

Suppression of Murine Experimental Autoimmune Optic Neuritis by Mature Dendritic Cells Transfected with Calcitonin Gene-Related Peptide Gene

Ryusaku Matsuda,^{1,2} Takeshi Kezuka,¹ Chiharu Nishiyama,² Yoshibiko Usui,¹ Yoshimichi Matsunaga,¹ Yoko Okunuki,¹ Naoyuki Yamakawa,¹ Hideoki Ogawa,^{2,3} Ko Okumura,² and Hiroshi Goto¹

PURPOSE. Calcitonin gene-related peptide (CGRP) exhibits prominent anti-inflammatory actions. We examined whether CGRP-transfected dendritic cells (DC) prevent the development of experimental autoimmune optic neuritis (EAON) and experimental autoimmune encephalomyelitis (EAE).

METHODS. A human CGRP-expressing plasmid was constructed, and used to transfect C57BL/6 mouse bone marrow-derived matured DC (mDC) by electroporation methods. Transfection efficiency was 50% with 80% cell viability. C57BL/6 mice were immunized with myelo-oligodendrocyte glycoprotein 35-55, and injected intravenously with CGRP-expressing mDC (CGRP gene-transfected group) or mock-transfected mDC (mock-transfected group) at the induction or effector phase. EAE was diagnosed clinically and EAON was assessed histopathologically. Delayed hypersensitivity was measured. Supernatants of spleen cell cultures were assayed for cytokines using ELISA. The CD4⁺CD25⁺Foxp3⁺ fraction in spleen cells was analyzed using flow cytometry.

RESULTS. For gene therapy in the induction phase, EAE developed in 50% of mice in the CGRP-transfected group compared with 80% in the mock-transfected group, and the mean pathological score for EAON was 1 in the CGRP-transfected group compared with 2 in the mock-transfected group ($P < 0.05$). For gene therapy in the effector phase, the mean EAE clinical score (1.5 vs. 3.0) and mean EAON pathological score (1.0 vs. 2.0) were both lower in the CGRP-transfected group compared with the mock-transfected group ($P < 0.05$). Delayed hypersensitivity was suppressed significantly in the CGRP-transfected group. IL-10 production by spleen cells in the CGRP-transfected group increased independent of MOG concentration, compared with the mock-transfected group. Interestingly, the proportion of

CD4⁺CD25⁺Foxp3⁺ cells increased significantly ($P < 0.05$) in the CGRP-transfected group compared with the mock-transfected group.

CONCLUSIONS. Gene therapy with CGRP-expressing mDC was effective in suppressing the development of EAON and EAE. (*Invest Ophthalmol Vis Sci.* 2012;53:5475-5485) DOI: 10.1167/iovs.12-9935

In Asian countries including Japan, multiple sclerosis (MS) often causes optic neuritis and myelitis and this form of MS is called optic-spinal multiple sclerosis (OSMS). In Europe and America, OSMS is called Devic disease or neuromyelitis optica (NMO).¹ NMO is often severe and more resistant to steroid pulse therapy than non-MS optic neuritis. The development of an optimal animal model is necessary to analyze the pathological conditions of an important disease such as NMO, and to develop a new cure. Experimental autoimmune encephalomyelitis (EAE) is a well-known animal model for MS, and the antigens used for immunization are myelin basic protein (MBP), proteolipid protein (PLP), and myelo-oligodendrocyte glycoprotein (MOG).²⁻⁵

Among these antigens, PLP and MOG antigen have been reported to induce optic neuritis. In particular, mouse EAE induced by MOG antigen often manifests inflammation of the spinal cord, optic nerve, and optic chiasm, and resembles human disease.³ To date, the onset rate of murine optic neuritis induced by MOG peptide immunization is approximately 60%,⁵ whereas the rate is approximately 60% to 70% in transgenic mice carrying T-cell receptor (TcR) specific for MOG.^{6,7}

The eye is an immune privileged site, in which the induction of conventional immunological 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 (α -MSH), vasoactive intestinal peptide, and calcitonin gene-related peptide (CGRP). These peptides have been shown to play important roles as immunomodulatory factors.¹¹⁻¹⁴

CGRP, a 37-amino acid peptide, is a neuropeptide that is expressed in the central and peripheral nervous systems,¹⁵ and constitutively in the sensory neurons of the iris and ciliary body.¹⁶ Specific receptors for CGRP are localized in most tissues, including the central nervous system, heart, liver, and spleen.¹⁷ A wide range of CGRP effects have been demonstrated in *in vitro* and *in vivo* experiments,¹⁸⁻²⁹ showing that CGRP influences key processes of the immune response and exerts an anti-inflammatory action. The main effect of both *in vitro* and *in vivo* CGRP administration is to dampen the immune response essentially by modifying antigen presentation in a variety of antigen-presenting cells (APCs), such as

From the ¹Department of Ophthalmology, Tokyo Medical University, Shinjuku-ku, Tokyo, Japan; the ²Atopy Research Center; and the ³Department of Immunology, Juntendo University School of Medicine, Bunkyo-ku, Tokyo, Japan.

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Corresponding author: Takeshi Kezuka, Department of Ophthalmology, Tokyo Medical University, 6-7-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan; tkezuka@tokyo-med.ac.jp.

dendritic cells (DCs), monocytes, macrophages, and Langerhans cells.^{23,24,26,30,31} Intracutaneously injected CGRP has been reported to impair the induction of contact hypersensitivity after acute, low-dose UV-B radiation due to immunological tolerance in normal and mast cell-deficient mice.³² We previously have shown that peritoneal exudate cells (PECs) cultured with CGRP suppress murine EAU in an antigen-specific manner, even in the efferent phase, and that IL-10 secreted from PEC plays an important role in CGRP-mediated suppression of murine EAU.³³ DCs are unique professional APCs capable of stimulating naïve T cells in primary immune response, and are more potent than monocyte/macrophages or B cells in the immune system.^{34,35} Normal mature DCs (mDCs) are potent APCs that enhance T-cell activation, whereas normal immature DCs (iDCs) are engaged in the induction of peripheral T-cell tolerance under steady-state conditions.^{34,36,37} However, clinical treatment with normal iDCs may not be appropriate for inflammatory diseases.³⁷ Therefore, further development of DCs 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 DCs to downregulate T-cell response by pharmacological methods or gene therapy, with the objective to generate regulatory DCs for the treatment of T-cell-mediated autoimmune disorders.³⁸ The latest DC-based approaches to treat autoimmune disorders focus on interfering with the ability of DCs to activate naïve T cells. Once T cells become activated, most conventional DC-based therapies are unable to inhibit the functions of the effector T cells. In human uveitis and other autoimmune disorders, the signs and symptoms that lead to diagnosis usually manifest after the onset of disease, when self-reactive T cells are already fully activated. Therefore, the potent regulatory effects of CGRP make this endogenous neuropeptide potentially useful in gene therapy using engineering DCs for the treatment of uveoretinitis.

In the current study, we hypothesized that the antigen-processing capacity of DCs may represent an appropriate endogenous system for delivery of therapeutic transgene proteins for the treatment of autoimmune diseases. Our results indicated that by injection of CGRP-expressing DCs, development of EAE and EAON and delayed hypersensitivity response were suppressed. These results suggest that *CGRP* gene transfer into DCs may be an effective therapy for human optic neuritis.

MATERIALS AND METHODS

Animals and Anesthesia

Six- to 8-week-old female C57BL/6 mice were obtained from Japan CLEA Inc. (Shizuoka, Japan). The mice were kept in an animal facility at the Tokyo Medical University in conformity with the administrative provision of Tokyo Medical University on animal management. All animals were treated according to the ARVO resolution on the use of animals in research. Intraperitoneal (IP) injection of a mixture of nembutal (30 mg/kg) and xylazine hydrochloride (125 mg/kg) was used for anesthesia.

Reagents

MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by conventional solid-phase techniques, as described elsewhere.⁵ Purified *Bordetella pertussis* toxin (PTX) was from Sigma Chemical (St. Louis, MO). Complete Freund's adjuvant (CFA) and *Mycobacterium tuberculosis* strain H37Ra were from Difco (Detroit, MI). Rat CGRP was purchased from Sigma Chemical. Murine granulocyte-macrophage

colony-stimulating factor (GM-CSF) was purchased from Pepro Tech (London, UK). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (Tokyo, Japan).

Generation of DCs

Murine DCs were generated according to a previous report.³⁹ Briefly, bone marrow cells from C57BL/6 mice were cultured with murine GM-CSF (20 ng/mL) in a bacteriologic Petri dish (BIO-BIK, Tokyo, Japan) for 6 days. Nonadherent cells were collected and subjected to negative selection with mAbs to Ly-76, CD2, B220, CD14, and Ly-6G (all from BD Pharmingen, San Jose, CA) 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 2 days. The stimulated DCs were washed and cultured with 10 µg/mL of MOG₃₅₋₅₅ in serum-free medium at 37°C in an atmosphere of 5% CO₂. After overnight culture, nonadherent cells were washed three times with culture medium to remove the antigen. These DC preparations were typically more than 95% pure as indicated by anti-I-A/I-E mAb and CD11c⁺ staining, and contained fewer than 0.1% erythrocytes, T cells, B cells, F4/80⁺ macrophages, NK cells, and neutrophils as assessed by fluorescence-activated cell sorting (FACS) analysis.

Construction of hCGRP-expressing Plasmid and Transfection in DCs

The plasmid for expressing human CGRP tagged with 2× Flag at the N-terminus was constructed as follows. The coding region of CGRP cDNA was amplified by PCR using cDNA synthesized from total RNA of ARPE-19 (human retinal pigment epithelial) cell line. Briefly, PCR was performed using primers 5'-actctagtcgccagagaggcggcatggg-3' (forward) and 5'-cctctctcctggagtcattcatctgctcag-3' (reverse), which correspond to just upstream of the translational initiation site (underlined portion of forward primer) and downstream of the translational stop codon (underlined portion of reverse primer) of human CGRP (accession number NM_000728).

The PCR product was cloned in pCR2.1 plasmid using a TOPO TA cloning kit (Invitrogen, Tokyo, Japan). An *EcoRI* site was introduced using a QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA) with the oligonucleotide 5'-ctagtcgccagagagaattcatgggtttccggaa-3' (introduced *EcoRI* site in italic and initiation codon underlined) and its complementary oligonucleotide. After confirming the nucleotide sequence, the CGRP cDNA fragment was prepared by *EcoRI* digestion and inserted into the *EcoRI* site of pCR3.1-2FL plasmid⁴⁰ using a Rapid DNA Ligation Kit (Roche, Mannheim, Germany). The resulting plasmid, pCR3.1-2FL-hCGRP or control vector, pCR3.1-2FL (mock), was transfected into mouse bone marrow-derived DCs by electroporation using Nucleofector II (Lonza Japan, Tokyo, Japan) according to manufacturer's instructions. Mature DCs transfected with pCR3.1-2FL-hCGRP or pCR3.1-2FL (mock control) were cultured with 10 µg/mL MOG₃₅₋₅₅ overnight before use.

To assess transfection efficiency, 2.5×10^5 cells were nucleofected with 2 µg pmaxGFP, and the cells were analyzed 24 hours post-nucleofection by flow cytometry. Cell viability was analyzed by propidium iodide (PI) staining (Sigma-Aldrich). Viability data are given in percent compared with PI-negative cells of the untreated mDC (negative control), mock-transfected mDC, or CGRP-transfected mDC.

EAE and EAON Induction and Histopathological and Clinical Evaluations

EAE and EAON were induced by the method described by Shao et al⁵ with modifications. The MOG₃₅₋₅₅ peptide was diluted in PBS (pH 7.35) to a concentration of 200 µg/50 µL/mouse. In another preparation, dimethyl sulfoxide (DMSO) was added to the MOG₃₅₋₅₅ peptide at 10 mg/mL, and then diluted in PBS to the same peptide content as above. The MOG₃₅₋₅₅ peptide was emulsified at a ratio of

TABLE. Disease Rate and Severity of EAE and EAON in Mice Immunized with the MOG Peptide, which Added DMSO

Disease	Disease Rate	Severity of Disease*
EAE	7/10	1.1 ± 0.9
EAON	9/10	1.7 ± 0.9

* The disease severity of EAE is expressed in clinical score, and the disease severity of EAON is expressed in histopathological score.

1:1 in CFA containing 600 µg/mL of *M. tuberculosis* H37 RA, and used to immunize C57BL/6 mice subcutaneously at the neck region. The mice were also injected IP with PTX (1 µg/100 µL/mouse) at the same time.

After the C57BL/6 mice were immunized with MOG35-55 to produce EAON and EAE, they were injected intravenously with mDCs transfected with pCR3.1-2FL-hCGRP (CGRP-transfected group) or pCR3.1-2FL (mock-transfected group) on day 0 for induction phase study or on day 9 for effector phase study.

A diagnosis of EAE was based on clinical signs. The clinical scores were as follows: 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, mice were killed at 10, 12, 13, and 14 days after immunization. The eyeballs were extracted and fixed in 4% paraformaldehyde followed by 10% formalin. Fixed and dehydrated tissue was embedded in methacrylate and 5-µm sections were cut through the pupillary-optic nerve plane and stained with hematoxylin and eosin. The pathological scores of the eye lesions were as follows: 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. In addition, Luxor fast blue (Fisher Scientific, Pittsburgh, PA) staining was performed to stain the myelin sheath of the optic nerve.

Measurement of Cytokine Production by Spleen Cells

Some mice were killed on day 30 after MOG immunization, and splenic lymphocytes were dissociated into single-cell suspensions by lysing erythrocytes with Tris-NH₄Cl. Spleen cells were cultured in flat-bottom 96-well plates at a density of 2×10^5 cells/200 µL in RPMI1640 medium supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 pg/mL streptomycin, 10% fetal calf serum (Biological Industries, Haemek, Israel), and 5×10^{-4} M 2-mercapto-ethanol. Spleen cells (2×10^6 cells/2 mL) were cultured with 10 µg MOG at 37°C in 6%

CO₂ in air. Supernatants were collected after 24 hours of culture and assayed for IL-10, IL-2, IL-6, TNF-α, and IFN-γ using the BD Cytometric Bead Array Flex Set (BD Biosciences, San Jose, CA), and IL-17 using Human IL-17A ELISA Ready-SET-Go! Kit (eBioscience, San Diego, CA) according to the manufacturers' instructions.

Measurement of Delayed-Type Hypersensitivity

We analyzed delayed hypersensitivity as an in vivo measure of the relative abilities of MOG-induced EAE and EAON mice to mount a Th1-dependent antigen recall response. On day 13 after immunization, mice were injected intradermally with 20 µg/10 µL of MOG35-55 suspended in PBS 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 follows: specific ear swelling = (24-hour measurement - 0-hour measurement) for test ear - (24-hour measurement - 0-hour measurement) for control ear, as described previously.³³

Measurement of In Vitro CGRP and IL-10 Production by ELISA

IL-10 concentrations in mDC culture supernatants were determined by ELISA using the Quantikine Mouse IL-10 Immunoassay (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. CGRP concentrations in mDC culture supernatants also were determined by ELISA using the human CGRP enzyme immunoassay kit (SPI-BIO, Montigny-le-Bretonneux, France). Briefly, the CGRP-expressing plasmid or control vector (mock) was transfected into mouse mDCs by electroporation. Then, cells were cultured in flat-bottom 24-well plates at a density of 2.5×10^5 cells/2 mL in RPMI1640 medium at 37°C in 6% CO₂/94% air. Supernatants were collected after 24-hour incubation and used for IL-10 and CGRP assay.

Quantification of mRNA by Real-Time PCR

Total RNA was prepared from CGRP-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 CGRP was quantified with the StepOne Real-Time PCR system (Applied Biosystems) using TaqMan Gene Expression Assay for CGRP

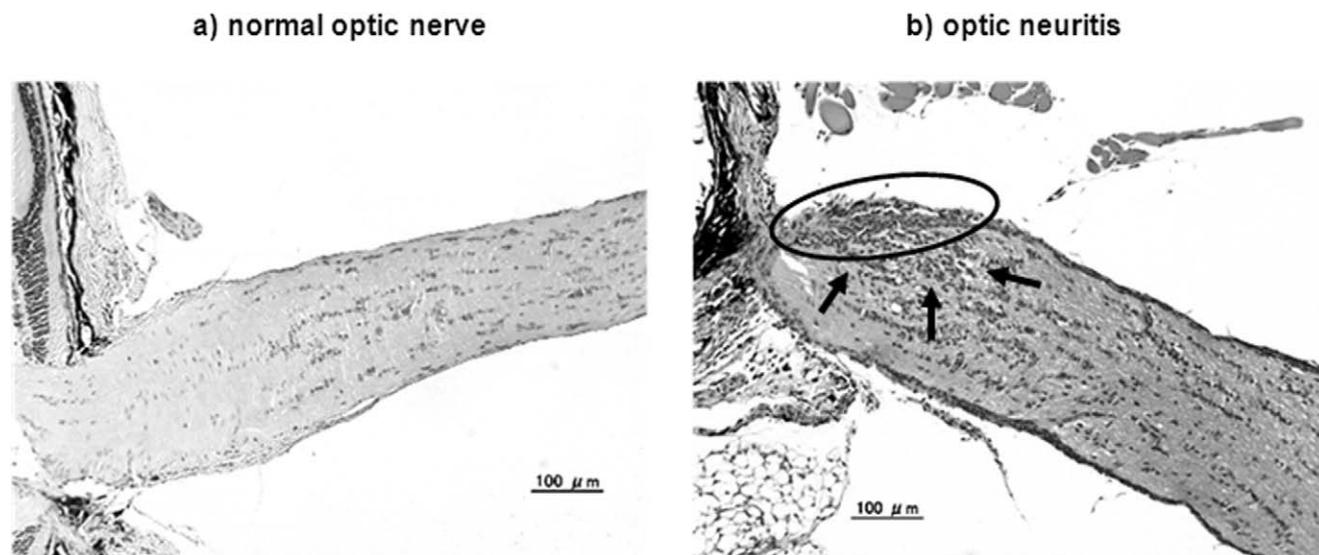


FIGURE 1. Histopathology of a normal optic nerve of control mouse (a) and histopathology of murine optic neuritis (b). In (b), the form of the optic nerve sheath has collapsed (circle), and cell infiltration is severe (arrows).

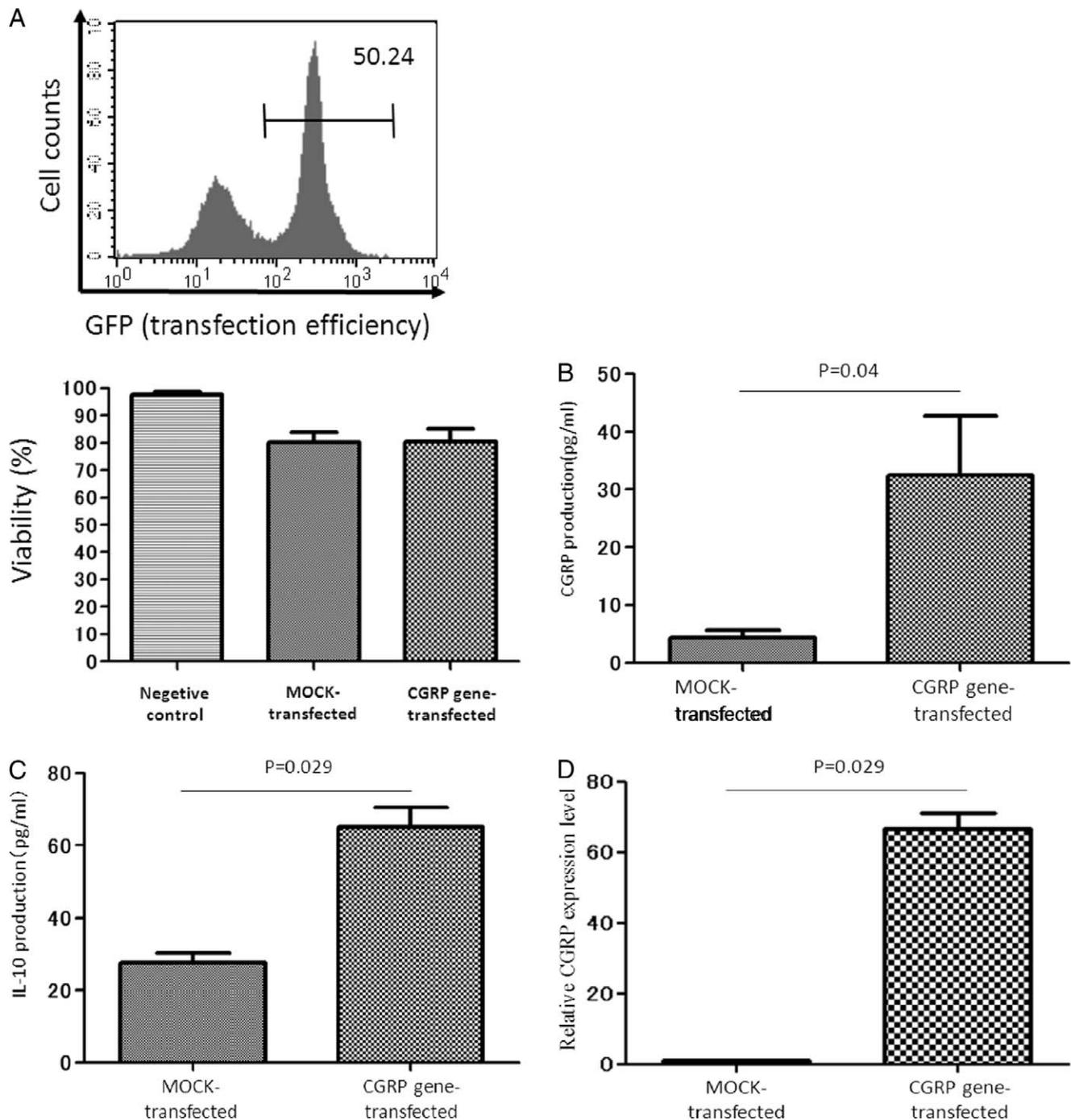


FIGURE 2. Results of transfection: transfection efficiency and cell viability of transfected mDC (A), CGRP (B), and IL-10 (C) production by *CGRP*-transfected DCs measured by ELISA, and mRNA expression of CGRP by *CGRP*-transfected DCs (D). (A) Gene transfer using mDC in suspension yielded 80% cell viability and 50% transfection efficiency. (B) Mean CGRP production was 32 pg/mL in *CGRP*-transfected DCs and 4 pg/mL (detection limit) in mock-transfected DCs. Mann-Whitney *U* test detected a significant difference between the two groups. (C) Mean IL-10 production was 80 pg/mL in *CGRP*-transfected DCs, and 25 pg/mL in mock-transfected DCs. Mann-Whitney *U* test detected a significant difference between the two groups. In (B) and (C), the data are expressed as mean \pm SD of at least five mice in each group, with similar results obtained in two independent experiments. (D) CGRP mRNA expression levels of *CGRP*-transfected mDCs and mock-transfected mDCs were analyzed by quantitative real-time PCR. Data are expressed as mean \pm SD ($n=5$). Mann-Whitney *U* test detected a significant difference between the two groups ($P=0.029$).

(Hs01100741) and TaqMan rodent GAPDH control reagents, together with TaqMan Universal Master Mix (all from Applied Biosystems).

Flow Cytometric Analysis

Single-cell suspension was prepared from the spleen by passing the tissue through a wire mesh. Cells were collected, washed, and

incubated with Fc block for 15 minutes at 4°C in FACS buffer (PBS supplemented with 1% BSA). For the analysis of cell surface marker expression, anti-CD4-fluorescein isothiocyanate (FITC; eBiosciences, San Diego, CA), anti-CD25-APC (clone PC61; eBiosciences) and anti-Foxp3-PE and isotype control (rat IgG2a-PE; eBiosciences) were used according to manufacturer's instructions. Cells were analyzed using a

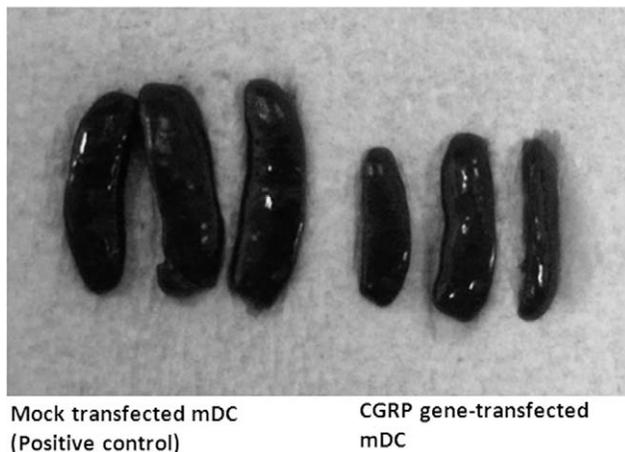


FIGURE 3. Spleens obtained in the induction phase. Mice were immunized with MOG and given one intravenous injection of 1×10^5 /mouse of cells for treatment. The spleens from mice injected with *CGRP*-transfected mDCs were smaller than the spleens from mice injected mock-transfected mDCs (positive control). The mean volume was 112.3 mg in the mock-transfected group versus 59.06 mg in the *CGRP*-transfected group, whereas the length was 15.6 vs. 8.7 mm.

FACS Calibur cytometer and the CellQuest Pro software (BD Biosciences).

Statistical Analysis

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

RESULTS

Induction of EAE and EAON

To examine disease rate and severity of EAE and EAON, C57BL/6 mice were immunized subcutaneously with MOG peptide with or without DMSO. Without DMSO, the time to disease onset was 14.5 ± 1.5 days for EAE, and 13.0 ± 1.5 days for EAON. With DMSO, the time to disease onset was 13.0 ± 2.0 days for EAE, and 12.5 ± 1.5 days for EAON. To obtain greater disease rate and severity of EAE and EAON, MOG peptide with added DMSO was used to immunize mice in subsequent experiments. EAE developed in seven (70%) of ten mice, and the disease severity score was 1.1 ± 0.9 . EAON developed in nine (90%) of ten mice, and the disease severity score was 1.7 ± 0.9 (the Table).

Figure 1a shows the histology of the normal optic nerve of a control mouse. Figure 1b shows the histology of a mouse with optic neuritis. The form of the optic nerve sheath collapsed, and cell infiltration was severe.

Properties of *CGRP*-Transfected Mature DCs

Gene transfer using mDCs in suspension yielded 80% cell viability and 50% transfection efficiency (Fig. 2A).

When we measured *in vitro* *CGRP* production from the *CGRP*-transfected mDCs using ELISA (Fig. 2A), the concentrations in culture supernatants ranged from 30 to 60 pg/mL (average; 32 pg/mL). On the other hand, *CGRP* production was 4 pg/mL, which was the detection limit of the assay, in mock-transfected mDCs ($P = 0.04$, versus *CGRP*-transfected mDCs).

In vitro IL-10 production increased significantly ($P = 0.029$) in *CGRP*-transfected mDCs compared with mock-transfected

cells (Fig. 2B). These findings indicate that IL-10 production is upregulated in *CGRP*-transfected mDC.

The *CGRP* mRNA expression was also markedly and significantly upregulated in *CGRP*-transfected mDCs compared with the mock-transfected cells (Fig. 2D).

Cytokine Production by Spleen Cells Derived from MOG-Immunized Mice

C57BL/6 mice were immunized subcutaneously with MOG peptide and then injected intravenously with 1×10^5 /mouse *CGRP*-transfected or mock-transfected mDCs once on day 1 (induction phase). Comparing the spleen size obtained on day 14 after immunization (Fig. 3), the spleens from mice injected with *CGRP*-transfected mDCs were smaller than those from mice injected with mock-transfected mDCs. The mean volume was 112.3 mg in mock-transfected group versus 59.06 mg in *CGRP*-transfected group, while the length was 15.6 vs. 8.7 mm. These findings suggest suppressed systemic inflammation in *CGRP*-treated mice.

We evaluated cytokine production from spleen cells collected from these mice on day 14 after immunization. The *in vitro* production of interleukins, TNF- α , and IFN- γ in response to MOG stimulation was measured using a CBA kit. In the *CGRP*-transfected group, IL-10 production increased compared with the mock-transfected group, independent of MOG concentration. On the other hand, IL-17, TNF- α , IFN- γ , IL-6, and IL-2 productions were reduced (Fig. 4). These changes resemble Th2 response.

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 intravenously with 1×10^5 /mouse *CGRP*-transfected or mock-transfected mDCs once on day 1 (induction phase). Clinical parameters were observed until day 14 after immunization.

Onset of EAE was observed from 11 to 13 days after immunization in the mock-transfected control group. Eighty percent of the control mice developed EAE. On the other hand, the rate of EAE was markedly lower in the *CGRP*-transfected group (50%). The average clinical score on day 14 after immunization was 3.0 in the control group, and 1.5 in the *CGRP*-transfected group ($P < 0.05$; Fig. 5a).

All the control mice developed EAON. On the other hand, the rate of EAON was lower in the *CGRP*-transfected group (71%). The average pathological score on day 14 after immunization was 2 in the control group, and 1 in the *CGRP*-transfected group ($P < 0.05$; Fig. 5b). Histologically, inflammatory cell infiltration in the optic nerve was evident in the mock-transfected group, whereas milder cell infiltration in the optic nerve was observed in the *CGRP*-transfected group (Fig. 5c).

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 intravenously with 1×10^5 /mouse *CGRP*-transfected or mock-transfected mDCs once on day 9 (effector phase). Clinical parameters were observed until day 14 after immunization.

All the mice in both groups developed EAE; however, the average clinical score on day 14 after immunization was 3.0 in the mock-transfected control group, and 1.5 in the *CGRP*-transfected group ($P < 0.05$; Fig. 6a). This finding indicates that *CGRP*-transfected mDCs ameliorate EAE when injected in the effector phase.

The severity of EAON appeared to be suppressed by injection of mDCs transfected with *CGRP* compared with

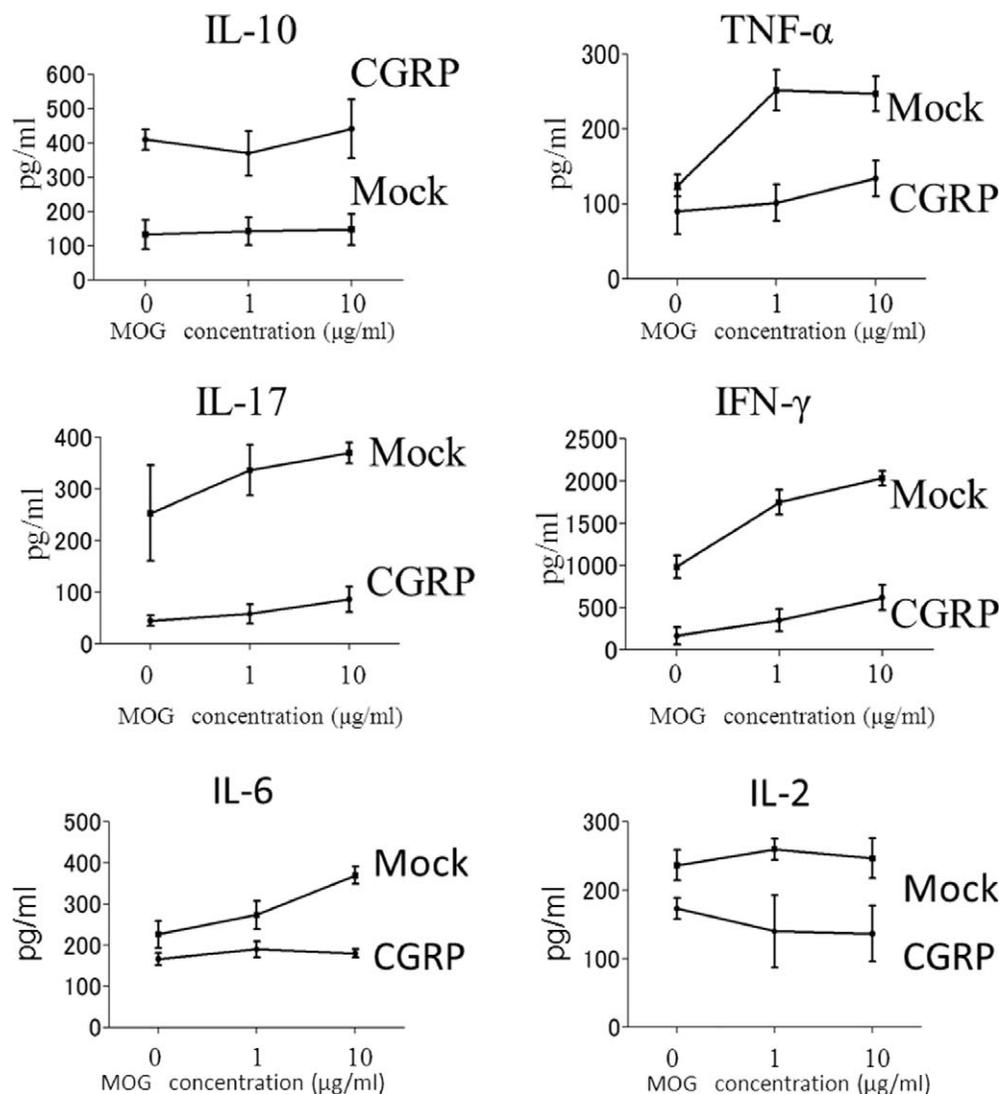


FIGURE 4. Cytokine productions from spleen cells collected from mice injected with *CGRP*-transfected cells in the induction phase. On day 14, spleen cells were collected from MOG-immunized mice injected with *CGRP*-transfected cells. Supernatants of cultured spleen cells were collected after 24-hour culture and assayed for IL-10, IL-2, IL-6, IL-17, TNF- α , and IFN- γ using a CBA kit. Data are expressed as mean and SE. Mann-Whitney *U* test detected significant differences in IL-10, IL-2, IL-6, IL-17, TNF- α , and IFN- γ between the two groups at a MOG concentration of 10 μ g/mL.

mock-transfected mDCs. EAE developed in 89% of the mice in the control group, and was reduced to 67% in the *CGRP*-transfected group (Fig. 6b). Histologically, infiltration of inflammatory cells in the optic nerve was observed in the mock-transfected group (Fig. 6c), while the infiltration was markedly milder in the *CGRP*-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 both induction phase and effector phase studies. In the induction study, delayed hypersensitivity was suppressed significantly ($P < 0.05$) in the group injected with *CGRP*-transfected mDCs compared with the mock-transfected control group (Fig. 7a). In the effector phase study, delayed-type hypersensitivity was also suppressed significantly ($P <$

0.05) by injection of mDCs transfected with *CGRP* compared with mock-transfected mDCs (Fig. 7b).

Measurement $CD4^+ CD25^+ Foxp3^+$ by Flow Cytometric Analysis

The proportions of $CD4^+ CD25^+ Foxp3^+$ cells in the spleens collected from mice on day 21 after immunization were studied by flow cytometry. The proportion of $CD4^+ CD25^+ Foxp3^+$ in the spleen was significantly higher ($P < 0.05$) in *CGRP*-transfected group compared with the mock-transfected group when DCs were injected in the induction phase (Fig. 8).

DISCUSSION

Previous studies have shown the feasibility of using immature bone marrow-derived DCs or mature bone marrow-derived DCs to suppress the development of EAE^{41,42} and EAU⁴³⁻⁴⁵ in

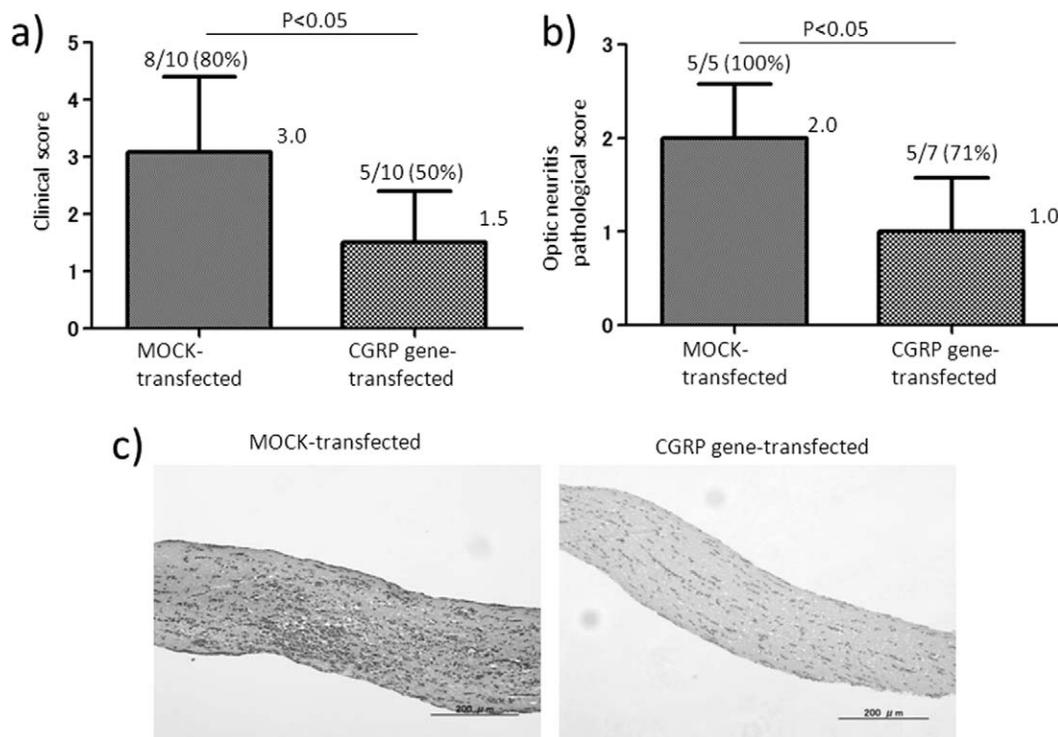


FIGURE 5. Incidence and severity of EAE (a) and EAON (b), and histopathology of EAON on day 14 (c) in the induction-phase study. C57BL/6 mice were immunized subcutaneously in the neck region with the MOG35-55 peptide. After immunization, the mice were injected intravenously with mDCs transfected with pCR3.1-2FL-hCGRP (*CGRP*-transfected group) or pCR3.1-2FL (mock-transfected group) on day 0. Mice were killed 14 days after immunization. (a) A diagnosis of EAE was based on clinical signs at day 14 after immunization. Mann-Whitney *U* test detected a significant difference between *CGRP*-transfected and mock-transfected groups. (b) A diagnosis of EAON was based on pathological score at day 14 after immunization. Mann-Whitney *U* test detected a significant difference between *CGRP*-transfected and mock-transfected groups. (c) In the mock-transfected group, inflammatory cell infiltration was apparent in the optic nerve. Optic nerves in the *CGRP*-transfected group showed less cell infiltration compared with the positive control group. The data are expressed as mean \pm SD of at least five mice in each group, with similar results obtained in three independent experiments.

mice. These DC-based therapies focused on generating immature DCs or mature DCs with the ability to induce Th2 differentiation. These therapeutic approaches are based on the concept that the “regulatory/tolerogenic” DCs induce differentiation of naïve T lymphocytes into Th2 or regulatory T cells.⁴⁶ The dual capabilities of CGRP to reduce Th1 response make the CGRP gene an ideal transgene to downmodulate T-cell response for treating human optic neuritis. When we synthesized human CGRP genes for insertion into plasmids, we used the human retinal pigment epithelial cell line ARPE19, as these cells carry the greatest amount of CGRP genes (S Sugita, personal oral communication in 2009).

A large amount of CGRP exists intra-ocularly, especially in the anterior chamber, and this compound is important for the maintenance of homeostasis in the eye. In the anterior chamber, TGF- β ,^{47,48} vasoactive intestinal peptide (VIP),⁴¹ and α -MSH⁴⁹ exist together with CGRP, and they also have immune regulatory function and suppress autoimmune diseases represented by uveoretinitis. In this study, it is not clear whether *CGRP*-transfected cells directly suppress cells that infiltrate the eye, or indirectly ameliorate cell infiltration in regional lymph nodes. From the observation that *CGRP*-transfected cells effectively inhibit EOAN even in the induction phase, these cells most likely act in regional lymph nodes. Further detailed studies using mDC isolated from green fluorescent protein-expressing mice are required in the future.

The results of the present *in vivo* and *in vitro* studies suggest that *CGRP*-transfected cells suppress EAON through mechanisms mediated by IL-10; however, the regulatory mechanism may involve not only soluble factors but also cell-

to-cell interaction. Particularly, the B7 family which is one of the adhesion molecules, is reported to be downregulated by CGRP stimulation,²⁴ and this evidence may support the cell-to-cell interaction theory as a mechanism of EAON suppression. In addition, as shown in Figure 3, the spleens of *CGRP*-transfected mice were markedly smaller than those of positive control mice, and maintained the size of normal spleens. This observation may be evidence that the inflammatory responses seen in positive controls are suppressed systemically in *CGRP*-transfected mice.

CGRP is able to modulate Langerhans cell function because a previous study showed that intracellular cAMP was significantly increased in cultured Langerhans cells after exposure to CGRP, and this increase was inhibited by a specific inhibitor of the CGRP receptor, demonstrating the existence of CGRP receptors on Langerhans cells.⁵⁰ We speculate that CGRP may modulate mature DC function in a similar manner. At day 14 after immunization, we found that *CGRP*-transfected mature DCs migrated to the optic nerve and lymph nodes (data not shown). Therefore, it is possible that *CGRP*-transfected mature DCs prevent cell infiltrations by acting both at the optic nerve and lymph node. Further studies are required to investigate whether CGRP directly prevents optic nerve fiber damage. CGRP appears to regulate immune functions, including macrophage activation and antigen-presenting capability, and T-cell proliferation.⁵¹ In a study that examined whether CGRP produces functional effects through regulating cytokines, such as IL-10, IL-1 β , and IL-12, in antigen-presenting Langerhans-like cells, CGRP augmented the LPS and GM-CSF-induced IL-10 expression but suppressed the LPS and

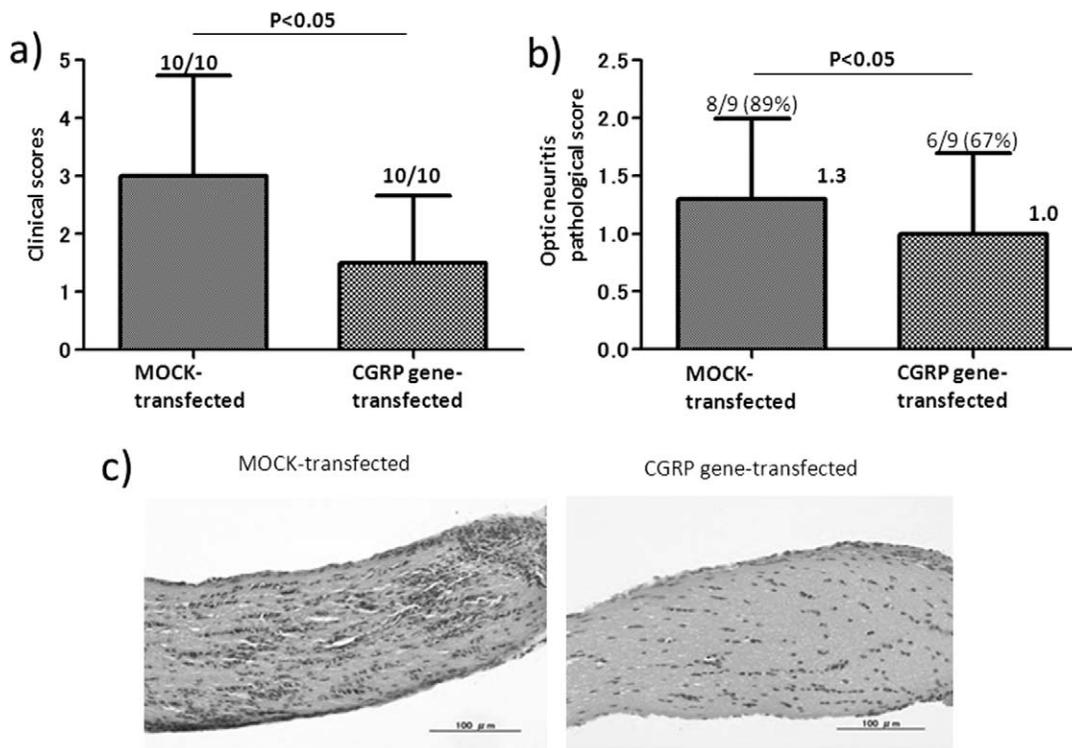


FIGURE 6. Incidence and severity of EAE (a) and EAON (b), and histopathology of EAON (c) in the effector phase study. After the C57BL/6 mice were immunized with MOG35-55, they were injected intravenously with mDCs transfected with CGPR plasmid (*CGRP*-transfected group) or vehicle plasmid (mock-transfected group) on day 9. Mice were killed 14 days after immunization. (a) A diagnosis of EAE was based on clinical signs at day 14 after immunization. Mann-Whitney *U* test detected a significant difference between *CGRP*-transfected and mock-transfected groups. (b) A diagnosis of EAON was based on pathological score at day 14 after immunization. Mann-Whitney *U* test detected a significant difference between *CGRP*-transfected and mock-transfected groups. (c) Inflammatory cells infiltrated the optic nerve in the mock-transfected group. There was less cell infiltration in the optic nerve in the *CGRP*-transfected group compared with the positive control group. The data are expressed as mean ± SD of at least five mice in each group, with similar results obtained in three independent experiments.

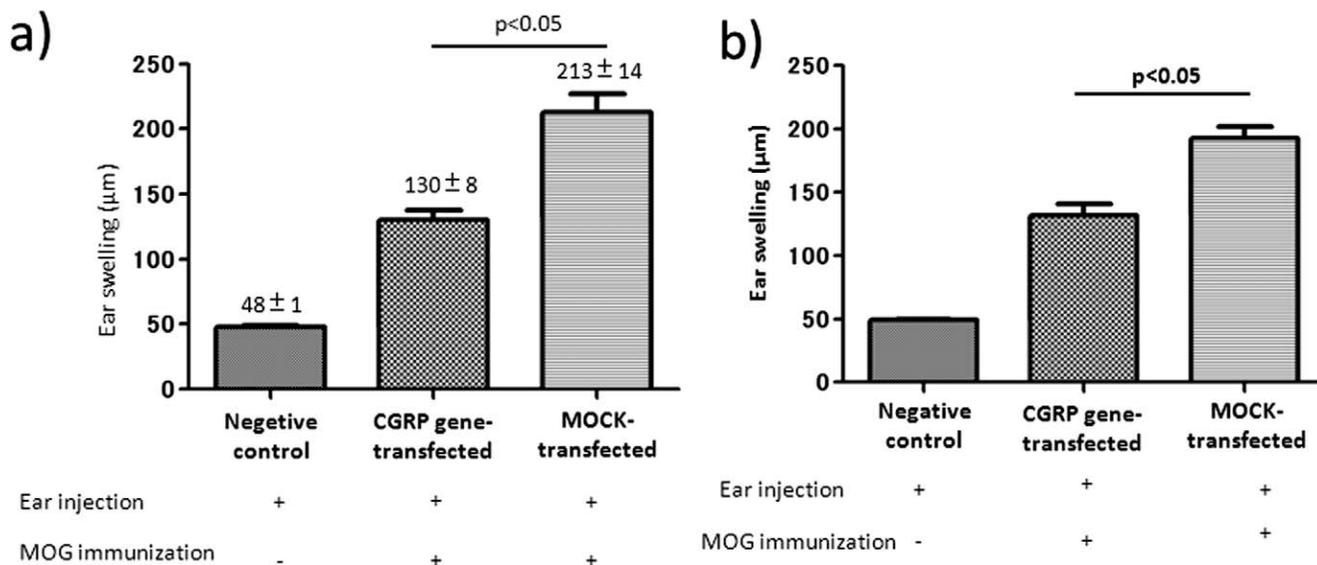


FIGURE 7. Delayed hypersensitivity response in the induction-phase study (a) and effector-phase study (b). In both induction-phase and effector-phase studies, mice were injected intradermally with MOG35-55 suspended in PBS into the pinna of one ear on day 13 after immunization. Ear swelling was measured 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) for test ear - (24-hour measurement - 0-hour measurement) for control ear. ANOVA and Scheffé test detected a significant difference between *CGRP*-transfected and mock-transfected groups in both studies.

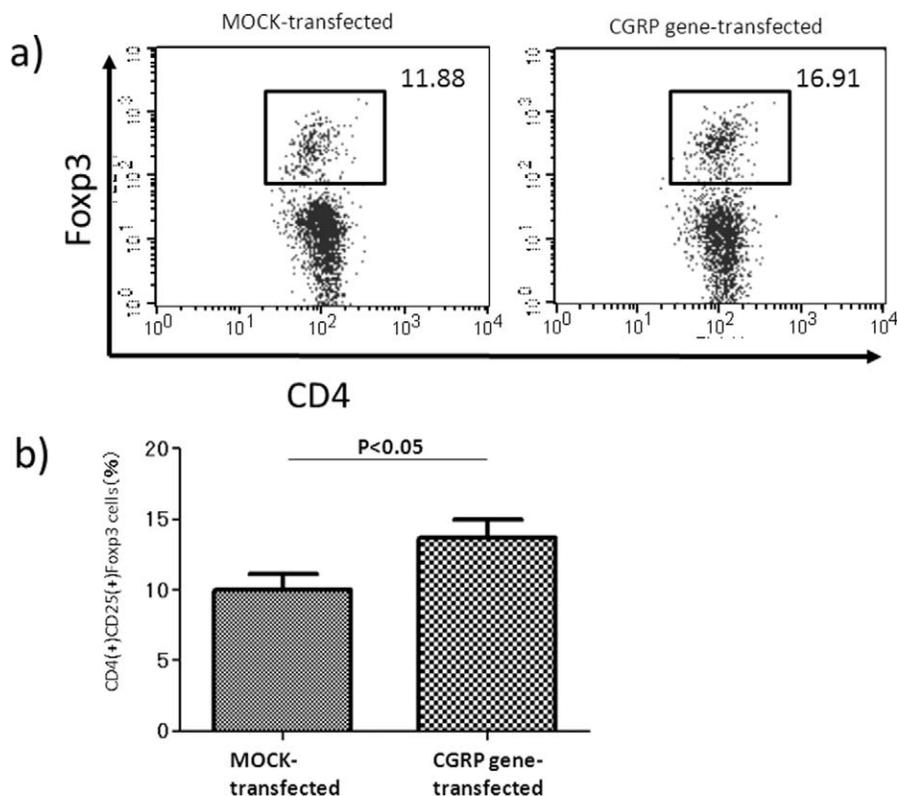


FIGURE 8. Expansion of CD4⁺CD25⁺Foxp3⁺ Treg cell population in the spleen cells injected with *CGRP*-transfected DCs in the induction phase. (a) Flow-cytometric characterization of the CD4⁺CD25⁺Foxp3⁺ Treg cells in the spleen at the onset of EAE/EAON. (b) Proportions of CD4⁺CD25⁺Foxp3⁺ Treg cells in the spleen. On day 21 after immunization, spleen cells were collected from MOG-immunized mice injected with *CGRP*-transfected DCs or mock-transfected DCs. Data represent mean \pm SD ($n = 5$). Mann-Whitney U test detected a significant difference between *CGRP*-transfected and mock-transfected groups.

GM-CSF-induced IL-1 and IL-12 expression. Furthermore, neutralization of IL-10 activity largely abrogated the suppressive effects of CGRP on B7-2 upregulation. These data suggest that 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 that CGRP induces production of IL-10, which, in a paracrine and/or autocrine fashion, inhibits antigen presentation for TH1-type immunity.³¹ Moreover, CGRP has been shown to suppress murine EAU in an antigen-specific manner, even in the efferent phase, and IL-10 secreted from PEC plays an important role in the CGRP-mediated suppression of murine EAU.³³ Extrapolating these findings to the present study, it is possible that *CGRP*-transfected mDCs might suppress murine EAE/EAON by augmenting IL-10 secretion, which inhibits the production of other cytokines, such as IL-17, TNF- α , and IFN- γ .

Therefore, it is possible that the *CGRP*-transfected cells are effective in treating not only optic neuritis, but also other autoimmune diseases. On the other hand, since a shift to Th2 response occurs after administration of *CGRP*-transfected cells, this therapy may not be effective in Th2-mediated diseases, such as asthma.

CD4⁺ CD25⁺ regulatory T (Treg) cells have received a great deal of attention as negative regulatory of MS pathogenesis. Treg cells regulate peripheral tolerance and autoimmunity, and abnormalities in Treg cell function may contribute to the development of autoimmune diseases. Thus, expansion of the Treg cell population could prevent autoimmune attacks, including EAE. Interestingly, we found that the proportion of CD4⁺CD25⁺Foxp3⁺ in the spleen was significantly increased in

CGRP-transfected group compared with the mock-transfected group. Our finding thus suggest that injection of *CGRP*-transfected DCs in the induction phase also enhance generation of Treg cells that may play an important role to protect the mice against the development of EAE and EAON.

In the present study, gene transfer into mDCs yielded 80% cell viability and 50% transfection efficiency. When transfection efficiency is low, fewer regulatory mDCs will be generated and suppression of inflammation may be less effective compared with higher transfection efficiency. Therefore, transfection efficiency is an important element in developing treatments using regulatory cells.

Usually intravenous injection of matured and antigen-pulsed DCs does not induce immune tolerance to EAE.⁵² In the present study, intravenous administration of *CGRP*-transfected cells both in the induction phase and effector phase ameliorated EAON. The finding that EAON is also suppressed by administration in the effector phase suggests that this cell 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.⁷ reported that IL-17 and IFN- γ -producing CD4-positive T cells affect the development of experimental optic neuritis. 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. Our results indicated that establishment of optic neuritis not only involves the Th1-Th2 theory, but is also associated with Th17.

EAE and EAON that affect the central nervous system can be defined as autoimmune diseases involving predominantly pathogenic T cells and/or B cells. When T cells from regional lymph node or spleen cells obtained from a mouse immunized with MBP or PLP are injected into a naive syngeneic mouse, EAE and EAON develop.⁵ Elucidation of these experiment models may lead to novel treatments for intractable diseases such as MS and neuromyelitis optica. Analysis of the onset mechanism of refractory optic neuritis has been accomplished through progress in experimental animal models in the past several years. This novel treatment using immunoregulatory cells acts specifically at the inflammatory site, and systemic adverse effects are expected to be fewer than the conventional therapies, such as steroids. The results presented in our study may provide a novel therapeutic strategy for refractory optic neuritis in humans.

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