Suppression of Experimental Autoimmune Uveoretinitis by Regulatory Dendritic Cells in Mice

Yoshihiko Usui, MD, PhD; Masaru Takeuchi, MD, PhD; Takaaki Hattori, MD, PhD; Yoko Okunuki, MD, PhD; Kazumi Nagasawa; Takeshi Kezuka, MD, PhD; Ko Okumura, MD, PhD; Hideo Yagita, PhD; Hisaya Akiba, PhD; Hiroshi Goto, MD, PhD

**Objective:** To examine the effects of bone marrow–derived regulatory dendritic cells (DCs) with potent immunoregulatory properties on the development of experimental autoimmune uveoretinitis (EAU).

**Methods:** Bone marrow cells obtained from C57BL/6 mice were treated with granulocyte-macrophage colony-stimulating factor, transforming growth factor-β, and interleukin (IL) 10 and stimulated with lipopolysaccharide to produce mature and regulatory DCs. Expression of major histocompatibility complex and costimulatory molecules was analyzed by flow cytometry. Then EAU was induced by immunization with human interphotoreceptor retinoid-binding protein (hIRBP) peptide 1-20, followed by intravenous injection of hIRBP peptide–pulsed regulatory DCs. Control mice received transforming growth factor-β and IL-10 nontreated mature DC or phosphate-buffered saline. We evaluated EAU clinically and histopathologically. Immunologic responses to hIRBP peptide were assessed by delayed-type hypersensitivity and T-cell proliferation and cytokine production.

**Results:** Regulatory DCs expressed comparable levels of major histocompatibility complex class II molecules but reduced levels of CD80, CD86, and CD40 compared with mature DCs. Delayed-type hypersensitivity to hIRBP peptide and the development of EAU were markedly suppressed in mice receiving regulatory DCs compared with control mice. Lymph node cells from regulatory DC–treated mice showed significantly reduced hIRBP-specific T-cell proliferation and interferon-γ production but increased IL-10 production.

**Conclusion:** Administration of regulatory DCs potentially inhibited the development of EAU.

**Clinical Relevance:** Application of regulatory DCs may be a novel candidate for immunotherapy for human endogenous uveitis.

focus of new therapies. Siepmann et al reported that a single injection of bone marrow–derived DCs matured by stimulation with lipopolysaccharide (LPS) led to peripheral tolerance by differentiation of regulatory T cells in EAU. In addition, DCs exposed to tumor necrosis factor α or estrogen have been shown to prevent experimental autoimmune encephalomyelitis. On the other hand, Sato et al demonstrated that DCs rendered mature by granulocyte-macrophage colony-stimulating factor (GM-CSF) in the presence of IL-10 and transforming growth factor β (TGF-β) possess immunoregulatory functions by generating CD+CD25+ or CD8+ CD25+ regulatory T cells, which they designated as regulatory DCs. The therapeutic effects of regulatory DCs on acute graft-vs-host disease, cutaneous chronic graft-vs-host disease, allergic airway inflammation, experimental endotoxemia, and bacterial peritonitis have been demonstrated. However, it remains unclear whether regulatory DCs confer protection against organ-specific autoimmune diseases. In the present study, we explored whether regulatory DCs have the capacity to provide immunoprotection against clinical murine EAU.

### METHODS

#### MICE

Seven- to 10-week-old female C57BL/6 mice were obtained (Charles River Japan Inc; Kanagawa, Japan). The mice were maintained in accordance with the statement of the Association for Research in Vision and Ophthalmology regarding the use of animals in research. A mixture of ketamine hydrochloride (10 mg/kg) and xylazine hydrochloride (100 mg/kg) (5:1 vol:vol) was administered intraperitoneally was used for anesthesia.

#### REAGENTS AND ANTIBODIES

Human IRBP (hIRBP) 1-20 peptide (GPTHLFQPSLVLDMAKV/GP) was synthesized by conventional solid-phase techniques using a peptide synthesizer. Freund complete adjuvant and Mycobacterium tuberculosis H37Ra were purchased from Difco Laboratories Inc (Detroit, Michigan). Purified Bordetella pertussis toxin was purchased from Sigma-Aldrich Corp (St Louis, Missouri). Murine GM-CSF, murine IL-10, and human TGF-β were purchased from PeproTech (London, England). Purified anti-CD16/32 (FcBlock); fluorescein isothiocyanate conjugated anti-I-A/I-E monoclonal antibodies; phycoerythrin-conjugated anti-CD11c and CD40 monoclonal antibodies; rat IgG isotype control; and hamster IgG control were purchased from BD Biosciences (San Jose, California). Lipopolysaccharide was purchased from Sigma-Aldrich Corp (St Louis).

#### EAU INDUCTION

Female C57BL/6 mice were immunized subcutaneously in the neck with 200 µg of hIRBP 1-20 peptide in Freund complete adjuvant containing 2.5 mg/mL of M tuberculosis H37Ra and then injected intraperitoneally with 1 µg of purified B pertussis toxin as additional adjuvant. Funduscopy examination was conducted in individual mice every 4 days as described previously and graded on a scale of 0 to 4. Two ophthalmologists (Y.U. and Y.O.) performed the clinical assessments in a masked fashion. The data from the assessment are presented as the mean clinical score for each group (n=10). In addition, EAU was assessed by histopathologic examination on day 21 after immunization. The enucleated eyes were fixed in 4% paraformaldehyde and then embedded in paraffin. Sections of the eyes were stained with hematoxylin-eosin. The severity of EAU was scored on a scale of 0 to 4 in half-point increments, according to a semiquantitative system described previously.

#### PREPARATION AND INJECTION of DCs

The generation of mature DCs and regulatory DCs was performed according to the previous reports. Briefly, mature DCs were generated by culturing bone marrow cells with murine GM-CSF (20 ng/mL) in a bacteriologic Petri dish (BIO-BIK, Tokyo, Japan) for 6 days. Nonadherent cells were carefully collected and subjected to negative selection with monoclonal antibodies to Ly-76, CD2, B220, CD14, and Ly-6G (BD Pharmingen, Inc; San Diego, California) plus sheep anti-rat IgG microbeads using autoMACS columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Purified cells were stimulated with LPS (1 µg/mL) for 2 days. Regulatory DCs were generated by culturing bone marrow cells with murine GM-CSF (20 ng/mL), murine IL-10 (20 ng/mL), and human TGF-β1 (20 ng/mL) for 6 days. The subsequent purification and LPS stimulation procedures were the same as for mature DCs. These DC preparations typically had purity of greater than 90% as estimated by anti-I-A/I-E or anti-CD11c monoclonal antibody staining and contained less than 0.1% erythrocytes, T cells, B cells, monocytes or macrophages, natural killer cells, and neutrophils as determined by flow cytometry. Regulatory or mature DCs were washed and resuspended, plated in culture plates, and treated with 4-µM of hIRBP peptide at 37°C in an atmosphere of 5% carbon dioxide for 12 hours. The cells were washed and resuspended in phosphate-buffered saline (PBS) and injected intravenously into mice at a dose of 5 × 10^6 cells per mouse on day 5 after immunization.

#### FLOW CYTOMETRIC ANALYSIS

For fluorescence activated cell sorter analysis, 10^6 cells were washed twice in PBS, preincubated with unlabeled anti-CD16/32 monoclonal antibody to avoid nonspecific binding of antibodies to FcγR, and then incubated with fluorescein isothiocyanate– or phycoerythrin-labeled monoclonal antibodies. After washing twice with PBS, the stained cells (live-gated on the basis of forward and side scatter profiles and propidium iodide exclusion) were analyzed on a flow cytometer (FACSCalibur; BD Biosciences). Data were processed using CellQuest software (BD Biosciences) and expressed as mean fluorescence intensity.

#### DELAYED-TYPE HYPERSENSITIVITY ASSAY

On day 19 after immunization, the mice received an intradermal injection of 10 µg of hIRBP peptide in PBS into the right ear pinnna. After 48 hours, ear swelling was measured using a micrometer (Mitutoyo, Tokyo, Japan). Antigen-specific delayed response was measured as the difference in ear thickness before and after challenge. Results were expressed as specific ear swelling calculated as (24-hour measurement − 0-hour measurement) for test ear − (24-hour measurement − 0-hour measurement) for control ear, as described previously.

#### IN VITRO PROLIFERATION AND CYTOKINE PRODUCTION

The DLN cells were removed from each group of regulatory DC–, mature DC–, or PBS-injected mice on day 21 after immunization. Cells were seeded in flat-bottom, 96-well microculture plates at a density of 6 × 10^3 cells per well in 200 µL of the culture me-
Exposure of major histocompatibility complex class II and costimulatory molecules on regulatory dendritic cells (rDCs) and mature dendritic cells (mDCs). Cells were stained with the indicated monoclonal antibodies or isotype-matched control immunoglobulin, and cell surface expression was analyzed by fluorescence activated cell sorter. Data are represented by a histogram showing cells stained with the indicated monoclonal antibody (bold lines) or isotype-matched control immunoglobulin (fine lines). The value shown in the upper righthand corner of each graph of the flow cytometry profile is the mean fluorescence intensity. The results are representative of 3 experiments with similar results.

To assess proliferative responses, cultures were pulsed with [3H]thymidine (0.5 µCi per well; DuPont NEN Research Products, Boston, Massachusetts) for the final 8 hours and harvested on a Micro 96 Harvester (Skatron Instruments, Lier, Norway). The incorporated radioactivity was measured using a microplate beta counter (Micro Beta Plus; Wallac, Turku, Finland). The data are presented as mean (SEM) counts per minute of triplicate cultures.

For DC-stimulated T-cell proliferation assay, mature or regulatory DCs (1 x 10^6 cells) were irradiated (30 Gy) and cocultured with DLN cells (5 x 10^6 cells) in the presence of hIRBP peptide (10 µg/mL) for 72 hours. Then proliferative response was assessed as described herein.

To assess cytokine production, cell-free supernatants were collected at 72 hours and assayed for IL-10 by enzyme-linked immunosorbent assay (Ready-SET-Go! kit; eBioscience Inc) and for interferon gamma (IFN-γ) using the OptEIA kits (BD Pharmingen Inc) and for interferon gamma (IFN-γ) using a mouse IFN-γ enzyme-linked immunosorbent assay (Ready-SET-Go! kit; eBioscience Inc, San Diego, California) according to the protocols recommended by the manufacturers.

**STATISTICAL ANALYSIS**

Differences between the 2 experimental groups were analyzed by the Mann-Whitney test. P < .05 was considered statistically significant.

**RESULTS**

**EXPRESSION OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II AND COSTIMULATORY MOLECULES ON REGULATORY DCs**

We first examined the cell surface expression of major histocompatibility complex (MHC) class II and various costimulatory molecules (CD80, CD86, and CD40) on regulatory and mature DCs generated from bone marrow cells from C57BL/6 mice. Compared with mature DCs, regulatory DCs showed almost comparable levels of the DC marker (CD11c) and MHC class II molecules (1-A/I-E), but the expression levels of CD80, CD86, and CD40 were greatly reduced (Figure 1). These phenotypes were consistent with those of regulatory DCs as reported previously.18-20

**EFFECT OF REGULATORY DCs ON THE DEVELOPMENT OF MURINE EAU**

We examined the in vivo effect of regulatory DCs on clinical EAU. Ten mice in each group were injected intravenously with hIRBP peptide–pulsed regulatory DCs or mature DCs (5 x 10^6 cells) or the same volume of PBS on day 5 after immunization. The PBS-injected control mice exhibited characteristic signs of EAU starting on day 8 after immunization and progression thereafter. Transfer of mature DCs had no significant effect on the clinical signs of EAU. However, transfer of regulatory DCs significantly alleviated the clinical signs of EAU compared with the mice injected with mature DCs or PBS on days 16 and 20 (Figure 2A). Figure 2B shows histologic scores of EAU eyes and the most severe representative histopathologic findings of the retina in these mice. In mice injected with mature DCs or PBS, inflammatory cells infiltrated the entire retina, and the anatomical structure of retinal layers was partially destroyed. In mice treated with regulatory DCs, however, cell infiltration into the retina was apparently reduced and retinal layer structures were mostly preserved.

We also analyzed delayed-type hypersensitivity response as a measure of in vivo IRBP-specific T_h1 responses. On challenge with hIRBP peptide, the regulatory DC–treated mice exhibited a significantly reduced response compared with mature DC– or PBS-treated mice (Figure 2C).

**EFFECT OF REGULATORY DCs ON THE DEVELOPMENT OF IRBP-SPECIFIC T_h1 CELLS IN VIVO**

We next examined in vitro proliferative response and cytokine production by DLN cells collected from hIRBP peptide–immunized and PBS–, mature DC–, or regulatory DC–
treated mice. The DLN cells derived from regulatory DC-treated mice showed significantly reduced T-cell proliferation in response to hIRBP peptide compared with mature DC- or PBS-treated mice (Figure 3A). Although proliferation was slightly higher in DLN cells from mature DC-treated mice compared with PBS-injected mice, no statistical difference was found. Production of IFN-γ was significantly reduced and IL-10 production was significantly increased in DLN cells from regulatory DC-treated mice compared with mature DC- or PBS-treated mice (Figure 3B and C). These results indicated that treatment with regulatory DCs inhibited the development and/or expansion of IRBP-specific Th1 cells in vivo.

EFFECT OF REGULATORY DCs ON THE PROLIFERATION OF IRBP-SPECIFIC Th1 CELLS IN VITRO

To determine the direct effect of regulatory DCs on the expansion of IRBP-specific T cells, DLN cells obtained from mice immunized with hIRBP peptide on day 21 after immunization were cocultured with mature DCs.
Proliferation was measured after 72 hours by [3H]thymidine uptake. The data
ported that regulatory T cells, especially CD4
and/or regulatory DCs in the presence of hIRBP peptide (10 µg/mL). As shown in Figure 4, although mature DCS increased the proliferation of uveitogenic T cells in vitro, regulatory DCS significantly inhibited the proliferation of uveitogenic T cells. Furthermore, regulatory DCS also significantly suppressed T-cell proliferation when added to a coculture of DLN cells and mature DCS. These results indicated that regulatory DCS possessed the capacity to suppress uveitogenic effector T cells, consistent with the in vivo results.

In the present study, we used regulatory DCS generated in vitro by treatment with TGF-β and IL-10 to suppress ongoing murine EAU. We studied the immunoregulatory role of regulatory DCS on EAU for the following reasons: (1) regulatory DCS exhibit reduced expression of costimulatory molecules (CD80, CD86, and CD40), decreased capability to activate T cells, and increased generation of CD4+CD25+ and CD8+CD25+ regulatory T cells; (2) regulatory DCS are mature and thus retain the immunoregulatory function in vivo even under inflammatory conditions; and (3) regulatory DCS generated from human monocytes have also been shown to have potent immunoregulatory functions in vitro. Therefore, an immunotherapeutic strategy using regulatory DCS is potentially useful for the development of a therapeutic approach clinically applicable to human uveitis.

Regulatory DCS suppressed the development of clinical EAU, which was accompanied by decreased proliferation and IFN-γ production but increased IL-10 production by IRBP-specific T cells. The decreased proliferation and IFN-γ production might be caused by the increased IL-10 production by regulatory DC–induced regulatory T cells. Regulatory T cells play an important role in induction of peripheral tolerance. We and others have recently reported that regulatory T cells, especially CD4+CD25+ regulatory T cells, play a critical role in suppressing murine EAU,26-28 and this suppressive effect is partly mediated by IFN-γ29 and IL-10.13 In this study, we showed that IFN-γ production was reduced whereas IL-10 production was increased in DLN cells derived from regulatory DC–treated mice. IL-10–producing T cells with in vitro regulatory properties have been generated using immature DCS29 or DCS treated with vitamin D3 or IL-10,30 suggesting that regulatory DCS induce tolerance, at least in part, by generating IL-10–producing regulatory T cells. Recently, a series of studies has shown that tolerogenic DCS not only produce inhibitory cytokines such as IL-10 and TGF-β but also can expand the number of CD4+CD25+Foxp3+ regulatory T cells.22,23,24 Therefore, the treatment of mice with regulatory DCS suppressed clinical EAU, possibly through the production of TGF-β and generation of CD4+CD25+Foxp3+ regulatory T cells in the DLN cells that were derived from regulatory DC–treated mice.

Jiang et al13 reported that immature DCS with low MHC class II and low CD86 expression markedly suppressed murine EAU when transferred to naive mice. This suppressive effect was lost when DCs underwent maturation in vitro exposure to LPS. A major purpose of studies on the pathogenesis of autoimmune uveoretinitis is to define the trigger events that lead to activation of autoreactive T cells. The mechanisms underlying DC–mediated tolerance are not completely understood. Further studies are needed to gain better understanding of the exact mechanism of how regulatory DCS suppress the T cells specific for retinal self-antigens.

In conclusion, our study demonstrated amelioration of ongoing EAU by regulatory DC transfer and suggests that regulatory DCS down-regulate antigen–specific response after immunization through suppression of T-cell expansion and TGF-β cytokine production. Therefore, in vitro manipulation of regulatory DCS would become a potential therapy for human uveitis with an autoimmune origin.

Submitted for Publication: May 22, 2008; final revision received August 25, 2008; accepted August 30, 2008.

Correspondence: Masaru Takeuchi, MD, PhD, Department of Ophthalmology, Tokyo Medical University, 6-7-1, Nishi-shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan (takeuchi@tokyo-med.ac.jp).

Financial Disclosure: None reported.

Funding/Support: This work was supported by Grant-in-Aid 16591769 and 19791294 for Scientific Research from the Japan Society for the Promotion of Science.

Additional Contributions: Katukari Sato, PhD, provided technical advice for the generation of regulatory dendritic cells, and Naoyuki Yamakawa, PhD, provided excellent technical assistance.

REFERENCES


