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Cutting Edge: In Vitro-Generated Tolerogenic APC Induce CD8\(^+\) T Regulatory Cells That Can Suppress Ongoing Experimental Autoimmune Encephalomyelitis

Douglas E. Faunce, Ania Terajewicz, and Joan Stein-Streilein

APC exposed to TGFβ2 and Ag (tolerogenic APC) promote peripheral Ag-specific tolerance via the induction of CD8\(^+\) T regulatory cells capable of suppressing Th1 and Th2 immunity. We postulated that tolerogenic APC might re-establish tolerance toward self-neuronal Ags and ameliorate ongoing experimental autoimmune encephalomyelitis (EAE). Seven days after immunization with myelin basic protein (MBP), mice received MBP-specific tolerogenic APC, and EAE was evaluated clinically. To test for the presence and the phenotype of T regulatory cells, CD4 and/or CD8 T cells from tolerogenic APC-treated mice were transferred to naive mice before their immunization with MBP. The MBP-specific tolerogenic APC decreased both the severity and incidence of ongoing EAE. Tolerance to self-neuronal Ags was induced in naive recipient mice via adoptive transfer of CD8\(^+\), but not CD4\(^+\) T cells. Rational use of in vitro-generated tolerogenic APC may lead to novel therapy for autoimmune disease. The Journal of Immunology, 2004, 172: 1991–1995.

Immunogenic inflammation serves to activate the adaptive immune system, clear foreign debris and organisms, and stimulate the healing process. The eye and the CNS are good examples of organs that are susceptible to damage during even minor inflammation. To prevent intraocular inflammation, the eye and the peripheral immune system cooperate in a model known as anterior chamber-associated immune deviation (ACAIID), whereby Ag entry via the anterior segment of the eye leads to a state of systemic peripheral tolerance or active suppression, rather than inflammation. ACAID is a complex, multicellular process involving communication between eye-derived APC and splenic T cells (1, 2), B cells (3, 4), and innate lymphocytes, including NKT cells (5) and γδ T cells (6, 7).

During the induction of ACAID, CD1d-restricted NKT cells are recruited to the spleen by eye-derived APC via chemokine (macrophage-inflammatory protein-2)-dependent mechanisms. Once in the spleen, the NKT cells and APC establish an immunoregulatory nanoenvironment that promotes the differentiation of CD8\(^+\) T regulatory (Tr) cells destined to suppress the efferent phase of cellular immunity (8, 9). The ability of eye-derived (or TGFβ2-treated/Ag-pulsed) APC to generate an Ag-specific CD8\(^+\) Tr cell has been extensively described by Streilein and colleagues (10, 11). The eye-derived APC are central to the development of this specialized form of tolerance and are known to acquire their tolerogenic capacity partly because of their exposure to an extraordinarily high concentration of TGFβ2 in the aqueous humor (12, 13). Experimentally, APC exposed to ocular factors suppress the activation of Ag-specific T cells in both naive and presensitized mice (4, 14, 15). Aside from an isolated report that used the ACAID system to suppress the elicitation of experimental uveitis (16), the ability of TGFβ-treated APC to suppress ongoing autoimmune diseases, such as arthritis, diabetes, or multiple sclerosis (MS), has not been reported.

Experimental autoimmune encephalomyelitis (EAE) is a prototypic experimental animal model for MS, an attack by the peripheral immune system on myelin components of the CNS. Like MS in humans, EAE in mice and other rodents is mediated by encephalitogenic CD4\(^+\) T cells that produce inflammatory cytokines such as IFN-γ and TNF-α (17, 18). Whereas MS in humans is a relapsing and remitting disease, EAE in rodent strains is characterized by a monophasic clinical course of ascending paralysis, followed by spontaneous complete recovery. The mechanisms involved in the recovery from EAE are not completely understood, but recent literature suggests a regulatory role for several different cell types including T cells of the CD4\(^+\), CD8\(^+\), and double-negative phenotypes (19–21). CD1d-restricted NKT cells have also been examined for their...
contribution to MS and EAE, although their precise role remains quite controversial and may depend largely on the specific immune microenvironment in which NKT cells interact with CD1d+ APC (22–25). APC that become tolerogenic after exposure to the immunosuppressive factors (i.e., TGFβ2 or α-melanocyte-stimulating hormone) in the ocular microenvironment in vitro have been shown to promote tolerance that can suppress both Th1- and Th2-mediated inflammation (15, 26). Given their extraordinary capacity to suppress different forms of immunogenic inflammation, we postulated that APC exposed to autoantigens and rendered tolerogenic by exposure to TGFβ2 would also suppress autoimmune inflammation. In this report, we present the novel observations that tolerogenic APC halt the progression of EAE, and that they do so via their induction of CD8+ Tr cells that suppresses myelin basic protein (MBP)-specific immunity in an Ag-specific fashion.

Materials and Methods

Mice

Female C57BL/6 wild-type mice used in these experiments were obtained from the Schepens Eye Research Institute Vivarium or Taconic Farms (Taconic, NY). Mice were housed on a 12/12-h light/dark cycle and provided food and water ad libitum. All animals were treated humanely and in accordance with the guidelines set forth by the Schepens Eye Research Institute Animal Care and Use Committee, and the National Institutes of Health guidelines.

Induction and measurement of EAE

EAE was induced with an adaptation of the method of Betelli et al. (27). Briefly, mice were immunized s.c. on each flank with 200 μg (400 μg total) of MBP (Sigma-Aldrich, St. Louis, MO) in CFA (Sigma-Aldrich) containing 4 mg/ml Mycobacterium tuberculosis H37ra (Difco Laboratories, Detroit, MI). Additionaly, all mice received i.p. injections of 200 ng of Bordetella pertussis toxin in PBS on days 0 and 2 (total, 400 ng). Evaluation of EAE was performed every other day for 33 days using the following clinical scores: 0, no overt signs of disease; 1, limp tail or hindlimb weakness; 2, limp tail and hindlimb weakness; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; and 5, moribund or death.

Cell-based treatment of EAE mice with tolerogenic APC

Tolerogenic, ACAID-like APC were prepared by a modification of methods reported by Wilbanks and Streilein (28). Briefly, thioglycolate-elicited peritoneal exudate cells (PEC) were cultured overnight in serum-free medium (SFM) containing 500 μg/ml bovine MBP (Sigma-Aldrich) and 5 ng/ml TGFβ2 (R&D Systems, Minneapolis, MN). Control (immunogenic) APC were prepared in the same manner, excluding the TGFβ2. After culture, the APC were washed three times in HBSS to remove excess TGFβ and MBP. The APC were placed at 4°C in PBS for 2 h and collected by vigorous pipetting. The cells were treated with DNaese, washed twice, and resuspended at a concentration of 5 × 10^5 cells/ml in HBSS. For cell-based treatment of EAE, each mouse was given 100 μl of cell suspension (5 × 10^5 cells) via the tail vein 7 days after immunization with MBP in CFA.

Preparation of Tr cell subsets for treatment of EAE

Thirty-three days after treatment of EAE mice with immunogenic vs tolerogenic APC, their spleens were collected and processed into erythrocyte-free single-cell suspensions. To enrich Tr cells, splenocytes were passed through Imuman goat anti-mouse IgG columns (Biotex Laboratories, Houston, TX). Enrichment efficiency was confirmed by flow-cytometric analysis of negatively selected cells. Depletion of either CD8+ or CD4+ Tr cells was achieved by incubation of Immunun-enriched fractions with either anti-CD8 (clone 2.43) or anti-CD4 antibodies (clone GK1.5) (both from American Type Culture Collection, Manassas, VA) plus LowTox baby rabbit complement (Cedar Lane Laboratories, Hornby, Ontario, Canada). Depletion of CD8 or CD4 subsets was confirmed by flow-cytometric analysis of cells immunostained with FITC-conjugated anti-CD8 mAb (Ly-2; clone 53-6.7) and PE-conjugated anti-CD4 mAb (L3T4; clone RM4–5) (both from BD PharMingen, San Diego, CA). For treatment with enriched cells, naïve C57BL/6 mice were given 5 × 10^6 of the respective cell type (i.v.) suspended in 100 μl of HBSS. The adoptive transfer of T cells from immunogenic vs tolerogenic APC-treated mice to naive recipients was done as donor equivalents (i.e., cells from one mouse were given to one recipient). Immediately after transfer of cells, Tr cell-treated mice were immunized with MBP in CFA plus pertussis toxin, and monitored for clinical symptoms every other day for 33 days.

Induction and measurement of OVA-specific delayed-type hypersensitivity (DTH)

Mice were inoculated (s.c.) with OVA in CFA (150 μg in 150 μl) and, 7 days later, received an intradermal injection in the ear pinnae (ear challenge) of 2.0 × 10^6 (in 10 μl of HBSS) PEC that had been cultured overnight with OVA (5 μg/ml in SFM). Mice that received ear challenge without prior s.c. sensitization served as a negative DTH control. Differences in DTH responses between the groups were determined by measuring ear thickness before challenge, and 24 and 48 h after challenge with an engineer’s micrometer (Mitutoyo, Paramus, NJ).

Statistical analyses

Statistical differences in the average day of onset and mean peak clinical scores between control vs experimental groups were determined by either nonparametric Mann-Whitney U tests, or t tests as required. Significance was considered at p ≤ 0.05 in all experiments.

Results

TGFβ2-treated APC modulate ongoing EAE

Previous studies by our laboratory and others showed that, when TGFβ2-treated APC were given to mice in vivo or used in coculture systems in vitro, they promoted the generation of CD8+ Tr cells that suppressed Ag-specific T cell immunity (4, 5, 26, 29). In this study, tolerogenic APC were established by culturing thioglycolate-elicited PEC in SFM containing MBP and TGFβ2, as previously described (4, 30). Immunogenic APC were cultured in SFM containing MBP, but without TGFβ2. The tolerogenic APC were injected (i.v.; 5 × 10^5 cells/mouse) into mice immunized with MBP in CFA 7 days earlier. A group of mice that received only MBP and CFA immunization (no cell treatment) served as an additional control. Mice that received MBP immunization plus treatment with tolerogenic APC exhibited a significant delay in disease onset (day 15 vs day 9 in controls) (Table I) and a significantly lower peak EAE score compared to controls (Fig. 1, Table I). Although both the untreated control mice and immunogenic APC-treated groups had 100% incidence of disease (15 of 15, and 14 of 14, respectively), we observed that the tolerogenic APC-treated group had lower incidence (10 of 13) (Table I). Moreover, whereas 90% of
mice that did not receive APC treatment (i.e., MBP immunization only) had a severity of score of ≥2, 62% of mice that received tolerogenic APC had a severity score of 1 or 0. The fact that the majority of mice given ACAID-like APC had peak scores of 1 or lower (mild or no disease), indicated suppression of EAE due to the tolerogenic, ACAID-like APC treatment. Thus, treatment with Ag-pulsed, tolerogenic APC 1 wk after immunization with MBP in adjuvant suppressed the development and severity of EAE, and delayed its onset.

*Tolerogenic APC suppress EAE through the development of CD8\(^+\) Tr cells*

Previously, we showed that tolerogenic APC suppressed T cell immunity via the CD1d/NKT cell-dependent generation of CD8\(^+\) Tr cells (4). Because the majority of tolerogenic APC-treated mice had delayed onset and lower magnitude of EAE, we proposed that the treatment reduced EAE through the production of Tr cells. To test for the presence of Tr cells, enriched splenic T cells from APC-treated mice that exhibited a peak EAE score of 0 or 1 (day 33 after MBP immunization), were transferred to naive, syngeneic recipients (5 \(10^6\) cells/mouse) 7 days before being immunized with MBP in CFA and monitored for EAE. To determine whether the Tr cells were CD4\(^+\) or CD8\(^+\), parallel groups of mice were given 5 \(10^6\) enriched T cells that were depleted of either CD4\(^+\) or CD8\(^+\) cells by specific Ab and complement (Fig. 2a). Mice that were immunized with MBP in CFA, but received either T cell transfer from immunogenic APC-treated EAE mice, or no cell transfers, were used as controls.

The control MBP-immunized mice developed typical EAE (Fig. 2b). In contrast, mice that received enriched T cells from experimental mice (i.e., recipients of tolerogenic APC) exhibited less severe and delayed EAE compared with the control groups (Fig. 2b), suggesting the presence of a Tr cell that suppressed disease. However, if the T cell preparations from tolerogenic APC-treated mice were first depleted of CD8\(^+\) T cells, the magnitude of EAE was identical with that of control mice (no cell treatment, MBP-immunized). In contrast, depletion of CD4\(^+\) cells from T cell preparations from tolerogenic APC-treated mice (i.e., only CD8\(^+\) T cells were transferred) resulted in even greater suppression of EAE than transfer of total tolerogenic cells, supporting the concept that the regulatory cell ca-

**Discussion**

In this report, we exploited the ability of in vitro-generated, MBP-pulsed tolerogenic APC (similar to ocular APC bathed in
TGFβ2 to promote the development of CD8+ Tr cells that conveyed peripheral tolerance and suppressed autoimmunity toward the myelin Ags. In our studies, the mice were given tolerogenic APC 7 days after being sensitized to MBP. Thus, a potent inflammatory response to Ag was presumably already well developed at the time of APC treatment. Compared with the untreated, MBP-immunized group, we observed that the majority of mice given tolerogenic ACAID APC either developed significantly less severe disease, or did not develop EAE at all. Together, these findings demonstrate the striking ability of in vitro-generated, tolerogenic APC to suppress an ongoing, robust inflammatory response. The fact that a small number of mice given TGFβ2-treated APC acquired disease may be due to differences in the kinetics of the inflammatory/immune response in individual mice, with regard to the timing of tolerogenic APC administration.

Previously, Okamoto et al. (16) transferred interphotoreceptor retinoid binding protein-pulsed, TGFβ2-treated APC to interphotoreceptor retinoid binding protein-immunized mice and successfully suppressed the induction of experimental uveoretinitis in naive mice. Our work extends the idea of tolerance generated by TGFβ2-treated APC in naive mice to show that the tolerogenic process works in previously immunized mice, because treatment of previously immunized mice with MBP-pulsed, tolerogenic APC markedly reduced the severity of EAE, even in the presence of a well-established inflammatory response. Similar to our findings, Menges et al. (31) showed that MOG-induced EAE could be completely prevented by repetitive administration of TNF-α-treated, MOG-pulsed bone marrow-derived dendritic cells (DC). In their report, Menges et al. (31) showed that TNF-α treatment of DC led to partial maturation, which resulted in their ability to induce an IL-10-producing CD4+ Tr cell that presumably suppressed disease. In contrast to their findings, we report in this study that treatment of mice with APC that were treated with TGFβ2 and pulsed with Ag leads to the generation of a CD8+ T cell with regulatory capacity. Moreover, unlike the TNF-α-treated DC discussed by Menges and colleagues, the TGFβ2-treated Ag-pulsed APC described here need to be given only once to alleviate ongoing EAE disease. Whether or not IL-10 is involved in CD8+ Tr cell-mediated suppression of EAE remains to be determined.

Although several studies showed that IL-10- and TGFβ-producing CD4+ Tr cells are capable of suppressing EAE (32), there are only a few reports studying the involvement of CD8+ Tr cells in EAE (33, 34). In this study, we show that transferring in vitro-manipulated APC may purposefully induce suppressive CD8+ Tr cells. The mechanisms used by the CD8+ Tr cells to control Th1 immunity may involve both soluble factors such as IL-10 and TGFβ (9, 35), and cell-cell contact (33). Jiang et al. (33, 34) showed that CD8+ Tr cells suppress MBP-reactive CD4+ T cells directly and provided compelling evidence that suppression of Ag-activated CD4+ T cells by CD8+ Tr cells occurred in a manner that required CD4:CD8 T cell contact and was dependent upon the MHC class 1b molecule, Qa-1. Interestingly, the generation of CD8+ Tr cells associated with ACAID has also been shown to be dependent upon the expression of Qa-1 (36).

Previous work by our laboratory supports the notion that the tolerogenic APC (either eye derived or i.v. injection) localize to the marginal zone of the spleen where they interact with CD8+ T cells, as well as IL-10-producing NKT cells (5, 8, 9). The possibility that tolerogenic cell interactions also occur in regions similar to the marginal zone (subcapsular sinus) in the lymph node has not been excluded. Discovery of the mechanism(s) responsible for CD8+ Tr suppression of effector cells in both primary and secondary immune responses is a current topic of ongoing investigation by our laboratory.

The novel findings presented in this report show that experimentally generated MBP-pulsed tolerogenic APC induced CD8+ Tr cells that suppressed immune responses to self Ags and that the Ag-specific CD8+ Tr cells were generated even in the presence of an established autoimmune inflammatory response. In addition, the effect of the ACAID-like APC in inducing tolerance was extremely powerful in that only one injection of cells was required for obvious modulation in the disease status of the EAE mice. Thus, the capability of Ag-pulsed tolerogenic APC to generate tolerance during ongoing immune inflammatory responses raises the possibility that cell-based therapeutics using in vitro-manipulated APC might be used to prevent the progression or recurrence of autoimmune disease.

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