and DC as a negative feedback mechanism to dampen uncontrolled production of inflammatory cytokines. In addition to innate cells, regulatory T-cells are able to produce IL-10 to inhibit the activation of antigen-specific cells and inflammatory response. Several recent studies indicate that type I IFN is able to exert its anti-inflammatory role via induction of IL-10 and IL-27 from macrophages and DC.[23–26] IL-10 activates the cytolytic function of NK cells as well as that mediated by activated/memory $CD8^+$ T-cells. Endogenous IL-10 often exerts a suppressive effect on $CD4^+$ T-cells and APC. The IL-10 effects to modulate hyperinflammation also may induce regulatory T-cells. IL-10 release regulates innate immune mechanisms and has an important role in the chronic inflammatory response associated with autoimmune disease.[27]

We have shown previously that CGRP gene-transfected mDC suppress murine experimental autoimmune uveoretinitis in an antigen-specific manner and ameliorate EAE even in the efferent phase, and that IL-10 secreted from DC has an important role in *CGRP*-mediated suppression of murine experimental autoimmune uveoretinitis[28] and EAE.[29] We hypothesized that the antigen-processing capacity of DC may represent an appropriate endogenous system to deliver therapeutic transgene proteins for the treatment of autoimmune diseases. In our study, we used the mouse EAE/EAON model induced by immunization with $MOG_{35–55}$ to examine whether IL-10–transfected mDC are able to prevent the development of EAE and EAON in these mice. Our results indicated that intravenous injection of IL-10–expressing mDC suppressed the development of EAE and EAON as well as delayed hypersensitivity (DH) response. Furthermore, IL-10–expressing GFP^+ mDC were recruited to the optic nerve and lymph nodes. The spleen and lymph nodes of these mice had an expanded $CD4^+CD25^+Foxp3$ cell population (regulatory T-cells; Treg), but reduced $CD80^+$, $CD86^+$, and MHC class II^+ cell populations. These data suggested that IL-10–transfected mDC may have an important role in the suppression of murine EAE and EAON, and IL-10 gene transfer into mDC could be a novel approach for the treatment of human MS or optic neuritis.

Materials and Methods

Animals and Anesthesia

Female C57BL/6 mice 6 to 8 weeks old were obtained from Charles River Laboratories Japan, Inc. (Ibaraki, Japan). Transgenic C57BL/6-Tg (CAG-EGFP) mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed according to the protocol approved by the Institutional Review Board of Juntendo University School of Medicine, Tokyo, Japan. All animals were treated according to the Association for Research in Vision and Ophthalmology (ARVO) Animal Statement for the Use of Animals in Ophthalmic and Vision Research. Intraperitoneal (IP) injection of a mixture of Nembutal (30 mg/kg) and xylazine hydrochloride (125 mg/kg) was used for anesthesia.

Reagents

The $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by conventional solid-phase techniques, as described previously.[7] Purified *Bordetella pertussis* toxin was obtained from Sigma Chemical (St. Louis, MO). Complete Freund's adjuvant (CFA) and *Mycobacterium tuberculosis* strain H37Ra were obtained from Difco (Detroit, MI). Murine GM-CSF was purchased from Pepro Tech (London, UK), and LPS was obtained from from Sigma-Aldrich (St. Louis, MO).

Culture Medium

Serum-free medium was used in all DC cultures. This medium was composed of RPMI 1640 (Sigma-Aldrich), 10 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), 0.1 mM nonessential amino acids (Gibco, Grand Island, NY), 1 mM sodium pyruvate (Sigma-Aldrich), 100 U/mL penicillin (Meiji Seika, Tokyo, Japan), 100 μg/mL streptomycin (Meiji Seika), 1×10^{-5} M 2-ME (Wako Chemicals, Richmond, VA), and 0.1% BSA (Wako). In some studies, RPMI 1640 containing 10% fetal bovine serum (FBS; Sigma-Aldrich) instead of 0.1% BSA was used.

Generation of Dendritic Cells

Murine DC were generated according to a previous report.[16] Briefly, bone marrow cells from C57BL/6 mice were cultured with murine GM-CSF (20 ng/mL) in a bacteriologic Petri dish (Asahi Glass Co., Ltd., Tokyo, Japan) for 6 days. Nonadherent cells were collected and subjected to negative selection with monoclonal antibodies (mAb) to Ly-76, CD2, B220, CD14, and Ly-6G (all from BD Pharmingen, Franklin Lakes, NJ) plus sheep anti-rat IgG mAb-conjugated immunomagnetic beads. The resultant cells were washed three times with cold PBS to prevent carryover of cytokines, and then stimulated with LPS (1 μg/mL) in a bacteriologic Petri dish for 1 day. The stimulated DC were washed and cultured with 10 μg/mL of $MOG_{35–55}$ in serum-free medium at 37°C in an atmosphere of 5% CO_2. After overnight culture, nonadherent cells were washed three times with culture medium to remove the antigen. These DC preparations typically were more than 95% pure as indicated by anti-I-A/I-E mAb and $CD11c^+$ staining, and contained less than 0.1% erythrocytes, T-cells, B-cells, $F4/80^+$macrophages, NK cells, and neutrophils as assessed by FACS analysis.

Generation and Transfection of mIL-10–Expressing Plasmid in DCs

The plasmid for expressing mouse IL-10 was constructed as follows. The coding region of IL-10 cDNA was amplified by PCR using cDNA synthesized from total RNA of mDC as template. Briefly, PCR was performed using primers 5′-gccttgcagaaaagagagctccatcatg-3′ (forward) and 5′-gcagactcaatacacactgcaggtgtt-3′ (reverse), which correspond to just upstream of the translational initiation site (underlined portion of forward primer) and the complementary sequence in 3′-

untranslated region of mouse IL-10 mRNA (accession number NM_010548), respectively. The PCR product was cloned into pCR2.1 plasmid using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). After confirming the nucleotide sequence, a *Hin*dIII/*Xho*I-digested fragment containing IL-10 cDNA was inserted into the *Hin*dIII/*Xho*I site of pCR3.1 (Invitrogen). The resulting plasmid was designated pCR3.1-mIL-10.

$CD11c^+$ DC were purified from the above-mentioned DC preparations using mouse CD11c MicroBeads and auto-MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). The IL-10–expressing plasmid pCR3.1-mIL-10 or vehicle plasmid pCR3.1 (mock transfection) was transfected into the purified $CD11c^+$ DC by electroporation using Nucleofector II (Amaxa GmbH, Koeln, Germany) according to manufacturer's instructions. Mature DC transfected with pCR3.1-mIL-10 or pCR3.1 were cultured with 10 μg/mL MOG_{35-55} overnight before use.

Measurement of In Vitro IL-10 Production and TGF-β1/2 by ELISA

IL-10 concentrations in mDC culture supernatants were determined by ELISA using the Quantikine Mouse IL-10 and Immunoassay (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. TGF-β1 and -β2 concentrations in mDC culture supernatants were determined using the Bio-Plex Pro TGF-β3-Plex assay (Bio-Rad Laboratories, Hercules CA) according to the manufacturers' instructions. Briefly, the IL-10–expressing plasmid or vehicle plasmid was transfected into mouse mDC by electroporation. Then, cells were cultured in flat-bottom 24-well plates at a density of 2.5×10^5 cells/2 mL of RPMI 1640 medium at 37°C in 6% CO_2/94% air. In separate wells, the IL-10–transfected mDC were cultured with 10 μg/mL of MOG_{35-55} in RPMI 1640 medium. Supernatants were collected after 24-hour incubation, and used for IL-10 and TGF-β1/2 assays.

Quantification of mRNA by Real-Time PCR

Total RNA was prepared from IL-10–transfected mDC or mock-transfected mDC using the RNeasy kit (Qiagen, Hilden, Germany), and was reverse transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The total mRNA level of IL-10 was quantified with the StepOne Real-Time PCR system (Applied Biosystems) using TaqMan Gene Expression Assay for IL-10 (Mm00439616_m1) and TaqMan rodent GAPDH control reagents, together with TaqMan Universal Master Mix (all from Applied Biosystems).

EAE and EAON Induction and Evaluation

EAE and EAON were induced by the method described by Shao et al.[7] with modifications. The MOG_{35-55} peptide was diluted in PBS (pH 7.35) to a concentration of 200 μg/50 μL/mouse. The MOG_{35-55} preparation was emulsified in CFA containing 600 μg/mL of heat-killed *M. tuberculosis* H37Ra (1:1), and used to immunize C57BL/6 mice subcutaneously in the neck region. The mice were also injected intraperitoneally with *B. pertussis* toxin (1 μg/100 μL/mouse) at the same time. After the C57BL/6 mice were immunized with MOG_{35-55} to produce EAON and EAE, they were injected intravenously with mDC transfected with pCR3.1–mIL-10 (IL-10–transfected group) or pCR3.1-2FL (mock-transfected group) on day 1 for the induction phase study, or on day 9 for the effector phase study.

A diagnosis of EAE was based on clinical signs. The clinical scores for EAE at day 28 after immunization were: 0, no symptom; 1, reduced tail tone; 2, weakened hind limbs or partial paralysis; 3, complete paralysis of hind limbs; and 4, paralysis of fore and hind limbs. In addition, EAON was evaluated pathologically. Mice were killed at 28 days after immunization. The eyeballs were extracted and fixed in 4% paraformaldehyde followed by 10% formalin. Fixed and dehydrated eyeballs were embedded in methacrylate, and 5 μm sections were cut through the pupillary–optic nerve plane, and stained with hematoxylin and eosin (HE). The pathologic scores for EAON were: 0, no lesion; 1, moderate cell infiltration in optic nerve; 2, strong cell infiltration in optic nerve; and 3, massive cell infiltration in optic nerve.

Measurement of Cytokine Production by Spleen Cells and Lymph Node Cells

Some mice were sacrificed on day 28 after MOG immunization, and spleens and lymph nodes were dissociated into single-cell suspensions by lysing erythrocytes with Tris-NH_4C1. Spleen cells and lymph node cells were cultured in flat-bottom 96-well plates at a density of 2×10^5 cells/200 μL of RPMI 1640 medium containing 10 μg MOG at 37°C in 6% CO_2 in air. Supernatants were collected after 24 hours of culture, and assayed for IL-10, IL-2, IL-6, IL-17, TNF-α, and IFN-γ using the Bio-Plex Pro Mouse Cytokine Standard Group I 23-Plex (Bio-Rad Laboratories) according to the manufacturers' instructions.

Measurement of Delayed-Type Hypersensitivity

We analyzed delayed hypersensitivity as an in vivo indicator of the relative abilities of MOG-induced EAE and EAON mice to mount a Th1-dependent antigen recall response. On day 13 after immunization, 20 μg/10 μL of MOG_{35-55} suspended in PBS were injected intradermally into the pinna of one ear. Ear swelling was measured after 24 hours using a micrometer (Mitutoyo, Tokyo, Japan). Antigen-specific delayed hypersensitivity was measured as the difference in ear thickness before and after challenge. Results were expressed as: Specific ear swelling = (24-hour measurement – 0-hour measurement) in test ear – (24-hour measurement – 0-hour measurement) in control ear, as described previously.[28]

Flow Cytometric Analysis

Single-cell suspensions were prepared from spleens and lymph nodes by passing the tissue through a wire mesh. Cells was collected, washed, and incubated with Fc Block (clone 2.4G2; BD Pharmingen) for 15 minutes at 4°C in FACS buffer (PBS supplemented with 1% BSA). Anti-CD4-FITC, anti-CD11c-FITC (FITC; eBiosciences, San Diego, CA), anti-CD80-PE, anti-CD86-PE, anti-$I\text{-}A^b$-PE, anti-Foxp3-PE (PE; eBiosciences), anti-CD25-APC (clone PC61; eBiosciences), and isotype control (rat IgG2a-PE; eBiosciences) were used for analysis of cell surface marker expression, according to manufacturer's instructions. Cell-surface fluorescence was analyzed with the FACSCalibur flow cytometer (BD Pharmingen).

Immunohistochemistry (IHC)

Mice were anesthetized and perfused transcardially with 10% neutral buffered formaldehyde. The eyes were enucleated and immersed in the same fixative overnight. Tissues were embedded in paraffin, and 4 μm sections were mounted onto New Silane III glass slides (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) and dried at 60°C. After deparaffinization in xylene and rehydration in graded ethanol series, endogenous peroxidases were quenched with 0.3% H_2O_2 in methanol for 15 minutes. Antigen retrieval was performed using a 10 M sodium citrate (pH 6.0) solution at 100°C (microwave) for 20 minutes. Primary antibodies were applied and incubated for 3 hours at room temperature. Swine polyclonal anti-rabbit IgG (Dako Japan, Tokyo, Japan) was used as secondary antibody. Horseradish peroxide (HRP)-streptavidin conjugate (Dako Japan), and 3-3′-diaminobenzidine tetrahydrochloride and HRP reaction were used for visualization. The following primary antibodies were used: Rabbit anti-Iba-1 antibody (1:1000; Wako Chemicals) and rabbit anti-myelin basic protein antibody (1:5; Histofine, Nichirei, Tokyo). After immunostaining, micrographs were acquired using a light microscope

equipped with a digital camera (BX50, DP70; Olympus, Tokyo, Japan).

Kinetics of IL-10–Transfected mDC

Mature DC were purified from the bone marrow of transgenic C57BL/6-Tg (CAG-EGFP) mice and transfected with IL-10 gene (IL-10–transfected GFP^+ mDC). After C57BL/6 mice were immunized with MOG_{35-55}, they were injected intravenously with 10^6 IL-10–transfected GFP^+ mDC on day 9 after immunization. The mice were killed at 28 days after immunization. Samples of spleen, lymph node, and optic nerve were collected for cryosection preparation. The tissues were removed, washed in PBS, and then embedded in Tissue Tek OCT compound (Sakura, Torrance, CA). Frozen sections were cut at a thickness of 5 μm on a cryostat (CM1850; Leica, Bannockburn, IL), and preserved at −80°C. To stain tissue for immunofluorescence, slides were brought to room temperature, dried, fixed in acetone for 10 minutes at 4°C, and then placed in PBS for 5 minutes to remove OCT compound. All slides were blocked with 5% BSA in PBS for 30 minutes. The slides were incubated overnight with anti-GFP Ab conjugated with Alexa 488 (Invitrogen) in a humidified chamber at 4°C and then washed in PBS three times for 5 minutes each. Slides were incubated with appropriate secondary Ab for 1 hour and then washed in PBS three times for 5 minutes each. To depict the nuclei, cells were mounted using VECTASHIELD with DAPI (Invitrogen). The sections were observed under a fluorescent microscope and a confocal laser scanning fluorescent microscope.

Quantification of IL-10 gene-transfected GFP^+ mDC was performed by counting green spots in a fixed area (a circle 250 μm in radius, original magnification ×20).

IL-10 mRNA Expression in Optic Nerve

Total RNA was extracted from optic nerves of mice treated with IL-10–transfected mDC or mock-transfected mDC using the RNeasy kit (Qiagen), and was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). IL-10 mRNA expression level was quantified with the StepOne Real-Time PCR system (Applied Biosystems) using TaqMan Gene Expression Assay for IL-10 (Mm00439616_m1) and TaqMan rodent GAPDH control reagents, together with TaqMan Universal Master Mix (all from Applied Biosystems).

Statistical Analysis

The significance of differences between means was determined using the Mann-Whitney *U* test or ANOVA followed by Sheffé test. *P* values less than 0.05 were considered as significant.

Results

Properties of IL-10–Transfected Mature Dendritic Cells

Gene transfer using mDC in suspension yielded 80% cell viability and 50% transfection efficiency. When we measured in vitro IL-10 production from the IL-10–transfected mDC using ELISA (Fig. 1a), the concentrations in culture supernatants ranged from 193.3 to 576.5 pg/mL (average 432.9 pg/mL). On the other hand, IL-10 production was 14.7 pg/mL in mock-transfected mDC ($P = 0.0286$ versus IL-10–transfected mDC). Moreover, IL-10 mRNA expression level in IL-10–transfected mDC was approximately 52-fold higher than that in mock-transfected mDC ($P = 0.0023$, Fig. 1b). Stimulation with 10 μg/mL of MOG_{35-55} did not enhance IL-10 production by mock-transfected mDC or IL-10–transfected mDC, compared to the corresponding cells without MOG_{35-55} stimulation (Fig. 1b).

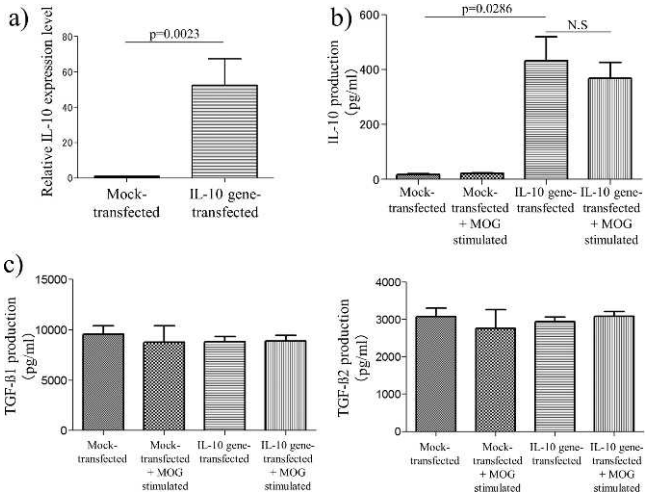

Figure 1. In vitro characteristics of IL-10–transfected mDC. (**a**) IL-10 mRNA expression levels of IL-10–transfected mDC and mock-transfected mDC were analyzed by quantitative real-time PCR. Data are expressed as mean ± SD ($n = 5$), $P = 0.0023$ analyzed by Mann-Whitney *U* test. (**b**) IL-10 productions by IL-10–transfected mDC and mock-transfected mDC were measured by ELISA. Data are expressed as mean ± SD ($n = 5$), $P = 0.0286$ analyzed by Mann-Whitney *U* test. (**c**) TGF-β1 and -β2 productions by IL-10–transfected mDC and mock-transfected mDC were measured by ELISA. Data are expressed as mean ± SD ($n = 5$), $P > 0.05$ analyzed by Mann-Whitney *U* test. Similar results were obtained in three independent experiments.

TGF-β1 and -β2 production was not significantly different between four groups (Fig. 1c).

Cytokine Production by Spleen Cells and Lymph Node Cells Derived from MOG-Immunized Mice

C57BL/6 mice were immunized subcutaneously with MOG peptide and then injected intravenously with IL-10–transfected or mock-transfected mDC on the same day (induction phase). On day 28 after immunization, the spleens of mice in the IL-10–transfected group were bigger than those in the mock-transfected group. In vitro productions of interleukins, TNF-alpha, and IFN-γ in response to MOG stimulation by spleen cells and lymph node cells collected from these mice on day 28 after immunization were measured. In the IL-10–transfected group, IL-2, IL-6, IL-17, IFN-γ, and TNF-alpha productions were significantly ($P < 0.05$) reduced compared to the mock-transfected group. On the other hand, IL-10 production was not significantly different, but tended to decrease in the IL-10–transfected group (Fig. 2a). Thus, cytokine production by spleen cells in response to MOG stimulation was markedly reduced in IL-10–transfected mice, suggesting that IL-10–transfected mDC suppressed MOG-specific systemic immunologic response. Additionally, in the IL-10–transfected group, IL-10 production from lymph node cells increased compared to the mock-transfected group, but TNF-alpha, IFN-γ, and IL-2 productions were reduced significantly (Fig. 2b).

Disease Rate and Severity in Induction Phase Study

Induction phase study was conducted by immunizing C57BL/6 mice subcutaneously with MOG peptide and then injecting IL-10–transfected or mock-transfected mDC intravenously on day 1 (induction phase). Clinical symptoms were observed until day 28 after immunization.

All the mice in the mock-transfected group had EAE (Fig. 3a), and onset of EAE was observed from 1 to 28 days after

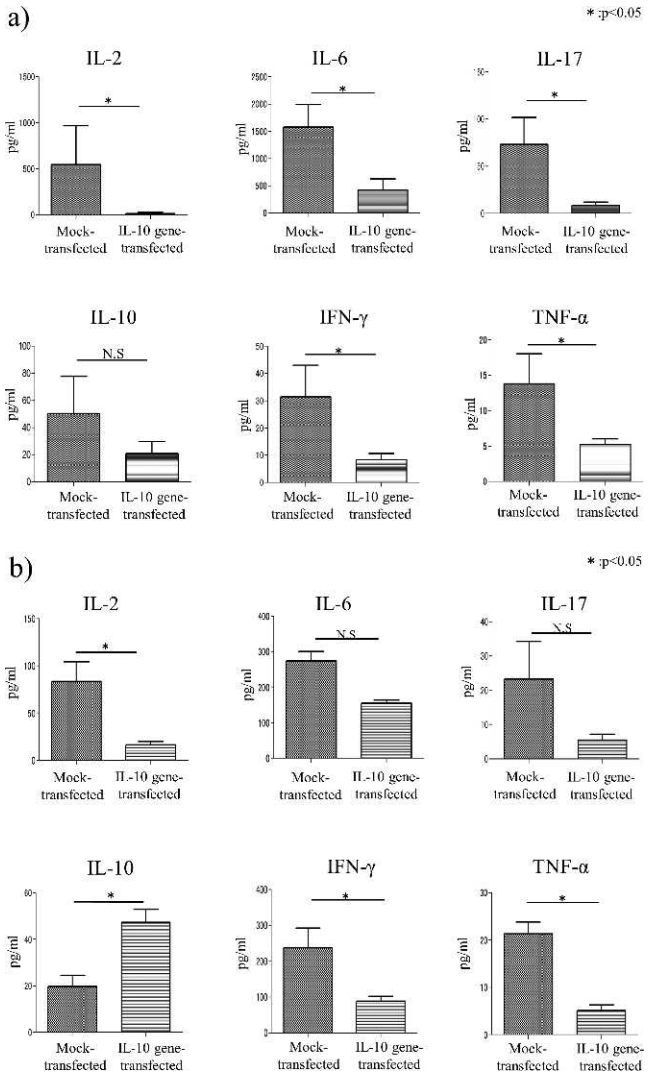

Figure 2. Cytokine productions from (**a**) spleen cells and (**b**) lymph node cells obtained from mice injected with transfected mDC in the induction phase. C57BL/6 mice were immunized subcutaneously in the neck region with the MOG_{35-55} peptide, and then injected intravenously with mDC transfected with the IL-10–expressing plasmid pCR3.1–mIL-10 (IL-10–transfected group) or vehicle plasmid pCR3.1 (mock-transfected group) on day 1. On day 28 after immunization, spleen cells and lymph node cells were obtained. Supernatants of cultured spleen or lymph node cells were collected after 24-hour culture, and assayed for IL-10, IL-2, IL-6, TNF-α, and IFN-γ using the Bio-Plex Cytokine Assay (Bio-Rad Laboratories). Data are expressed as mean ± SD ($n = 4$), $P < 0.05$ analyzed by Mann-Whitney *U* test, with similar results obtained in three independent experiments.

immunization. On the other hand, the rate of EAE was markedly lower in the IL-10–transfected group (60%). The average clinical score on day 28 after immunization was 2.1 in the control group and 1.1 in the IL-10–transfected group ($P < 0.05$, Fig. 3b). This finding indicated that intravenous injection of IL-10–transfected mDC in the induction phase ameliorated EAE.

All (100%) the mice in the mock-transfected group compared to only 57% in the IL-10–transfected group had EAON. The average pathologic score on day 28 after immunization was 2.1 in the mock-transfected group and 1.1 in the IL-10–transfected group ($P < 0.05$, Fig. 3c).

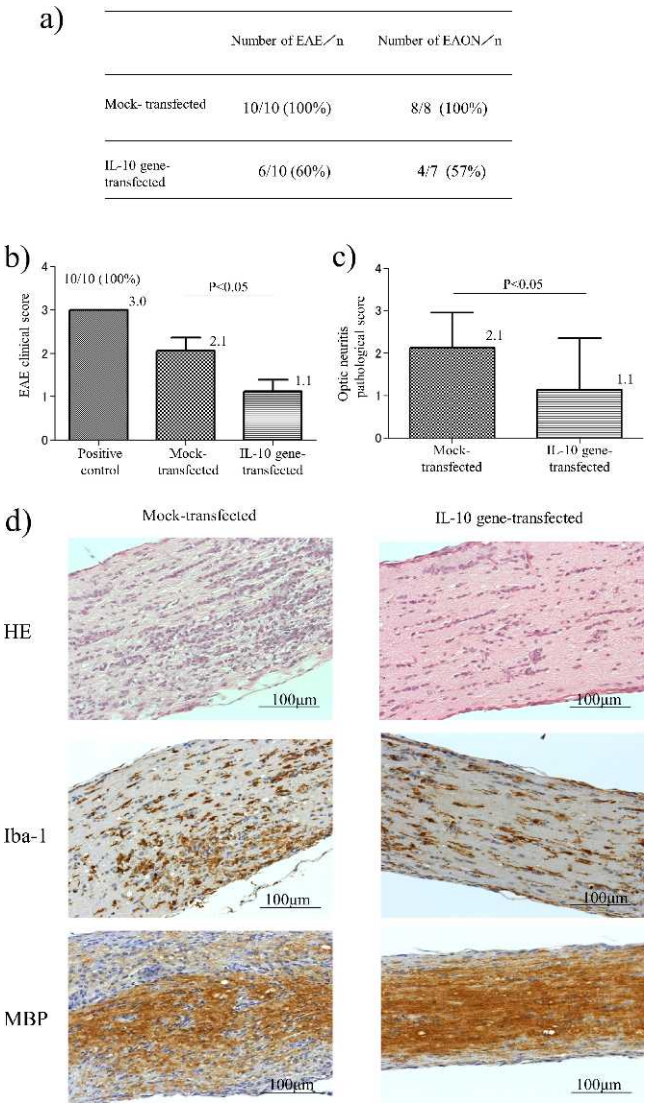

Figure 3. Induction phase study: Incidence of EAE and EAON (**a**), severity of EAE (**b**) and EAON (**c**), and histopathology of EAON on day 28 (**d**). C57BL/6 mice were immunized subcutaneously in the neck region with the MOG_{35-55} peptide, and then injected intravenously with IL-10–transfected mDC or the mock-transfected mDC on day 1 (induction phase). The mice were observed for clinical symptoms, and then killed at day 28 after immunization for pathologic studies. (**a**) All the mice in mock-transfected group had EAE and EAON. On the other hand, the rate of EAE was 60% and the rate of EAON was 57% in the IL-10–transfected group. (**b**) A diagnosis of EAE was based on clinical signs at day 28 after immunization. Mann-Whitney U test detected a significant difference between IL-10–transfected and mock-transfected groups. (**c**) A diagnosis of EAON was based on pathologic score at day 28 after immunization. Mann-Whitney *U* test detected significant difference between IL-10–transfected and mock-transfected groups ($P < 0.05$). (**d**) Representative micrographs of optic nerve stained with HE, and immunostained for Iba-1 and MBP are shown. Marked inflammatory cell infiltration in the optic nerve is observed in the mock-transfected group, but cell infiltration is less severe in the IL-10–transfected group. Microglia cell infiltration as indicated by Iba-1 immunostaining is less intense in the IL-10–transfected group. Microglia cell infiltration tended to accumulate in the local inflammation of optic nerve in the mock-transfected group. MBP immunostaining is denser in the IL-10–transfected group. Values represent mean ± SD results from ≥10 mice in each group, with similar results obtained in three independent experiments.

a)

	Number of EAE/n	Number of EAON/n
Mock- transfected	5/5 (100%)	8/9 (89%)
IL-10 gene-transfected	4/5 (80%)	6/9 (67%)

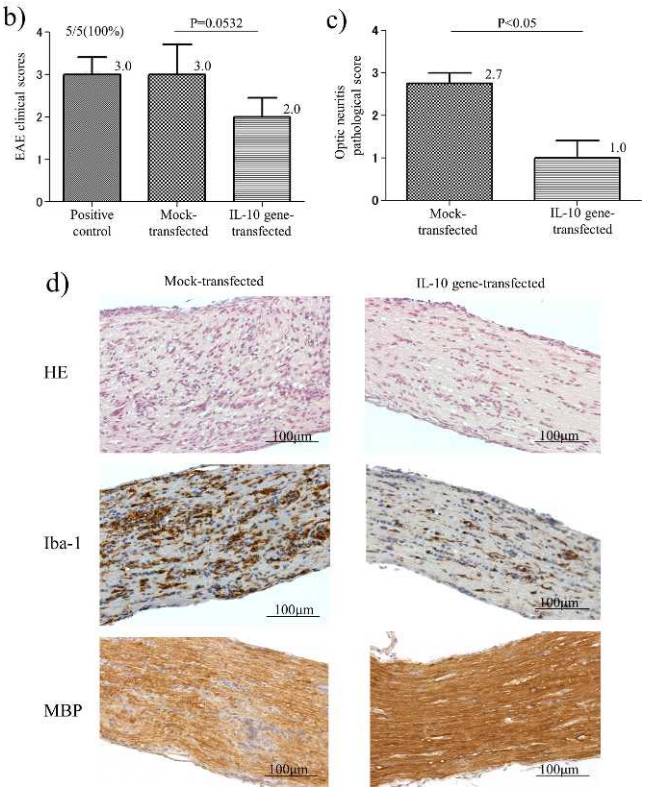

Figure 4. Effector phase study: Incidence of EAE and EAON (**a**), severity of EAE (**b**) and EAON (**c**), and histopathology of EAON (**d**) on day 28. C57BL/6 mice were immunized with MOG_{35-55}, and then injected intravenously with IL-10–transfected mDC or mock-transfected mDC on day 9 (effector phase). The mice were observed for clinical symptoms, and then killed at day 28 after immunization for pathologic studies. (**a**) All the mice in the mock-transfected group had EAE, and 89% had EAON. On the other hand, the rate of EAE was 80% and the rate of EAON was 67% in the IL-10–transfected group. (**b**) A diagnosis of EAE was based on clinical signs at day 28 after immunization. Mann-Whitney *U* test detected no significant difference between IL-10–transfected and mock-transfected groups. (**c**) A diagnosis of EAON was based on pathologic score at day 28 after immunization. Mann-Whitney *U* test detected a significant difference between the IL-10–transfected and mock-transfected groups. (**d**) Representative micrographs of optic nerve stained with HE, and immunostained for Iba-1 and MBP. Many inflammatory cells infiltrate the optic nerve in the mock-transfected group, but there is less cell infiltration in the IL-10–transfected group. Microglia cell infiltration as indicated by Iba-1 immunostaining is milder in the IL-10–transfected group. Moreover, Microglia cell infiltration tended to accumulate in the local inflammation of optic nerve in the mock-transfected group. MBP immunostaining is denser in the IL-10–transfected group. Values represent mean ± SD results from ≥10 mice in each group, with similar results obtained in three independent experiments.

Histopathologically, inflammatory cell infiltration in the optic nerve was marked in the mock-transfected group and was milder in the IL-10–transfected group (Fig. 3d). Microglia cell infiltration in the optic nerve as indicated by Iba-1 immunostaining was less intense in the IL-10–transfected group. Moreover, infiltration of microglia cells tended to accumulate in the local inflammation of optic nerve in the mock-transfected group. MBP immunostaining in the optic nerve was denser in the IL-10–transfected group compared to the mock-transfected group. This result suggests that treatment with IL-10–transfected mDC may prevent tissue damage caused by demyelination of the optic nerve.

Disease Rate and Severity in Effector Phase Study

Effector phase study was conducted by immunizing C57BL/6 mice subcutaneously with MOG peptide and then injecting IL-10–transfected or mock-transfected mDC intravenously on day 9 (effector phase). Clinical symptoms were observed until day 28 after immunization.

All (100%) the mice in the mock-transfected group had EAE, compared to 80% in the IL-10–transfected group (Fig. 4a). However, the average clinical score on day 28 after immunization was 3 in the mock-transfected group and 2 in the IL-10–transfected group, which tended to be different between the two groups ($P = 0.0532$, Fig. 4b). EAON developed in 89% of the mice in the mock-transfected group and decreased to 67% in the IL-10–transfected group. The pathologic score was 2.7 in the mock-transfected group and was significantly reduced to 1 in the IL-10–transfected group (Fig. 4c). These results indicated that injection of IL-10–transfected mDC in the effector phase ameliorated the severity of EAON.

Histologically, marked infiltration of inflammatory cells in the optic nerve was observed in the mock-transfected group (Fig. 4d), while the infiltration was considerably milder in the IL-10–transfected group. Microglia cell infiltration in the optic nerve as indicated by Iba-1 immunostaining was less intense in the IL-10–transfected group. Moreover, infiltration of microglia cells tended to accumulate in the local inflammation of optic nerve in the mock-transfected group. MBP immunoreactivity in the optic nerve was denser in the IL-10–transfected group than in the mock-transfected group.

Delayed Type Hypersensitivity Response in Induction and Effector Phase Studies

To evaluate whether cell-mediated immunity was suppressed, we measured delayed type hypersensitivity response on day 13 after immunization in the induction phase and effector phase studies. In the induction phase study, delayed hypersensitivity was suppressed significantly ($P < 0.05$) in the group injected with IL-10–transfected mDC compared to the mock-transfected group (Fig. 5a). In the effector phase study, delayed-type hypersensitivity also was suppressed significantly ($P < 0.05$) by injection of mDC transfected with IL-10 compared to mock-transfected mDC (Fig. 5b).

Expression of MHC Class II and Co-Stimulatory Molecules on Spleen and Lymph Node Cells Derived from MOG-Immunized Mice

C57BL/6 mice were immunized subcutaneously with MOG peptide and then injected intravenously with IL-10–transfected or mock-transfected mDC on day 1 (induction phase). We examined the cell surface expression of MHC class II and various co-stimulatory molecules (CD80, CD86) on spleen cells and lymph node cells collected from these mice on day 28 after immunization. Compared to the mock-transfected group, the IL-10–transfected group showed greatly reduced ($P < 0.05$) expression levels of CD80, CD86, and MHC class II molecules ($I\text{-}A^b$, Fig. 6).

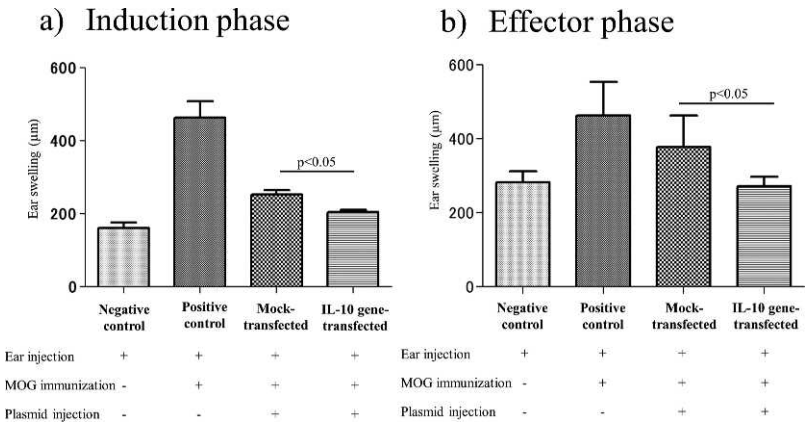

Figure 5. Delayed hypersensitivity response in induction phase study (**a**) and effector phase study (**b**). In the induction phase and effector phase studies, C57BL/6 mice were immunized with MOG, and injected intravenously with IL-10–transfected mDC or mock-transfected mDC either on day 1 (induction phase) or day 9 (effector phase). On day 13 after immunization, the mice were injected intradermally with MOG_{35-55} suspended in PBS into the pinna of one ear. Ear swelling was measured after 24 hours using a micrometer. Delayed hypersensitivity was measured as the difference in ear thickness before and after challenge. Ear swelling = (24-hour measurement – 0-hour measurement) in test ear – (24-hour measurement – 0-hour measurement) in control ear. Data are expressed as mean ± SD ($n = 10$). ANOVA followed by Scheffé test detected significant differences between IL-10–transfected and mock-transfected groups in both studies, with similar results obtained in three independent experiments.

Recruitment of IL-10–Transfected GFP$^+$ mDC to Spleen, Lymph Node, and Optic Nerve from MOG-Immunized Mice

In the next series of experiments, we sought to determine whether the injected IL-10–transfected mDC migrate to the spleen, lymph node, and optic nerve. C57BL/6 mice were immunized subcutaneously with MOG peptide and then injected intravenously with IL-10–transfected GFP$^+$ mDC on day 9 after immunization. Confocal laser scanning fluorescent microscope analysis demonstrated the presence of IL-10–transfected GFP$^+$ mDC immunostained by anti-GFP antibody in the spleen, lymph node, and optic nerve (Fig. 7). Furthermore, we counted the numbers of IL-10–transfected GFP$^+$ mDCs recruited to the spleen, lymph node, and optic nerve. The numbers of IL-10–transfected GFP$^+$ mDC were significantly ($P < 0.05$) higher in the lymph node and optic nerve compared to the spleen (Fig. 7d).

Moreover, IL-10 mRNA expression level in the optic nerves of mice treated with IL-10–transfected mDC was approximately 7-fold higher than that of mice infected with mock-transfected mDC ($P < 0.05$, Fig. 7e).

IL-10–Transfected mDC Treatment-Induced Treg Cells in the Spleen and Lymph Node

Accumulation of Treg cells in CNS of EAE mice has been shown to correlate with natural recovery from the disease.[30] The

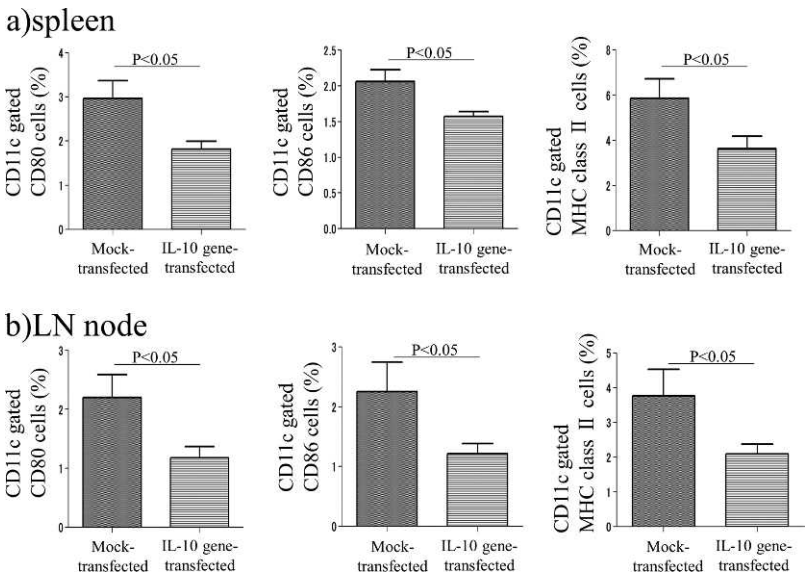

Figure 6. Flow-cytometric studies of CD80, CD86, MHC class II expression in spleen cells (**a**) and lymph node cells (**b**) of C57BL/6 mice immunized with MOG_{35-55} and injected with IL-10–transfected mDC in the induction phase. On day 28 after immunization, spleen and lymph node cells were collected from MOG-immunized mice injected with IL-10–transfected mDC or mock-transfected mDC. Data are expressed as mean ± SD ($n = 10$). Mann-Whitney U test detected significant differences between IL-10–transfected and mock-transfected groups in the spleen and lymph node cells ($P < 0.05$), with similar results obtained in three independent experiments.

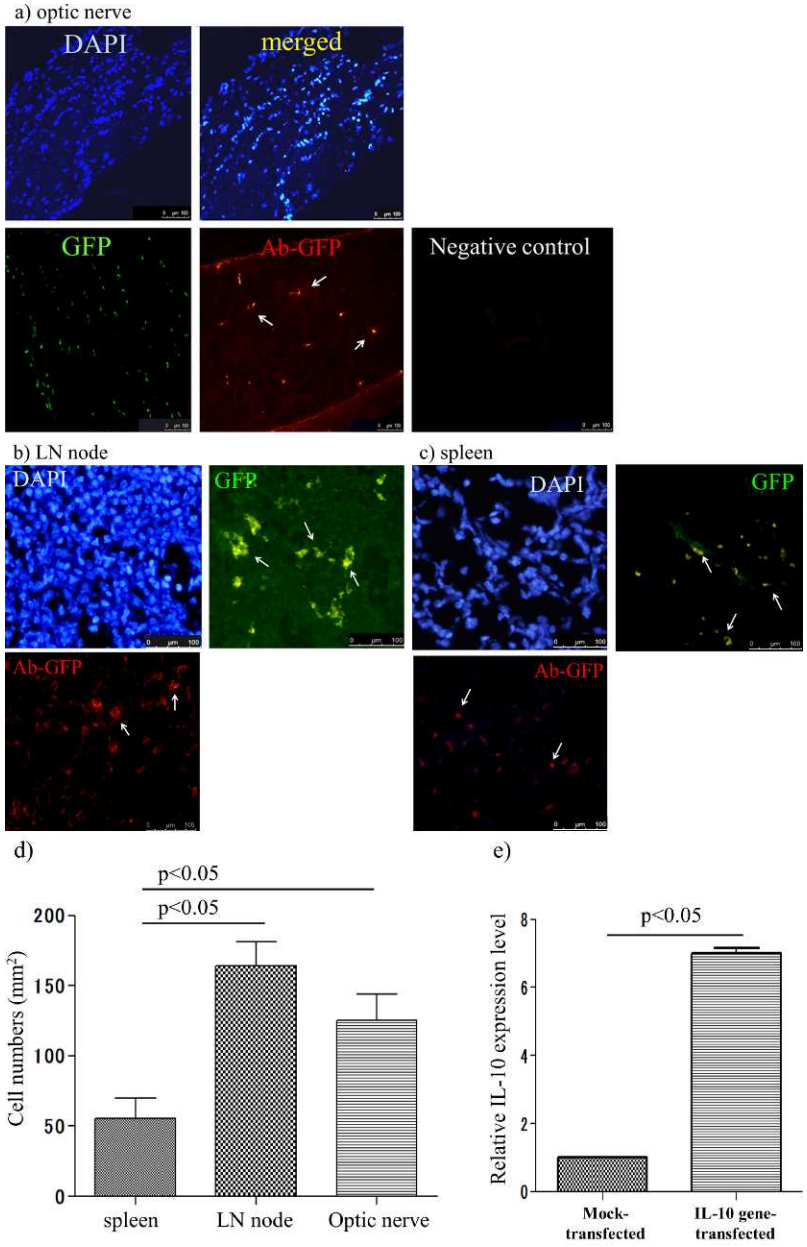

Figure 7. Representative fluorescence micrographs demonstrating recruitment of IL-10–transfected mDC to the optic nerve (**a**), secondary lymph node (**b**) and spleen (**c**). Mature DC were purified from the bone marrow of transgenic C57BL/6-Tg (CAG-EGFP) mice and transfected with IL-10 gene (IL-10–transfected GFP$^+$ mDC). After C57BL/6 mice were immunized with MOG_{35-55}, they were injected intravenously with 10^6 IL-10–transfected GFP$^+$ mDC. On day 28 after immunization, optic nerve, lymph node, and spleen were collected and cryosections were prepared. GFP$^+$ cells were observed unstained (*green florescence*) or immunostained with anti-GFP Ab (*red fluorescence*). Nuclei were stained using with DAPI (*blue fluorescence*). Sections were observed using a confocal laser scanning fluorescent microscope. (**a**) IL-10–transfected GFP$^+$ mDC (*green*) are detected throughout the optic nerve, and are immunostained by anti-GFP Ab (*red*). (**b**) Recruitment of IL-10–transfected GFP$^+$ mDC also is observed in the lymph node. (**c**) IL-10–transfected GFP$^+$ mDC are detected in the spleen. *White arrows* indicate IL-10–transfected mDCs, (**d**) Quantification of IL-10–transfected GFP$^+$ mDC was performed by counting the fluorescent spots in the optic nerve, lymph node and spleen. The number of spots in five fixed areas (a fixed area is defined as a circle with a radius of 250 μm under ×20 magnification) was counted and presented as mean ± SD. The analysis was performed in at least five sections per organ ($P < 0.05$). (**e**) IL-10 mRNA expression levels in optic nerves of mice treated with IL-10–transfected mDC or mock-transfected mDC were analyzed by quantitative real-time PCR. Data are expressed as mean ± SD ($n = 5$), $P = 0.01511$ analyzed by Mann-Whitney *U* test, with similar results obtained in three independent experiments.

proportions of CD4$^+$ cells and CD4$^+$CD25$^+$Foxp3$^+$ cells in the spleens collected from mice on day 21 after immunization were studied by flow cytometry. The proportions of CD4$^+$CD25$^+$Foxp3$^+$ cells in the spleen and lymph nodes were significantly higher ($P < 0.05$) in the IL-10–transfected group compared to the mock-transfected group when the mDC were injected in the induction phase (Figs. 8a, 8b). Moreover, we examined spleen and lymph nodes isolated from IL-10–transfected mDC-treated mice and found significantly ($P < 0.05$) decreased numbers of CD4$^+$cells in the spleen and lymph nodes (Figs. 8a, 8b).

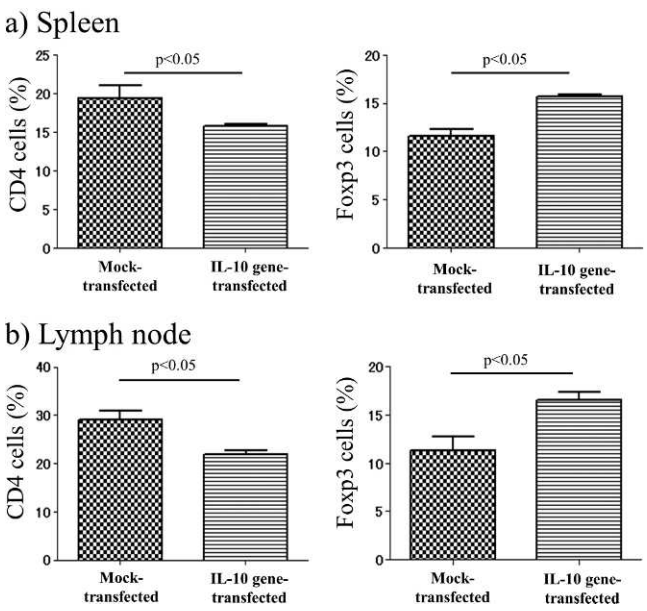

Figure 8. Populations of CD4 T-cells and $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen (**a**) and lymph node cells (**b**). C57BL/6 mice were immunized with MOG_{35-55} and injected with IL-10–transfected mDC or mock-transfected mDC in the induction phase. On day 21 after immunization, spleen cells and lymph node cells were collected and studied by flow cytometry. Data represent mean ± SD ($n = 5$). Mann-Whitney *U* test detected significant differences between IL-10–transfected and mock-transfected groups ($P < 0.05$), with similar results obtained in three independent experiments.

Discussion

Autoimmune diseases, such as MS, may result from failure of tolerance mechanisms to prevent the expansion of inflammatory autoreactive T-cells. $CD4^+$ Treg cells are considered to be the key mediator in the maintenance of immune tolerance and potent modulators of T-cell–mediated immune responses.[31]

Previous studies have shown the feasibility of using immature bone marrow–derived DC or mature bone marrow–derived DC to suppress the development of EAE[19,31–33] and EAU[34–36] in mice. These DC-based therapies focus on generating immature or mature DC with the ability to induce Th2 differentiation, and the approaches are based on the concept that "regulatory/tolerogenic" DC induce differentiation of naïve T lymphocytes into Th2 or Treg.[37]

Regulatory T-cells are critical for maintaining immune tolerance and preventing autoimmune reactions.[38] The suppression and/or dysregulation of Treg cells can induce autoimmune and inflammatory disorders, such as MS.[39–42] Many studies suggest that suppression of EAE is associated with an upregulation of IL-10 and $CD4^+CD25^+Foxp3^+$ Treg cells in the CNS and lymphoid organs.[43,44] Moreover, the ability of Treg cells to mediate spontaneous recovery from an active disease process in EAE and MS patients suggests the therapeutic potential of restoring Treg homeostasis.[45,46]

The role of IL-10 as suppressive mediators is well known, and IL-10 produced by Treg cells is essential for the prevention of autoimmunity in mouse models, and in IL-10–mediated tolerance.[47–49] Usually intravenous injection of matured and antigen-pulsed DC does not induce immune tolerance to EAE.[50] In our study, intravenous administration of IL-10–transfected mDC in the induction phase and effector phase ameliorated EAON. The finding that EAON also is suppressed by administration in the effector phase suggests that this DC therapy may be effective even when given after clinical onset of the disease. Various studies have examined cytokine production from pathogenic cells in experimental optic neuritis. Bettelli et al. reported that IL-17– and IFN-γ–producing $CD4^+$ T-cells modified the development of experimental optic neuritis.[51] IL-17 produced by Th17 cells is regarded to be a cytokine that is important for the development of autoimmune disease. Unlike Th2 cells, Th17 cells have the property of mediating allergic disease.

In our study, mock-transfected mDC injected in the induction phase did not protect the mice from suffering EAE and EAON, but slightly ameliorated the severity of EAE (Fig. 3b) and reduced delayed type hypersensitivity compared to positive control (Fig. 5b). These results suggested some potential effect of mock-transfected mDC in suppressing EAE although the effect is significantly less than the IL-10–transfected mDC. The IL-10–transfected mDC that we produced directly expressed a substantial level of IL-10. Our results clearly demonstrated that injection of IL-10–transfected mDC even in the induction phase suppressed cells that infiltrate the eye, and ameliorated EOAN/EAE. We also showed that Th1 cytokine production was reduced in the spleen and lymph node. IL-10 production was reduced in the spleen but increased significantly in lymph nodes in mice treated with IL-10–transfected mDC. We speculated that IL-10–transfected mDC may have had a role in immune suppression in an autocrine manner, a mechanism of action similar to negative-feedback or inhibition of the NFκB pathway.[52] As shown in Figure 7, IL-10–transfected mDC effectively migrated to regional lymph nodes and the optic nerve. While recruitment of IL-10–transfected mDC is low in the spleen on day 28 after MOG immunization, recruitment was significantly higher in the lymph nodes (Fig. 7e). Therefore, the level of IL-10 production may differ in the spleen and lymph nodes. Additionally, as shown in Figure 1b, IL-10 production increased significantly in IL-10–transfected mDCs compared to mock-transfected mDCs. However, MOG_{35-55} stimulation did not enhance IL-10 production by IL-10–transfected mDC. Thus, IL-10–transfected mDCs may not function in an antigen-specific manner. Moreover, as shown in Figures 7d and 7e, the numbers of IL-10–transfected GFP^+ mDC were significantly higher in the lymph node and optic nerve compared to the spleen, and IL-10 mRNA expression in the optic nerve of mice treated with IL-10–transfected mDC was markedly higher than that of mice injected with mock-transfected mDC. These findings indicated that IL-10–transfected mDC were recruited not only to primary and secondary lymphoid organs but also to local inflammatory sites, probably regulating inflammatory responses locally and systemically. Furthermore, as shown in Figure 8, the proportions of $CD4^+CD25^+Foxp3^+$ cells in lymph nodes were significantly higher in the IL-10–transfected group compared to the mock-transfected group. Therefore, it is possible that the Treg cells have a role in suppressing inflammatory responses in optic nerve lesion as well as in draining lymph nodes. The mechanisms by which Treg cells suppress inflammation and ameliorate EAE and EAON will be studied further.

Definitely, treatment with IL-10–transfected mDC suppressed Th1 cytokine productions in spleen and lymph nodes. However, the regulatory mechanism involved not only soluble factors, but also cell-to-cell interaction. Particularly, in our study, the B7 family (CD80, CD86) that consists of adhesion molecules was decreased by injection of IL-10–transfected mDC. This finding may support the cell-to-cell interaction theory as a mechanism of EAON suppression. Moreover, we showed that IL-10–transfected mDC reduced the severity of EAE and EAON associated with expansion of the Treg population in the spleen and lymph nodes, and reduced number of CD4 cells in these tissues. DC migrate to secondary

lymphoid tissues, such as the lymph nodes and spleen. This pathway is modulated subsequently during DC–T-cell interaction, and involves a combination of signals resulting from the interaction of costimulatory molecules as well as the production of cytokines. In addition, as shown in Figures 3d and 4d, the optic nerve of mice treated with IL-10–transfected mDC showed markedly reduced demyelination compared to control mice treated with mock-transfected DC. A recent study showed that neural stem cells engineered to express IL-10 had enhanced ability to induce immune suppression and remyelination in EAE.[53] Another study showed that implantation of DC into the site of spinal cord injury activated neural stem/progenitor cells and promoted functional recovery in marmoset.[54] Extrapolating this finding to our study, the inflammatory responses in our EAON model may have been suppressed systemically by the IL-10–transfected mDC that have migrated to the optic nerve. The results of the present in vivo and in vitro studies suggested that IL-10–transfected DC suppress EAON through mechanisms mediated by IL-10. Therefore, it is possible that IL-10–transfected mDC are effective in treating not only optic neuritis, but also other autoimmune diseases. DCs undergoing maturation can differentiate to become exceptionally good APCs with the capacity to stimulate naïve T-cells. In addition to activating T-cell immunity, stimulation of T-cells by mature, antigen-presenting DCs also is required for tolerance induction. Our finding suggested that injection in the IL-10–transfected mDC group in the induction phase also enhance generation of Treg cells, which may have an important role to protect the mice against the development of EAE. Moreover, DH, inflammatory cytokine, and CD4 T-cells were significantly decreased in the IL-10–transfected group compared to the mock-transfected group. Thus, regarding administration of IL-10–transfected mDC, this therapy may be effective in EAE, leading to demyelination, axonal damage, and neurologic deficits.

We recently reported that *CGRP*-transfected DC also have the potential to suppress EAE and EAON.[29] CGRP-induced suppression of antigen-presenting function is mediated, at least in part, by changes in cytokine expression that favor less robust antigen presentation for cell-mediated immunity, and CGRP induces the production of IL-10 which, in a paracrine and/or autocrine fashion, inhibits antigen presentation for TH1-type immunity. Therefore, *CGRP*-transfected mDCs are IL-10–specific. Our present findings suggested that injection of IL-10–transfected mDC also enhance generation of Treg cells, which may have an important role to protect the mice against the development of EAE and EAON. After injecting IL-10–transfected mDC, the DC migrated to the lymph node in the neck (Fig. 7), and the lymph nodes in the neck region of EAE/EAON model mice were swollen from inflammation. Therefore, IL-10–transfected mDC are organ-specific because IL-10–transfected mDC migrate to the optic neuritis lesion. The protective effect of IL-10–transfected mDC was of similar magnitude to that of *CGRP*-transfected mDC, although IL-10–transfected mDC had the potential of suppressing EAE and EAON to a greater extent in the induction phase.

In conclusion, our study demonstrated amelioration of ongoing EAON and EAE by intravenous injection of IL-10–transfected mDC and suggested that IL-10–transfected mDC down-regulate antigen-specific response to the immunized peptide through suppressing T-cell expansion and Th1 cytokine production. Further elucidation of the mechanisms using this experiment model may lead to novel treatments for intractable diseases, such as MS and neuromyelitis optica. Analysis of the pathogenesis of refractory optic neuritis has been accomplished through knowledge acquired from experimental animal models in the past several years. The results presented in our study may provide insight into a novel therapeutic strategy of targeted cell therapy for refractory optic neuritis in humans.

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