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TGF-β-treated antigen presenting cells suppress collagen-induced arthritis through the promotion of Th2 responses

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Abbreviations: CIA, collagen induced arthritis; Imm-APC, immunogenic antigen presenting cell; Tol-APC, tolerogenic antigen presenting cell

Abstract

Collagen-induced arthritis (CIA) is mediated by self-reactive $CD4^+$ T cells that produce inflammatory cytokines. TGF-β_2-treated tolerogenic antigen-presenting cells (Tol-APCs) are known to induce tolerance in various autoimmune diseases. In this study, we investigated whether collagen-specific Tol-APCs could induce suppression of CIA. We observed that Tol-APCs could suppress the development and severity of CIA and delay the onset of CIA. Treatment of Tol-APCs reduced the number of IFN-γ- and IL-17-producing $CD4^+$ T cells and increased IL-4- and IL-5-producing $CD4^+$ T cells upon collagen antigen stimulation *in vitro*. The suppression of CIA conferred by Tol-APCs correlated with their ability to selectively induce IL-10 production. We also observed that treatment of Tol-APCs inhibited not only cellular immune responses but also humoral immune responses in the process of CIA. Our results suggest that *in vitro*-generated Tol-APCs have potential therapeutic value for the treatment of rheumatoid arthritis as well as other autoimmune diseases.

Keywords: antigen-presenting cells; arthritis, experimental; autoimmune diseases; immune tolerance; Th1 cells; Th2 cells

Introduction

Collagen-induced arthritis (CIA) is an animal model for human rheumatoid arthritis (RA). CIA can be induced by immunization with type II collagen (CII), the major protein constituent of articular cartilage (Courtenay *et al*., 1980). Susceptibility to CIA is associated with murine MHC class II haplotypes $H\text{-}2^q$ and $H\text{-}2^r$, whereas susceptibility to RA is associated with human MHC class II haplotypes DR1 and DR4 (Zanelli *et al*., 1995). The main histopathological features of the resulting joint inflammation in CIA are similar to those in RA, including proliferative synovitis, pannus formation, erosion of cartilage and bone, and fibrosis (Trentham *et al*., 1977). Additionally, CIA and RA are both mediated by the dominant activation of helper T cells expressing pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-1β, IL-6 and IL-17 (Mauri *et al*., 1996; Miossec and van den Berg, 1997; Ortmann and Shevach, 2001). Recently, Th17 cells were identified by a profile of cytokines that is distinct from those observed in Th1 or Th2 subsets, and they could have a crucial role in chronic inflammatory or autoimmune diseases, such as colitis, asthma, experimental allergic encephalitis (EAE), and CIA (Nakae *et al*., 2003; Harrington *et al*., 2005; Langrish *et al*., 2005). Unbalanced Th1/Th2 T-cell polarization has been suggested to play a pathogenetic role in the development of autoimmune diseases. Therefore, modulation of T-cell polarization is a potential therapeutic target in the various autoimmune diseases.

Professional APCs can either be immunogenic or tolerogenic depending on their stage of maturation or level of activation (Mahnke *et al*., 2002). APC function can also be modified by cytokine treatment, including TGF-β_2 and IL-10 (Wilbanks *et al*., 1992; Steinbrink *et al*., 1997). TGF-β_2 is a major immunosuppressive cytokine in the aqueous humor of the anterior chamber (a.c.) of the eye and is known to modulate the function of thioglycolate-induced peritoneal exudate cells (PECs) *in vitro*. PECs obtained from mice injected with thioglycollate medium, a well known macrophages inducer (Baron and Proctor, 1982; Leijh *et al*., 1984). Macrophages that make up the majority of the PECs are identified as expressing F4/80, CD11b, and c-Fms markers (Hirsch *et al*., 1981;

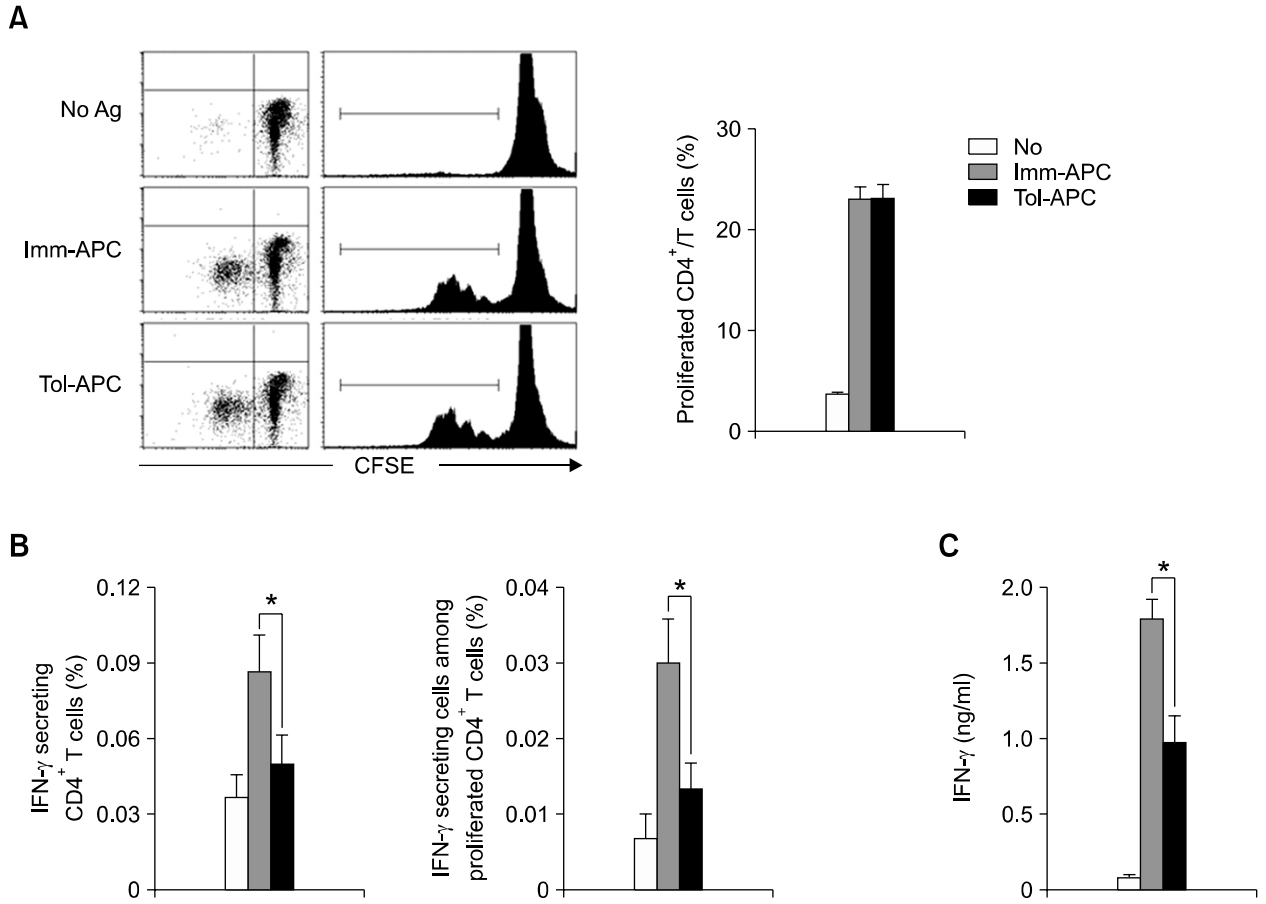

Figure 1. Effect of Imm-APCs vs. Tol-APCs on Ag-specific $CD4^+$ T cell responses. PECs derived from BALB/c WT mice were loaded with OVA protein with or without TGF-β_2. Tol-APCs or Imm-APCs were cultured with CFSE-labeled $CD4^+$ T cells from OVA-primed DO11.10 mice. (A, B) After 72 h, the cultured cells were stained for proliferation of Ag-specific $CD4^+$ T cells with anti-TCRβ and anti-CD4 and for intracellular cytokine staining with anti-IFN-γ. (C) IFN-γ production in the culture supernatants was measured by ELISA. The results represent the mean $\pm$ SEM (3-4 mice per group). Similar results were obtained in two independent experiments. *$P < 0.05$ versus Tol-APC treatment.

Remold-O'Donnell, 1988). TGF-β_2-treated PECs are functionally similar to the "eye-derived" APCs and play a role in the generation of T regulatory (T reg) cells both *in vitro* and *in vivo* (Wilbanks *et al*., 1991, 1992; Wilbanks and Streilein, 1991; Katagiri *et al*., 2002). Since APCs interact directly with antigen-specific T cells, APCs that can induce specific tolerance could be a very effective and specific means of targeting autoreactive T cells. Therefore, Tol-APCs have great potential to be used for the prevention and treatment of T cell-mediated autoimmune diseases. Previous studies reported that TGF-β_2-treated Tol-APCs induce tolerance as well as suppression of EAE and airway pulmonary inflammation (Faunce *et al*., 2004; Zhang-Hoover *et al*., 2005). The ability of TGF-β_2-treated APCs to suppress ongoing autoimmune diseases, such as arthritis and diabetes, has not been reported.

In this study, we investigated whether tolerance induced by Tol-APCs inhibits ongoing CIA. The suppression of cellular and humoral responses by Ag-specific Tol-APCs in the CIA was examined.

Our study shows that *in vitro*-generated Tol-APCs could suppress the development and severity of CIA through the increase of Th2 responses against the CII antigen.

Results

Suppression of OVA-specific $CD4^+$ Th1 cell responses by Tol-APCs

The effect of Tol-APCs on the Th1-biased $CD4^+$ effector T cells was analyzed by using a model $CD4^+$ T cell clone, DO11.10 TCR Tg T cells. Ova-specific $CD4^+$ T cells were isolated from

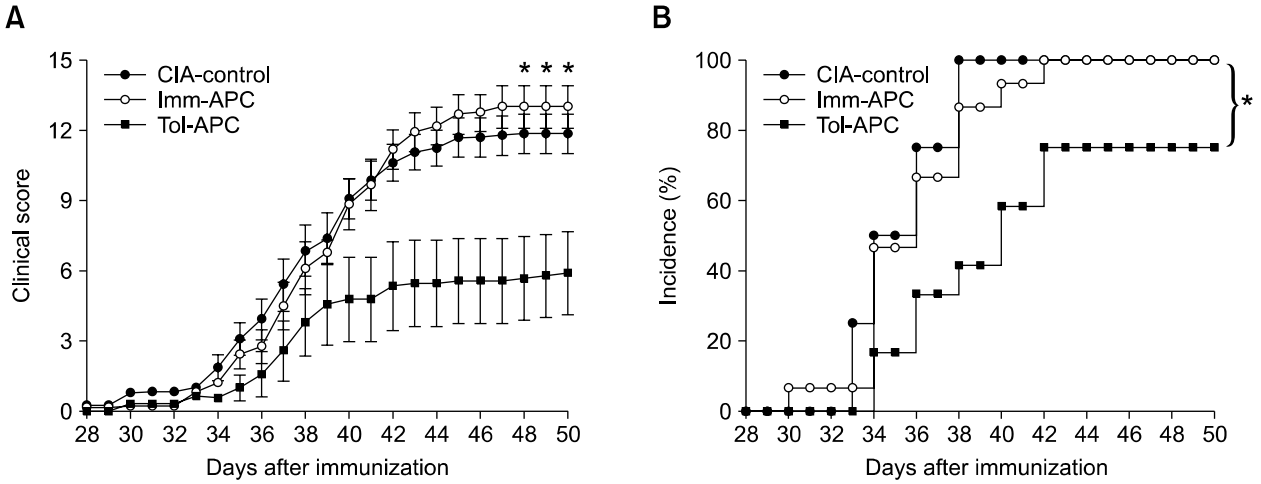

Figure 2. Treatment with Tol-APCs reduces the severity and inhibits the onset of CIA. To induce CIA, mice were immunized i.d. at the base of the tail with 100 μg of chicken CII emulsified with an equal volume of CFA. The mice were boosted by i.d. injection with 100 μg of CII in IFA. Seven days later, mice received i.v. injections of 1×10^6 Imm-APC (○) or Tol-APC (■) or no APC transfer as the CIA control (●). (A) The clinical scores of arthritis in each group. Each paw was scored from 0 to 5 according to the severity of arthritis, with a maximal score of 20 per mouse. (B) The percentages of arthritic mice. Results are representative of three independent experiments with similar results. Bars show the mean ± SEM (6-8 mice per group). ***$P < 0.001$, *$P < 0.05$ versus Tol-APCs-treated mice.

immunized DO11.10 Tg mice and co-cultured with OVA-loaded Tol-APCs or Imm-APCs for 72 h to evaluate T cell proliferation and IFN-γ production.

As shown in Figure 1A, no obvious difference was found in the proliferation of OVA-specific $CD4^+$ T cells when they were stimulated with Tol-APCs (23.3 ± 1.1%) or Imm-APCs (23.3 ± 1.3%). However, the frequency of IFN-γ-producing $CD4^+$ T cells was higher in the Imm-APCs-treated group than in the Tol-APCs-treated group (Figure 1B). Furthermore, the level of IFN-γ in the culture supernatants was also markedly higher in the Imm-APCs-treated group compared to the Tol-APCs-treated group (Figure 1C).

These results indicated that TGF-β_2-driven Tol-APCs could induce the down-regulation of the Th1 response and have the potential to ameliorate autoimmune conditions driven by self-reactive Th1 CD4 T cells.

Tol-APCs ameliorate ongoing CIA

Based on the previous reports on the effect of TGF-β_2-treated APCs against EAE (Faunce *et al*., 2004) and airway hyperreactivity (Zhang-Hoover *et al*., 2005) and our results described above, we investigated whether Tol-APCs could induce the suppression of CIA.

Mice immunized with CII to induce CIA received i.v. injections of Tol-APCs or Imm-APCs seven days after the completion of the immunization protocol. CII-immunized mice treated with Tol-APCs showed significantly less severe disease development and delayed onset of CIA compared to those treated with Imm-APCs or those mock treated (CIA control) (Figure 2). Although both the Imm-APCs-treated group and CIA control group had 100% incidence of the disease, the Tol-APCs-treated group had slightly reduced disease incidence (100% vs. 75%, $P < 0.05$) with significantly lower severity (13.0 ± 0.9 vs. 5.9 ± 5.1, $P < 0.001$) and slightly delayed disease onset (32.8 ± 2.6 vs. 34.7 ± 4.9) compared to those in the Imm-APCs-treated group.

Tol-APCs reduce CII-specific Th1 responses

Next, we analyzed the effect of Tol-APCs on CII-specific T cell responses. We isolated splenic cells from CIA-induced mice at day 45 after immunization with CII and stimulated the cells with CII-loaded APCs *in vitro*. As shown in Figure 3A and 3B, the percentage of IFN-γ-producing $CD4^+$ T cells was higher in Imm-APCs-treated mice than in Tol-APCs-treated mice. The frequency of IL-17-producing $CD4^+$ T cells was also higher in Imm-APCs-treated mice than in Tol-APCs-treated mice but the difference was marginal. In contrast, Tol-APCs-treated mice showed higher frequencies of IL-4-producing $CD4^+$ T cells and IL-5-producing $CD4^+$ T cells than did Imm-APCs-treated mice.

The cytokine levels in the culture supernatants showed similar patterns to the frequencies of the cytokine-producing cells. The levels of IFN-γ, IL-1β and IL-17, which are related to the severity of the

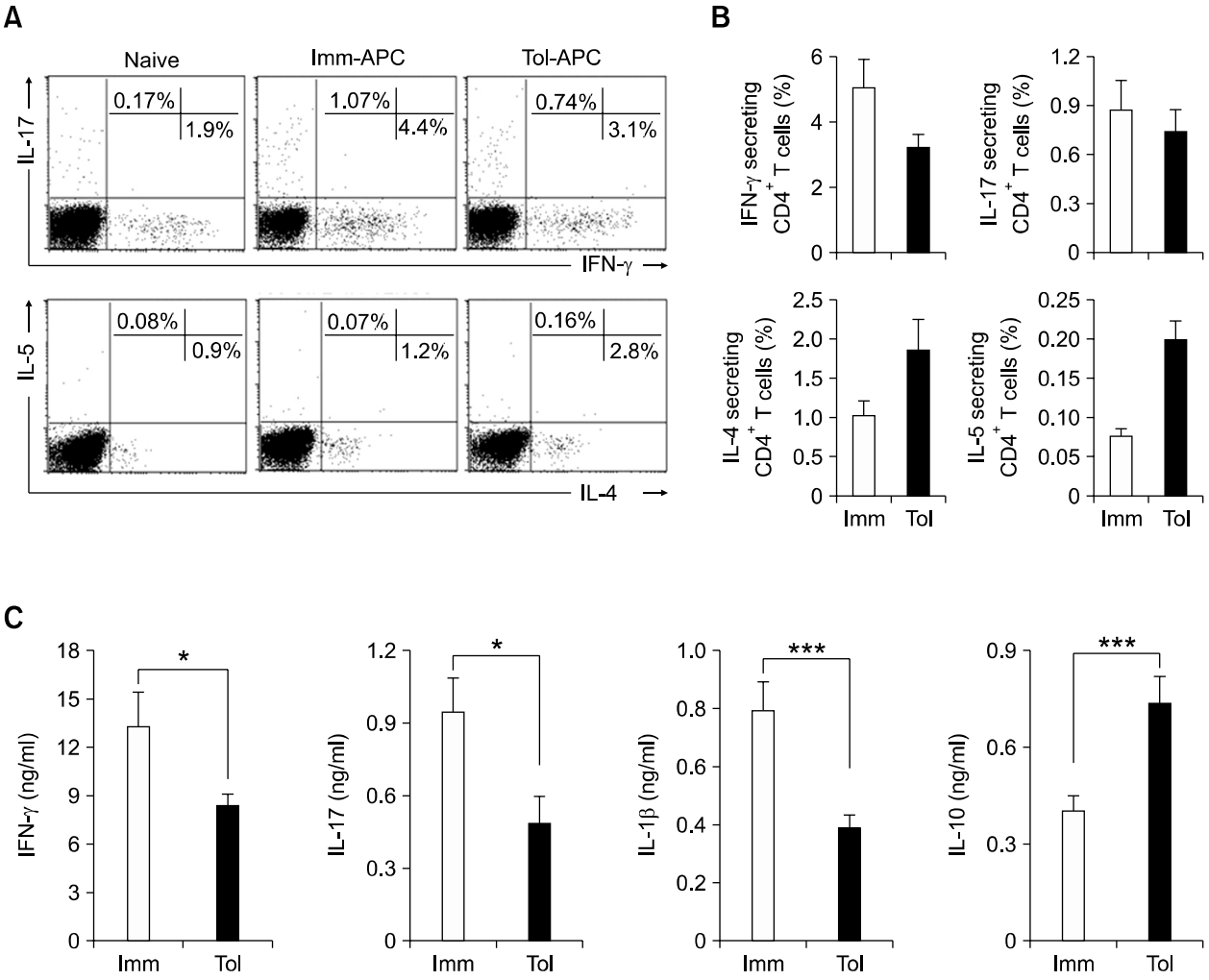

Figure 3. CII-specific T cell responses in Tol-APCs-treated and Imm-APCs-treated mice. Mice were immunized as previously described. Splenic cells were collected from CIA-induced mice on 45 days after the first immunization and re-stimulated *in vitro* with 100 μg/ml of CII. After 72 h, the cells were stained with anti-TCRβ, anti-CD4, anti-IFN-γ and anti-IL-17 mAbs or anti-IL-5 and anti-IL-4 mAbs for intracellular cytokine staining. (A) Dot plots show IFN-γ, IL-17, IL-5 and IL-4 secretion of gated $CD4^+$ T cells. (B) Bars represent the percentage of the cytokine-secreting $CD4^+$ T cells after re-stimulation for 72 h. (C) Cytokines in the culture supernatants were measured by ELISA. The results represent the mean ± SEM (6-8 mice per group). Similar results were obtained in three independent experiments. ***$P < 0.005$, *$P < 0.05$ versus Tol-APCs-treated mice.

disease (Katz *et al*., 2001; Nakae *et al*., 2003), were significantly lower in Tol-APCs-treated mice than in Imm-APCs-treated mice (Figure 3C). However, IL-10 production was meaningfully increased in the cells from Tol-APCs-treated mice when compared with those from Imm-APCs-treated mice (Figure 3C).

The concentrations of IL-4 and TGF-β_2 in the culture supernatants were not detectable in either group (data not shown). Together, these results indicated that the CII-specific Tol-APCs could control not only the suppression of inflammatory cytokines, but also the induction of inhibitory cytokines, such as IL-10, upon re-stimulation with CII antigen.

Tol-APCs reduce serum levels of anti-CII antibodies

To address whether serum levels of CII-specific Abs were altered after treatment of Tol-APCs, we measured CII-specific IgG2a and IgG1 antibody levels at day 45 after immunization with CII.

As shown in Figure 4, CII-specific total IgG was significantly lower in Tol-APCs-treated mice than in Imm-APCs-treated mice (OD: 0.66 ± 0.03 vs. 0.96 ± 0.05, $P < 0.005$). Th1 immune response-related IgG2a (OD: 0.19 ± 0.02 vs. 0.32 ± 0.02, $P < 0.005$) was significantly reduced in Tol-APCs-treated mice compared with Imm-APCs-treated mice. However, the Th2 response-related IgG1 (OD: 0.35 ± 0.06 vs. 0.19 ± 0.01, $P < 0.05$) was meaningfully elevated in Tol-APCs-treated mice compared

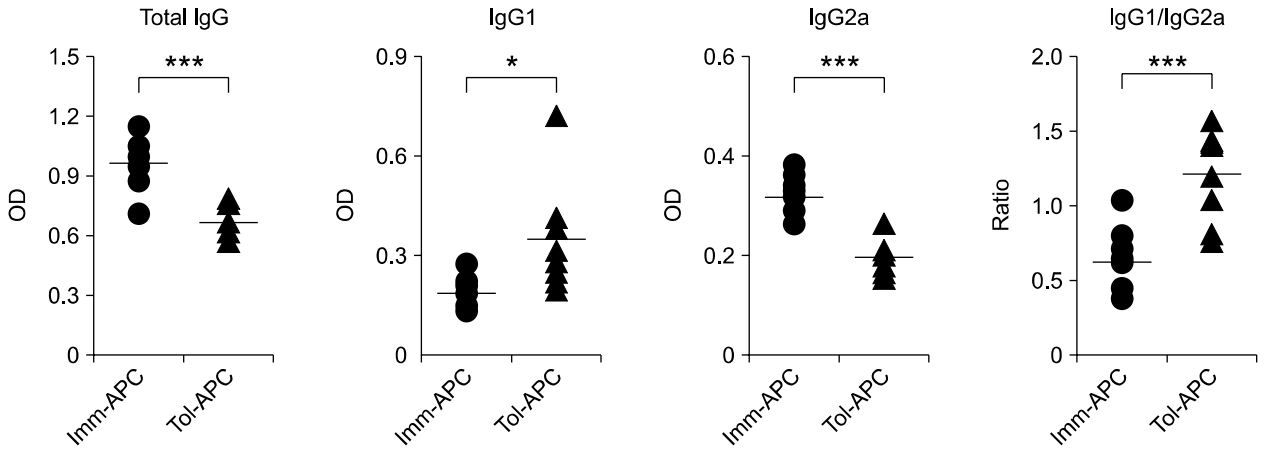

Figure 4. CII-specific antibody responses in Tol-APCs-treated and Imm-APCs-treated mice. Mice were immunized i.d. at the base of the tail with 100 μg of CII emulsified with an equal volume of CFA and boosted with 100 μg of CII in IFA on day 21. Seven days later, mice received i.v. injections of 1×10^6 Tol-APCs or Imm-APCs. The levels of CII-specific total anti-IgG, -IgG1 and -IgG2a in the serum collected at day 45 were determined by ELISA. These results are representative of three independent experiments with similar results. ***$P < 0.005$, *$P < 0.05$ versus Tol-APCs-treated mice.

with Imm-APCs-treated mice. As a consequence, the relative ratio of Th2 vs. Th1 type antibodies (IgG1/IgG2a) was also significantly elevated in Tol-APCs-treated mice compared to Imm-APCs-treated mice. These results indicate that the suppression of CIA by Tol-APCs treatment is associated with a Th2 bias of CII-reactive B cells.

Discussion

CIA has been known as a model of Th1-mediated autoimmune disease (Mauri *et al*., 1996). In this model, Th2-derived cytokines have been shown to ameliorate the disease. It has been proposed that increasing Th2 function and suppressing Th1 cells could be beneficial for the treatment of CIA (Morita *et al*., 2001; Nakajima *et al*., 2001). The induction of Ag-specific tolerance is critical for the prevention of autoimmunity and maintenance of immune tolerance. Previous studies reported that bone marrow immature dendritic cells (BMiDCs) generated in the presence of GM-CSF and IL-4 are potent immunomodulatory cells able to induce protection against autoimmune diseases (Feili-Hariri *et al*., 1999; Menges *et al*., 2002). Another study showed that repeated treatment of bovine CII-pulsed TNF-α-treated DCs were able to inhibit the development of CIA by production of Th2 cytokines, such as IL-4 and IL-5 (van Duivenvoorde *et al*., 2004).

TGF-β_2-treated Tol-APCs are known to induce anterior chamber-associated immune deviation (ACAID)-like tolerance, which is a kind of peripheral tolerance to protect the eye from destructive inflammation, that is mainly mediated by a multicellular process involving eye-derived APCs and splenic T cells (Wilbanks and Streilein, 1991), B cells (D'Orazio and Niederkorn, 1998), γδ T cells (Xu and Kapp, 2001) and NKT cells (Sonoda *et al*., 1999). Sonoda *et al*. (Sonoda*et al*., 1999) showed that CD1d-reactive NKT cells are required for the efficient development of $CD8^+$ T reg cells during the induction of ACAID and ACAID-like tolerance. It has been shown that NKT cells can not only secrete suppressive cytokines, such as IL-10 and TGF-β, to promote an immunosuppressive environment (Sonoda *et al*., 2001, 2007), but also contribute to promote immune inflammation in various disease models (Akbari *et al*., 2003; Chiba *et al*., 2005). Based on these results, NKT cells have been proposed to both promote and suppress immune responses, depending on the APC-NKT engaged environment. Several studies have shown that IL-10- and TGF-β-producing $CD4^+$ T reg and $CD8^+$ T reg cells induced by Tol-APCs are capable of suppressing autoimmune disease models (Van de Keere and Tonegawa, 1998; Menges *et al*., 2002; Faunce *et al*., 2004; Zhang-Hoover *et al*., 2005). Tol-APCs are an attractive target for immunotherapy in a variety of autoimmune diseases because their suppressive function in an Ag-specific manner can modify functions of autoantigen-specific T cells. The treatment of Tol-APCs has been shown to modulate T cell function in mice, shifting a Th1 cytokine response to a Th2-like response. The effect of Tol-APCs on the Th1/Th2 balance has been proven in a murine model of EAE (Faunce *et al*., 2004).

Therefore, we investigated whether tolerance induced by Tol-APCs could inhibit arthritis and its related systemic immune response in the murine

arthritis model. In this study, we showed that the ACAID-like tolerance by Tol-APCs could suppress the development of arthritis and its related systemic immune response in CIA. Unlike the suppression of CIA by the repeated transfer of TNF-α-treated DCs, the TGF-β_2-treated Tol-APCs could induce suppression by a single injection. We observed that the treatment of Tol-APCs reduced Th1 cytokine (IFN-γ) production and increased Th2 cytokines, such as IL-4, IL-5 and IL-10, compared with that of Imm-APCs. In addition to CII-specific T cell responses, the production of proinflammatory cytokines, such as IL-1β and IL-17, was significantly reduced in the amelioration of arthritis in Tol-APCs-treated mice. We also observed that the relative ratio of IgG1/IgG2a was meaningfully increased in Tol-APCs-treated mice. B cells also play an essential role in CIA by secreting CII-specific Abs, especially complement-fixing isotypes (Svensson *et al*., 1998). It has been shown that the levels of autoantigen CII-specific antibodies are correlated with the severity and development of arthritis (Stuart and Dixon, 1983; Wang *et al*., 1995).

Our results demonstrate that the protection from CIA using Tol-APCs was associated with a shift from Th1 responses to Th2 responses by CII-specific T cells and B cells. These types of tolerance might lead to a new therapeutic approach for rheumatoid arthritis. In conclusion, we have demonstrated that the CII-specific Tol-APCs contribute to amelioration of CIA through the inhibition of CII-specific Th1 responses during disease development.

Methods

Mice

BALB/c and DO11.10 TCR transgenic (Tg) mice (BALB/c background) were purchased from Jackson Laboratories (Bar Harbor, ME) and DBA/1 mice from Charles River Laboratories (Japan). The animals were kept under specific pathogen-free conditions and studied at 7-10 weeks of age. The experimental protocols adopted in this study were approved by the Laboratory Animal Care and Use Committee of Korea University.

CIA induction and measurement of clinical score

DBA/1 mice were immunized intradermally (i.d.) at the base of the tail with 100 μg of chicken type CII (Sigma-Aldrich) emulsified with an equal volume (50 μl) of complete Freund's adjuvant (CFA; Sigma-Aldrich) according to a standard method (Brand *et al*., 2007). The mice were boosted by i.d. injection with 100 μg of CII emulsified with incomplete Freund's adjuvant (IFA; Sigma-Aldrich) on day 21. Seven days later, the mice received intravenous (i.v.) injections of Tol-APCs (1×10^6 cells/mouse) or immunogenic-APCs (Imm-APCs; 1×10^6 cells/mouse). Mice were monitored on alternate days for arthritis development until the end of the experiment. The clinical severity of arthritis was graded as follows: 0 = normal paws, 1 = edema and erythema in only one digit, 2 = slight edema or erythema in at least some digits, 3 = slight edema involving the entire paw, 4 = moderate edema and erythema involving the entire paw, and 5 = severe edema and erythema involving the entire paw and subsequent ankylosis. The average of the macroscopic score was expressed as the cumulative value of all paws, with a maximum score of 20.

In vitro generation of immunogenic-APCs and tolerogenic-APCs

PECs were obtained from collections of peritoneal washes of BALB/c or DBA/1mice 3 days after they received an intraperitoneal (i.p.) injection of 3 ml of 3% thioglycolate solution (Sigma-Aldrich). The collected PECs were cultured overnight in serum-free medium (SFM) containing 100 μg/ml ovalbumin (OVA) protein or chicken CII (Sigma-Aldrich) and 5 ng/ml TGF-β_2 (R&D system) to generate Tol-APCs. Imm-APCs were prepared in the same manner, excluding the TGF-β_2. After culture, the APCs were washed three times with Hank's balanced salt solution (HBSS) to remove free antigens and TGF-β_2.

The remaining adherent cells were incubated at 4°C in PBS for 2 h and collected by vigorous pipetting. The cells were washed three times with HBSS and resuspended at a concentration of 1×10^7 cells/ml in HBSS (Wilbanks and Streilein, 1992). The cells were stained with anti-CD11b and CD86 antibodies and confirmed that more than 97% of the cells show typical peritoneal macrophage phenotype ($CD11b^+CD86^+$). For the transfer of APCs to CIA-induced mice, each group was given 100 μl of a cell suspension (1×10^6 cells/mouse) via the tail vein 7 days after the second immunization with CII in IFA.

In vitro OVA-specific $CD4^+$ T cell responses

Effector $CD4^+$ T cells were obtained from DO11.10 transgenic mice immunized subcutaneously (s.c.) with 100 μg of OVA protein in CFA. After 2 weeks, the primed $CD4^+$ T cells were sorted with antibody-coated magnetic beads and labeled with 5 μM CFSE. Purified $CD4^+$ T cells (5×10^5 cells/well) were added to 96-well plates containing OVA-specific Tol-APCs or Imm-APCs (5×10^4 cells/well). After 3 days, the culture supernatants were collected and analyzed for cytokines by ELISA. The culture cells were harvested for intracellular cytokine staining and proliferation assays through CFSE dilution.

Measurement of CII-specific T cell responses in vitro

To analyze the CII-specific T cell cytokine response, splenocytes were harvested from mice at day 45 after the first immunization with CII. Single-cell suspensions were prepared, and then the cells (5×10^5 cells/well in 96-well

flat-bottom plates) were re-stimulated in triplicate with 100 μg/ml of chicken CII. After 72 h, culture supernatants were collected and assessed for the presence of cytokines by ELISA (OPTEIA Mouse cytokine set: BD Pharmingen).

For intracellular cytokine staining, at day 45 after the first immunization, splenocytes were isolated from the immunized mice, and the cells (5×10^5 cells/well in 96-well flat-bottom plates) were re-stimulated with 100 μg/ml of chicken CII for 72 h and then treated with Golgi stop (BD Pharmingen). After six hours, the cells were harvested for intracellular cytokine staining. To determine the levels of intracellular cytokines, the cells were initially stained with the appropriate mAbs, fixed, permeabilized with Cytofix/Cytoperm solution (BD Pharmingen), and finally stained with APC or R-PE conjugated anti-IL-4, anti-IL-5, anti-IL-17 or anti-IFN-γ mAbs for 45 minutes at 4°C. The percentage of cells expressing cytoplasmic IL-4, IL-5, IL-17 or IFN-γ was determined via flow cytometry.

Flow cytometric analysis

Cells were stained in FACS staining buffer (PBS containing 0.1% BSA and 0.01% sodium azide), incubated for 20 min at 4°C with an anti-FcR-γ mAb (2.4G2), and labeled for an additional 30 min with appropriate mAbs (all from BD Biosciences). The following mAbs were used: TCRβ (H57), CD4 (RM4-5), CD8α (53-6.7), IFN-γ (XMG1.2), IL-4 (11B11), IL-5 (TRFK5), and IL-17 (TC11-18H10). The stained cells were analyzed on a FACSCalibur using CellQuest software (BD Biosciences).

Measurement of collagen-specific IgG titers

At day 45 after the first immunization with CII, sera were collected from immunized mice. Immunoplates (Nunc) were coated with 10 μg/ml of CII in PBS at 4°C overnight. After blocking with 5% bovine serum albumin in PBS, serially diluted (1:5,000-1:200,000) serum samples were added to CII-coated wells and incubated for 1 hour at RT. The plates were incubated with biotinylated anti-IgG1, anti-IgG2a or anti-IgG antibodies (all from BD Biosciences), and avidin-HRP was added and incubated for one hour at RT. Following a series of washes with PBS, plates were developed with TMB, and reactions were stopped by the addition of a stop solution. Absorbance values were measured using an ELISA reader (Bio-Rad) at 450 nm.

Statistical analysis

Student's *t*-tests were used to determine statistical differences between the two groups. Throughout the text, figures and legends, the following symbols are used to denote statistical significance: ***, $P < 0.001$; **, $P < 0.01$, *, $P < 0.05$.

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