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The requirement of natural killer T-cells in tolerogenic APCs-mediated suppression of collagen-induced arthritis

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Abbreviations: ACAID, anterior chamber-associated immune deviation; CIA, collagen induced arthritis; PEC, peritoneal exudate cells; NKT cells, natural killer T-cells; Tol-APC, tolerogenic antigen presenting cell; α-GalCer, α-galactosylceramide

Abstract

TGF-β-induced tolerogenic-antigen presenting cells (Tol-APCs) could induce suppression of autoimmune diseases such as collagen-induced arthritis (CIA) and allergic asthma. In contrast, many studies have shown that NKT cells are involved in the pathogenesis of Th1-mediated autoimmune joint inflammation and Th2-mediated allergic pulmonary inflammation. In this study, we investigated the effect of CD1d-restricted NKT cells in the Tol-APCs-mediated suppression of autoimmune disease using a murine CIA model. When CIA-induced mice were treated with Tol-APCs obtained from $CD1d^{+/-}$ or $CD1d^{-/-}$ mice, unlike $CD1d^{+/-}$ APCs, $CD1d^{-/-}$ Tol-APCs failed to suppress CIA. More specifically, $CD1d^{-/-}$ Tol-APCs failed to suppress the production of inflammatory cytokines and the induction of Th2 responses by antigen-specific CD4 T cells both *in vitro* and *in vivo*. Our results demonstrate that the presence of CD1d-restricted NKT cells is critical for the induction of Tol-APCs-mediated suppression of CIA.

Keywords: antigen-presenting cells; antigens; arthritis; CD1d; experimental; immune tolerance; natural killer T-cells

Introduction

Antigen presenting cells (APCs) can be either immunogenic or tolerogenic depending on their stage of maturation and their level of activation. APCs function can also be modified by treatment with cytokines such as TGF-β_2 and IL-10. TGF-β_2 is a major immunosuppressive cytokine that is present in the aqueous humor of the anterior chamber (a.c.) of the eye; it is also known to modulate the function of thioglycolate-induced peritoneal exudate cells (PECs) *in vitro* (Wilbanks and Streilein, 1992; Steinbrink *et al*., 1997). Since APCs interact directly with antigen-specific T cells, APCs that induce specific tolerance could be a very effective and specific means of targeting autoreactive T cells.

Natural killer T (NKT) cells are a unique subset of lymphocytes that co-express the T cell receptor (TCR) and NK cell receptors. NKT cells recognize glycolipid antigens such as α-GalCer, a glycosphingolipid originally isolated from marine sponges; these antigens are presented by the non-polymorphic, MHC class I-like molecule CD1d. A key property of NKT cells is their ability to secrete large amounts of cytokines rapidly, including IFN-γ and IL-4, upon stimulation with glycolipid antigens (Park *et al*., 1998; Park and Bendelac, 2000). These unique properties, among others, make NKT cells a potential therapeutic target in various infectious and autoimmune diseases (Hong *et al*., 2001; Jahng *et al*., 2001). Many studies have reported that defects or dysfunctions of NKT cells are important in autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis (MS), and Type I diabetes (Sumida *et al*., 1995; Gombert *et al*., 1996; Kojo *et al*., 2001). Furthermore, it is well known that NKT cells secrete suppressive cytokines, such as IL-10 and TGF-β (Bendelac *et al*., 1997; Steinbrink *et al*., 1997; Hong and Van Kaer, 1999; Margalit and Ilan, 2005; Sonoda *et al*., 2007), which enhance the immunosuppressive environment after the TCR

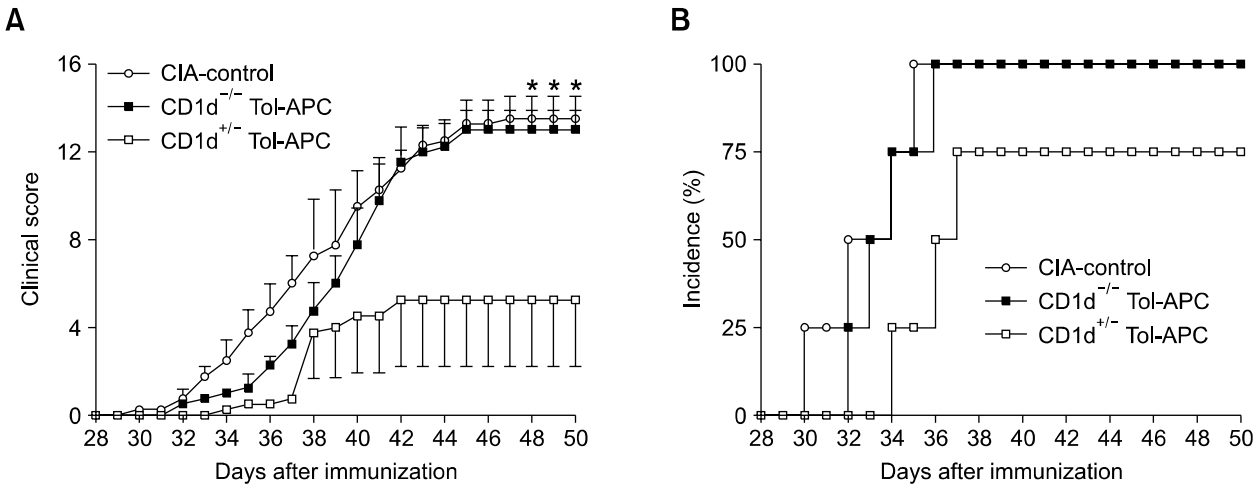

Figure 1. Treatment of Tol-APCs derived from $CD1d^{-/-}$ mice failed to reduce the severity of CIA. To induce CIA, mice were immunized by i.d. injection at the base of the tail with 100 μg of chicken CII emulsified with an equal volume of CIA. Three weeks later, the mice were boosted intradermally with 100 μg of CII in IFA. Seven days later, mice received i.v. injections of 1×10^6 $CD1d^{-/-}$ Tol-APCs (■) or $CD1d^{+/-}$ Tol-APCs (□), or no APCs 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. Bars show the mean ± SEM (6-8 mice per group). *** = $P < 0.001$ versus $CD1d^{+/-}$ Tol-APCs- treated mice.

binds to CD1d molecules on the APCs and marginal zone B cells (Sonoda *et al*., 2001; Faunce and Stein-Streilein, 2002). However, there are enough data supporting the role of NKT cells in the pathogenesis of autoimmune joint inflammation, such as seen in CIA (Chiba *et al*., 2005; Kim *et al*., 2005). Our previous study also showed that NKT cells are associated with acceleration and perpetuation of arthritic disease in a DBA/1 genetic background (Jung *et al*., 2009).

Although the effects of Tol-APCs and NKT cells on CIA have been investigated independently, their coordinated functions have not been studied.

In this study, we investigated whether CD1d-reactive NKT cells affect the course of Tol-APCs-mediated suppression of CIA. Surprisingly, our study showed that CD1d-reactive NKT cells were essential for the suppression of Th1 responses to antigen-specific $CD4^+$ T cells through Tol-APCs. This is in sharp contrast to data suggesting that NKT cells alone have a pathogenic role in CIA (Chiba *et al*., 2005; Ohnishi *et al*., 2005).

Results

Tol-APCs derived from $CD1d^{-/-}$ mice did not suppress CIA

To determine the effect of NKT cells on Tol-APCs-mediated suppression of CIA, disease-induced DBA/1 mice received i.v. injections of 1×10^6 $CD1d^{+/-}$ Tol-APCs or $CD1d^{-/-}$ Tol-APCs 28 days after immunization with chicken type II collagen (CII). Regarding disease severity, mice injected with $CD1d^{+/-}$Tol-APCs showed significantly reduced symptoms of CIA compared to those injected with $CD1d^{-/-}$ Tol-APCs (13.5 ± 1.0 vs. 5.3 ± 3.0, $P < 0.001$); reduced disease onset was also observed in these mice (33.7 days ± 0.8 vs. 35.7 days ± 0.8; Figure 1). Although both $CD1d^{-/-}$ Tol-APCs-treated and CIA control mice showed 100% incidence of disease, $CD1d^{+/-}$ Tol-APCs-treated mice showed reduced disease incidence (75%). Thus, treatment with $CD1d^{+/-}$, but not $CD1d^{-/-}$, Tol-APCs suppressed development and severity of CIA and delayed onset.

$CD1d^{-/-}$ Tol-APCs failed to reduced inflammatory cytokines and anti-CII antibodies in the serum

Next, we analyzed the amounts of inflammatory cytokines and CII-specific antibodies in the serum of CIA-induced mice after treatment with $CD1d^{+/-}$ or $CD1d^{-/-}$ Tol-APCs. As shown in Figure 2, 45 days after immunization with CII, the amounts of IFN-γ and IL-17 in the sera of CIA control mice and $CD1d^{-/-}$ Tol-APCs-treated mice were not different. However, in $CD1d^{+/-}$ Tol-APCs-treated mice, the amounts of IFN-γ and IL-17 were significantly reduced. mRNA levels of IL-17 measured from affected joints by RT-PCR also showed reduced local production of IL-17 in the $CD1d^{+/-}$ Tol-APCs-treated mice (data not shown). In addition, CII-specific total IgG was significantly lower in $CD1d^{+/-}$ Tol-APCs-treated mice than in $CD1d^{-/-}$

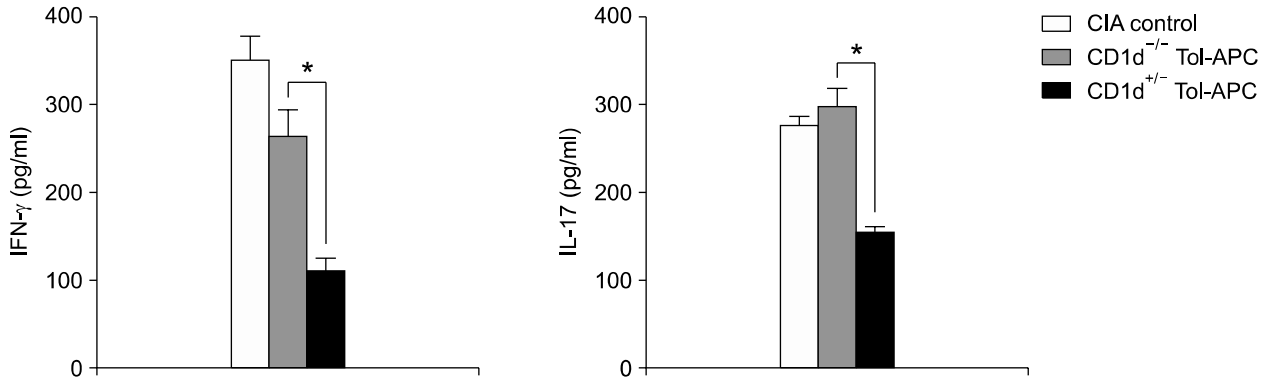

Figure 2. CD1d$^{-/-}$ Tol-APCs failed to reduce inflammatory cytokines in the serum. Sera were collected from the mice (in Figure 1) 45 days after the first immunization and their IFN-γ and IL-17 levels were measured by ELISA. These results are representative of three independent experiments. * = $P < 0.005$ versus CD1d$^{+/-}$ Tol-APCs treated mice.

Tol-APCs-treated mice (OD: 0.55 ± 0.06 vs. 0.9 ± 0.07, $P < 0.005$). More specifically, while Th1-related IgG2a (OD: 0.17 ± 0.02 vs. 0.26 ± 0.04, $P < 0.05$) was significantly reduced in CD1d$^{+/-}$ Tol-APCs-treated mice, Th2-related IgG1 (OD: 0.28 ± 0.06 vs. 0.18 ± 0.03, $P < 0.05$) was significantly elevated, compared to CD1d$^{-/-}$ Tol-APCs-treated mice (Figure 3). These results suggested that the suppression of CIA by CD1d$^{+/-}$ Tol-APCs treatment may be associated with the inhibition of Th1 or Th17 immune responses.

CD1d$^{-/-}$ Tol-APCs failed to reduce CII-specific Th1 responses

To analyze the effect of NKT cells on Th1 responses in the context of Tol-APCs treatment, CII-specific T cell responses were assessed *in vitro*. Splenocytes from CD1d$^{+/-}$ or CD1d$^{-/-}$ Tol-APCs-treated mice were isolated at 45 days after CIA-induction and were stimulated for 72 h with CII *in vitro*. The amounts of inflammatory cytokines in the culture supernatants of restimulated splenocytes showed similar patterns to those in the serum of Tol-APCs-treated animals. The levels of IFN-γ, IL-1β and IL-17, which are related to the severity of the disease (Mauri *et al.*, 1996; Miossec and van den Berg, 1997; Nakae *et al.*, 2003), were significantly lower in the culture supernatants of splenocytes from CD1d$^{+/-}$ Tol-APCs-treated mice than in those from CD1d$^{-/-}$ Tol-APCs-treated mice or CIA control mice (Figure 4). In contrast, the levels of Th2 cytokines (IL-4 and IL-10) were significantly higher in culture supernatants of splenocytes from CD1d$^{+/-}$ Tol-APCs-treated mice than in those from CD1d$^{-/-}$ Tol-APCs-treated mice (Figure 4).

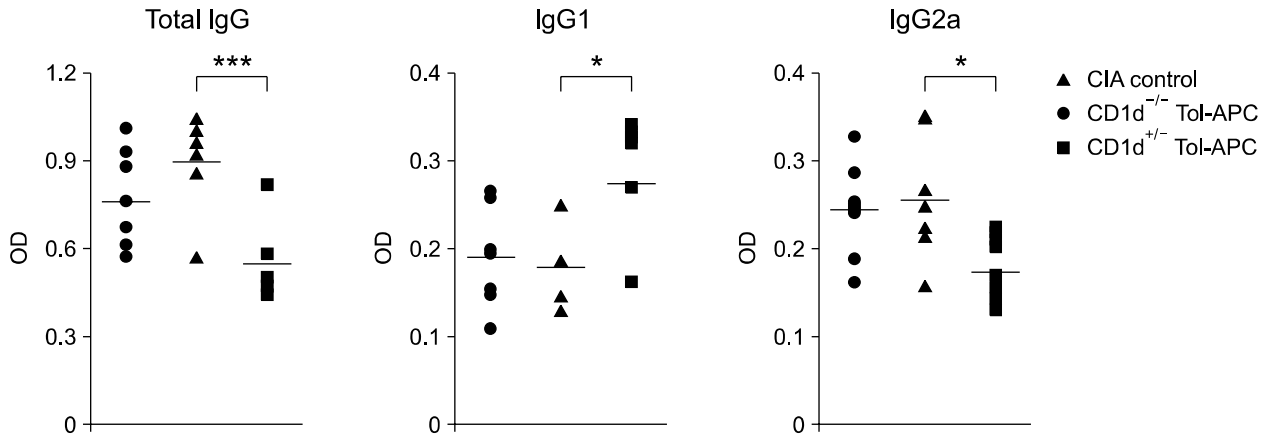

Figure 3. CII-specific antibody responses in Tol-APCs-treated mice. Mice were immunized and received Tol-APCs as described in Materials and Methods. The levels of CII-specific total anti-IgG, -IgG1 and -IgG2a in the serum collected at day 45 were determined by ELISA. These results are representative of three independent experiments with similar results. *** = $P < 0.005$, * = $P < 0.05$ versus CD1d$^{+/-}$ Tol-APCs treated mice.

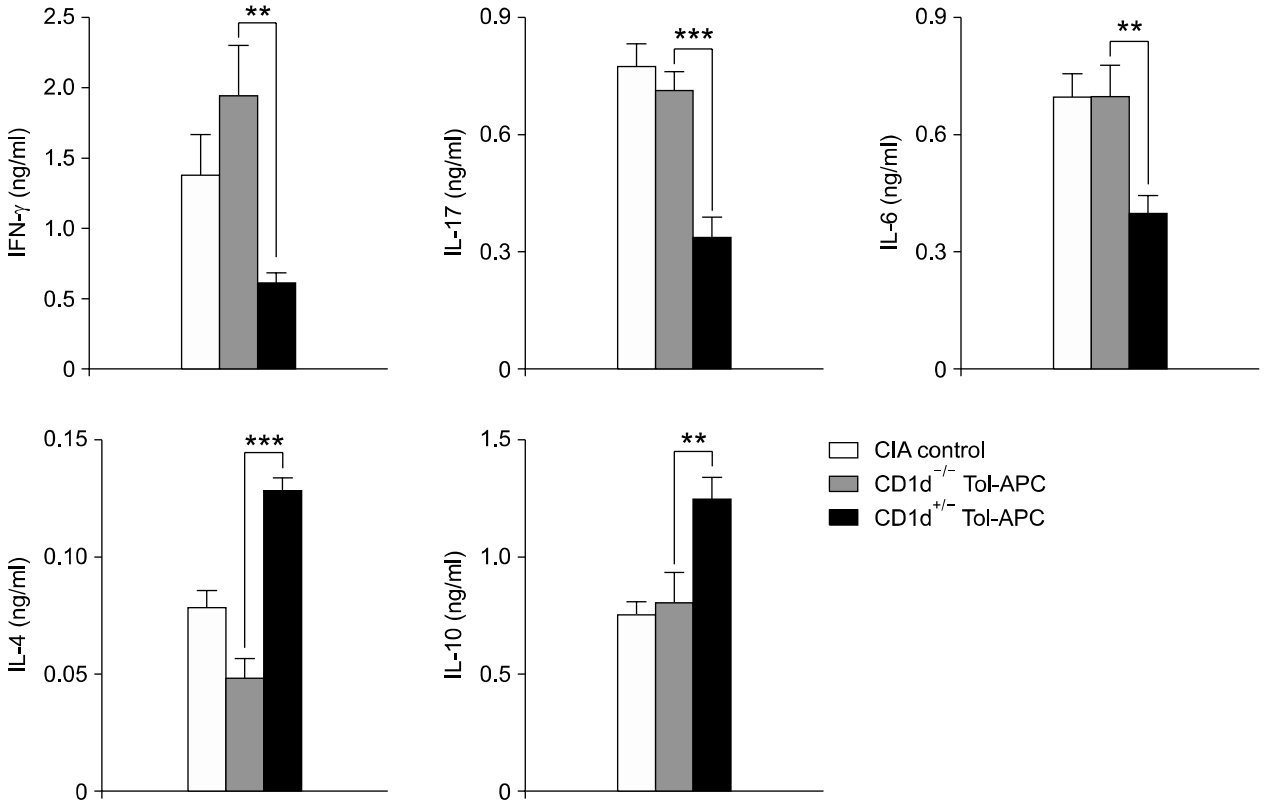

Figure 4. CII-specific T cell responses in $CD1d^{+/-}$ or $CD1d^{-/-}$ Tol-APCs-treated mice. Mice were immunized and received Tol-APCs as previously described. Splenic cells were collected from CIA-induced mice 45 days after the first immunization and were restimulated *in vitro* with 100 μg/ml of CII. After 72 h, the culture supernatants were collected and analyzed for cytokines by ELISA. The results represent the mean ± SEM (6-8 mice per group). Similar results were obtained in three independent experiments. * = $P < 0.005$, ** = $P < 0.01$ versus Tol-APCs-treated mice.

Discussion

This study demonstrated that the presence of NKT cells is required for the Tol-APCs-mediated suppression of CIA. This was rather unexpected because NKT cells are known to contribute for the pathogenesis of CIA, a Th1-mediated autoimmune joint inflammation (Chiba *et al*., 2005; Kim *et al*., 2005; Ohnishi *et al*., 2005). Treatment of CIA-induced mice with Tol-APCs obtained from DBA/1$CD1d^{+/-}$ mice reduced disease severity and delayed its onset, compared to $CD1d^{-/-}$ Tol-APCs. We also found that $CD1d^{+/-}$ Tol-APCs mediated not only the inhibition of inflammatory cytokines and the production of the anti-CII-specific antibody, but also the induction of Th2 responses of the antigen-specific $CD4^+$ T cells.

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). CIA and RA are both mediated by the dominant activation of Th1 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). Unbalanced Th1/Th2 T-cell polarization has been suggested to play a pathogenic role in the development of this type of disease. It has also been proposed that increasing Th2 function and suppressing Th1 cells could be beneficial to the treatment of CIA (Morita *et al*., 2001; Nakajima *et al*., 2001).

NKT cells rapidly secrete large amounts of various cytokines, characteristic of both Th1 and Th2 responses after activation by their ligands, including α-GalCer and its analogs (Mars *et al*., 2004; Van Kaer, 2004). The rapid production of cytokines by activated NKT cells has been shown to ameliorated or worsen the progression of several diseases, including type I diabetes, colitis, asthma, EAE, tumor rejection, and various infectious diseases (Hong *et al*., 2001; Jahng *et al*., 2001; Singh *et al*., 2001). On the other hand, studies aimed at defining the role of NKT cells and the protective effects of their ligands in CIA have generated conflicting results (Chiba *et al*., 2004; Miellot *et al*., 2005). In addition, many studies suggested that NKT cells may be involved in the

pathogenesis of autoimmune joint inflammation (Chiba *et al*., 2005; Kim *et al*., 2005; Ohnishi *et al*., 2005). Our recent results using DBA/1 mice, a well-known model of CIA, also showed that NKT cells contributed to disease progression in the natural state.

Because NKT cells show either protective or pathogenic effects on various autoimmune diseases, it is possible that there are functionally distinct subsets of NKT cells. In fact NKT cells could be classified into several subsets based on their phenotypes and their ability to secrete cytokines (Park *et al*., 2001; Jahng *et al*., 2004; Michel *et al*., 2007). Furthermore, it seems that they contribute to various diseases depending on the prevailing conditions of the activation state, residing organs, cell subset and the APC-NKT-engaged environment (Coppieters *et al*., 2007; Pichavant *et al*., 2008). However, the obvious discrepancy of the NKT cell effects on the murine CIA disease model requires clarification of the situations in which these cells can exert their protective or pathologic effects.

Therefore, it is informative that NKT cells are known to be crucial for the development of anterior chamber-associated immune deviation (ACAID) and are required for induction of mature T reg cells by secretion of IL-10 (Sonoda *et al*., 2001). In addition, TGF-β_2-treated Tol-APCs are known to induce ACAID-like tolerance, a type of peripheral tolerance that protects the eye from destructive inflammation and that is mainly mediated by multicellular processes involving eye-derived APCs and splenic T (Wilbanks and Streilein, 1991), B (D'Orazio and Niederkorn, 1998), $\gamma\delta$ T (Xu and Kapp, 2001) and NKT cells (Sonoda *et al*., 1999).

Induction of antigen-specific tolerance is critical for the prevention of autoimmunity and maintenance of immune tolerance. Tol-APCs are an attractive target for immunotherapy in a variety of autoimmune diseases because the treatment of Tol-APCs may modulate T cell function in mice in an antigen-specific manner, shifting a Th1 response to a Th2-like response (Faunce *et al*., 2004).

In contrast, a recent report clearly demonstrated that NKT cells strengthen and optimize ongoing immune responses of antigen-specific $CD8^+$ cytotoxic T lymphocytes, (Hong *et al*., 2009) as well as pre-established Th1 and Th2 $CD4^+$ T cells (Shin *et al*., 2010).

However, in a specific immunological intervention as the interaction with TGFβ_2-induced Tol-APCs, NKT cells can be transformed to tolerogenic status in which they increased the production of IL-10 and decreased inflammatory cytokines such as IFN-γ and IL-17.

These findings, although NKT cells by themselves can strengthen the autoantigen-specific Th1 $CD4^+$ T cell response in CIA, suggested that NKT cells enhance Tol-APCs-induced antigen-specific Th2 responses in the same disease condition. The balance between their effects on pathogenesis and protection of CIA might explain the outcome of the disease in a specific pathophysiological condition.

Although NKT cells can contribute to the development of CIA, the presence of NKT cells is critical for the disease suppression induced by Tol-APCs. Furthermore, because these results were obtained without using artificial NKT cell ligands such as α-GalCer, they probably reflect the physiological functions of NKT cells. Thus, the use of Tol-APCs coupled with the modulation of NKT cell functions might lead to a new therapeutic approach for chronic autoimmune diseases such as human RA.

Methods

Mice

DBA/1 mice were purchased from Charles River Laboratories (Japan). $B6CD1d^{-/-}$ mice were backcrossed more than eight times into DBA/1 mice to generate $DBA/1CD1d^{-/-}$ mice. All results reported in this study were derived from comparative analyses of littermates expressing +/- vs. -/- genotypes. The animals were kept under specific pathogen-free conditions and were 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 by intradermal (i.d.) injection at the base of the tail with 100 μg of chicken type II collagen (CII; Sigma-Aldrich) emulsified with an equal volume (50 μl) of complete Fruend's adjuvant (CFA; Sigma-Aldrich). The mice were boosted by i.d. injection of 100 μg of CII emulsified with incomplete Fruend's adjuvant (IFA; Sigma-Aldrich) on day 21. Seven days later, the mice received intravenous (i.v.) injections of 1×10^6 Tol-APCs obtained from $CD1d^{+/-}$ or $CD1d^{-/-}$ mice. Mice were monitored for arthritis development on alternate days 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 Tol-APCs

PECs were obtained from collections of peritoneal washes

of $CD1d^{+/-}$ and $CD1d^{-/-}$ of DBA/1 mice 3 days after intraperitoneal (i.p.) inoculation with 3 ml of 3% thioglycolate solution (Sigma-Aldrich). The collected PECs were cultured overnight in serum-free medium (SFM) containing 100 μg/ml chicken CII and 5 ng/ml TGF-β_2 (R&D Systems) to generate Tol-APCs. After culturing, the APCs were washed three times with Hank's balanced salt solution (HBSS) to remove free antigen and TGF-β_2. The remaining adherent cells were incubated at 4°C in PBS for 2 h and then collected by vigorous pipetting. Cells were washed three times with HBSS and resuspended at a concentration of 1×10^7/ml in HBSS. The phenotypes and purities of resulting APCs were confirmed by FACS. More than 97% of APCs from both CD1d+ and CD1d- mice showed CD11b+ MHC-II+ macrophage phenotype. For the transfer of APCs to CIA-induced mice, each mouse 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.

Measurement of serum cytokines and collagen-specific Ig titers

Forty-five days after the first immunization with CII, sera were collected from immunized mice for measurement of serum cytokines and anti-CII specific antibodies. Individual serum samples from the immunized mice were collected and assessed for the presence of IFN-γ (OPTEIA Mouse cytokine set, BD Pharmingen) and IL-17 (R&D Systems) by enzyme-linked immunosorbant assay (ELISA).

Immunoplates (Nunc) were coated with 10 μg/ml of CII in PBS and incubated 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 h at RT. The plates were incubated with biotinylated anti-IgG1, anti-IgG2a or anti-IgG antibodies (all from BD Biosciences). Avidin-HRP was then added and incubated for 1 h at RT. Following a series of washes with PBS, plates were developed with TMB and the reactions were terminated by adding stop solution. Absorbance values were measured using an ELISA reader (Bio-Rad) at 450 nm.

Measurement of CII-specific T cell responses *in vitro*

To analyze the CII-specific T cell cytokine response, splenocytes were harvested from mice 45 days after the first immunization with CII. Single-cell suspensions were prepared, and the cells (5×10^5 cells/well in 96-well flat-bottom plates) were restimulated in triplicate with various concentrations of chicken CII (0, 25, 50 and 100 μg/ml). After 72 h, culture supernatants were collected and assessed for the presence of cytokines by ELISA (OPTEIA Mouse cytokine set, BD Pharmingen).

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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