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A distinct tolerogenic subset of splenic IDO$^+$CD11b$^+$ dendritic cells from orally tolerized mice is responsible for induction of systemic immune tolerance and suppression of collagen-induced arthritis

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ABSTRACT

In oral tolerance, locally instigated tolerance in the gut propagate to systemic tolerance. In order to investigate the mechanism, we analyzed indoleamine 2,3–dioxygenase (IDO) expression in splenic dendritic cell (DC) subsets and tested whether DCs suppress collagen-induced arthritis (CIA) by inducing regulatory T cells (Tregs). The proportion of IDO-expressing cells was higher in the CD11b$^+$ subset of splenic DCs from orally tolerized CIA mice. These DCs suppressed type II collagen-specific T cell proliferation and promoted Treg induction from CD4$^+$CD25$^-$ T cells using transforming growth factor-β. These DCs also increased the expression of cytotoxic T lymphocyte antigen-4 and programmed death-1 on Tregs. When adoptively transferred, spenic IDO-expressing CD11b$^+$ DCs from tolerized animals suppressed the development of arthritis, increased the Treg/Th17 cell ratio, and decreased the production of inflammatory cytokines in the spleen. Taken together, a distinct subset of splenic IDO$^+$CD11b$^+$DCs is responsible for the systemic immune regulation in oral tolerance.

1. Introduction

Oral administration of antigen suppresses the immune response to the fed antigen by stimulating the gut-associated lymph tissue (GALT) such as Peyer's patches (PPs), lamina propria, and mesenteric lymph nodes (mLNs) [1–4]. Dendritic cells (DCs) are the major player in this type of immune suppression and DCs in the GALT display tolerogenic characteristics. However, little is known about the biological characteristics of DCs in the spleen and how they exert their systemic effect after induction of oral tolerance.

Regulatory T cells (Tregs) play an important role in immune regulation and in preventing autoimmunity, and are indispensable to the development of oral tolerance [5,6]. Tregs are usually induced by DCs in GALT [7]. We reported previously that the DCs with the CD11b$^+$ phenotype in PPs can induce CD4$^+$ T cells to differentiate into antigen-induced Tregs in orally tolerized mice with collagen-induced arthritis (CIA) [8]. However, the biological characteristics of the major tolerance-inducing DC subsets in the spleen, and whether these cells exert their regulatory function under inflammatory conditions such as CIA remain unknown.

DC populations in each specific lymphoid tissue possess distinct characteristics that reflect the disparity in their immunological environments [9]. Each lymphoid organ is thought to prime different helper T cell responses. For example, freshly isolated DCs from PPs, especially the CD11b$^+$ myeloid subset, produce IL–10 and induce the differentiation of Th2-type cells, whereas splenic DCs induce mainly Th1 cytokines [10,11]. However, injection of splenic DCs purified from ovalbumin-fed mice transfers the immune suppression of oral tolerance toward this antigen [12]. These seemingly contradictory results may be explained by more detailed characterization of tolerogenic DCs. We have demonstrated that among DCs from PPs, the subset expressing the immune-regulatory enzyme

Abbreviations: APCs, antigen-presenting cells; CIA, collagen-induced arthritis; CII, type II collagen; DCs, dendritic cells; ELISA, enzyme-linked immunosorbent assay; GALT, gut-associated lymph tissue; IDO, indoleamine 2,3-dioxygenase; MFI, mean fluorescence intensity; mLN, mesenteric lymph node; 1-MT, 1-methyltryptophan; pDCs, plasmacytoid dendritic cells; PPs, Peyer's patches; Th17, T helper 17 cells; Tregs, regulatory T cells.

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indoleamine 2,3–dioxygenase (IDO) is crucial for the induction of type II collagen (CII)-mediated oral tolerance and suppression of arthritis in a CIA mouse model [13]. We were interested in identifying the subset of splenic DCs mainly responsible for the IDO production, Treg differentiation and consequent induction of systemic immune tolerance.

The potential role of IDO in immune suppression first gained attention because of its involvement in Treg induction [14]. IDO is an enzyme that catabolizes tryptophan and produces several metabolic products including kynurenine, which inhibits the proliferation of effector T cells [15]. One subset of GALT DCs that expresses IDO is the $CD103^+$ population, which was reported to induce $Foxp3^+$ Treg differentiation and to inhibit IL–17 production [16]. The same study showed that deregulation of IDO activity caused impaired oral tolerance and increased intestinal inflammation. IDO has also been linked to the tolerogenic properties of plasmacytoid DCs (pDCs) isolated from tumor-draining lymph nodes [17]. By contrast, IDO-expressing cells are found only in the mLNs and not in the spleens of normal mice under physiological conditions [18]. In the CIA mouse model, the incidence and severity of CIA was significantly higher in mice treated with 1–methyl tryptophan (1–MT), a chemical inhibitor of IDO [19]. In another study, IDO-deficient mice had a higher incidence of arthritis and exacerbated disease severity compared with IDO-competent mice [20]. These results emphasize the importance of IDO to immune tolerance. However, studies of IDO have examined its local expression only, for example in the gut or a tumor, and the pattern of IDO expression in the peripheral immune system under inflammatory conditions is unknown.

To understand the biological characteristics of tolerogenic DCs in peripheral lymphoid organs, we examined the characteristics of IDO-expressing DC subsets in the spleens of orally tolerized CIA mice. We focused on whether the IDO^+ DCs subset from tolerized CIA mice can promote Treg differentiation and thereby regulate the immune response. We found that a splenic subset of IDO-expressing $CD11b^+$ DCs is a major player in the immune regulation in response to experimental arthritis after induction of oral tolerance.

2. Materials and methods

2.1. Mice

Male DBA1/J mice (SLC, Inc., Shizuoka, Japan), 7 weeks of age, were maintained under specific pathogen-free conditions and fed standard laboratory mouse chow (Ralston Purina, St. Louis, MO) and water *ad libitum*. All mice were treated according to the regulations of the Catholic Ethics Committee of the Catholic University of Korea.

2.2. Preparation of CII

Bovine CII was kindly provided by Professor Andrew Kang of the University of Tennessee. CII was extracted in its native form from the articular cartilage of fetal calf and was purified as described previously [21].

2.3. Induction of oral tolerance and arthritis in DBA/1 mice

The mice used in this study were divided into three groups: wild-type, tolerized (CII-fed) CIA, and untolerized (saline-fed) CIA mice. To induce CIA, DBA1/J mice were injected in the base of the tail with 100 μg of CII emulsified in complete Freund's adjuvant (CFA). The tolerized group was fed 100 μg of bovine CII using an oral Zonde needle (Natsume, Tokyo, Japan) every 2 days for 2 weeks, beginning 2 weeks before the tail injection of CII to induce CIA. Mice in the untolerized CIA group were fed an equal volume of saline instead of CII through the same administration schedule.

2.4. Cell isolation

Mononuclear cells from the spleens were incubated with anti-mouse CD11c-conjugated microbeads (Miltenyi Biotec, Auburn, CA) and subjected to positive selection through magnetic-activated cell sorting. The DCs obtained were incubated with anti-CD11c and anti-CD11b after FcR blocking. $CD11b^+$ and $CD11b^-$ DC subsets were sorted on the basis of their expression of CD11c and CD11b using a Vantage FACSorter (BD Biosciences). The purity of the sorted DCs was > 95%.

To isolate $CD4^+CD25^-$ T cells, mononuclear cells from the spleens of tolerized CIA mice were stained with a mixture of anti-CD4 and –CD25 monoclonal antibodies (mAbs) (BD Pharmingen, San Diego, CA) and sorted. The purity of the sorted $CD4^+CD25^-$ cells was 95–99% as evaluated by flow cytometry.

2.5. Flow cytometry

Single mononuclear cells were prepared from the spleens of each group of mice, stained with mAbs to CD11c, CD11b, CD8α, CD19, pDC, MHC II, CD80, CD86, programmed death ligand 1 (PD–L1), and PD–L2 after FcR blocking, permeabilized, and fixed with CytoPerm/CytoFix (BD Pharmingen) as instructed by the manufacturer. Cells were stained further with rabbit anti-IDO polyclonal antibody (Transgenic Inc., Kobe, Japan), followed by PE-conjugated goat anti-rabbit Ig, and then subjected to flow cytometric analysis (FACSCalibur, BD Biosciences, San Jose, CA). Rabbit IgG was used as the corresponding isotype antibody control.

To identify Tregs, expanded T cells were stained first with mAbs to CD4, CD25, Inducible costimulator (ICOS), Glucocorticoid induced TNF related (GITR), and Programmed death-1 (PD–1), and then with mAbs to cytotoxic T lymphocyte antigen 4 (CTLA–4) and Foxp3 using the regulatory T Cell Staining Kit (eBioscience, San Diego, CA). Events were collected and analyzed with FlowJo software (TreeStar).

2.6. Confocal microscopy

Spleens were removed 5 weeks after tail injection of CII and were snap-frozen in liquid nitrogen and stored at −80 °C. Tissue sections (7 mm) of spleens were fixed in 4% paraformaldehyde and stained using FITC-labeled anti-CD11b mAb (BD Biosciences, San Diego, CA), biotinylated anti-IDO mAb (BD Biosciences, San Jose, CA), streptavidin–Cy3 in PBS, and allophycocyanin-labeled anti-CD11c mAb (BD Biosciences). After overnight incubation at 4 °C, stained sections were analyzed by confocal microscopy (LSM 510 Meta; Carl Zeiss, Göttingen, Germany).

2.7. Measurement of the CII-specific T cell proliferative response

Mice were euthanized 5 weeks after CII injection in the tail. $CD11b^+$ DCs (1×10^4 cells) isolated from splenic mononuclear cells of CII-fed tolerized or saline-fed untolerized CIA mice were cultured for 3 days with irradiated antigen-presenting cells (APCs) (1×10^5 cells) and CII–reactive $CD4^+$ T cells (1×10^5 cells) obtained from the spleens of untolerized CIA mice. Cells were pretreated with 1–MT (250 μM), an IDO–specific inhibitor, 2 h before CII stimulation. Eighteen hours before the termination of culture, 0.5 μCi of [^{3}H] thymidine (New England Nuclear, Boston, MA) was added to each well. Cells were harvested onto glass fiber filters and counted in a Matrix-96 direct ionization counter (Packard Instrument Co., Downers Grove, IL). Data are presented as the mean cpm of triplicate cultures.

2.8. Measurement of IDO enzymatic activity

The IDO enzyme assay was performed as reported previously [22]. In brief, the concentration of kynurenine, a tryptophan metabolite, was measured in the collected coculture supernatants. For the assay, 30 μl of 30% trichloroacetic acid was added to 60 μl of culture supernatant, and the mixture was vortexed and centrifuged at 12,000 rpm for 5 min. 40 μl of supernatant was added to an equal volume of Ehrlich reagent (5 μl of glacial acetic acid and *p*–dimethylaminobenzaldehyde). The OD was measured at 492 nm. Purified l-kynurenine (0–500 μM; Sigma–Aldrich, St Louis, MO) was used as a standard.

2.9. Detection of cytokine production by ELISA

The concentrations of IL-1β, IL-6, IL-10, IL-12, IL-21, TNF-α, and TGF-β in the culture supernatant were measured using an ELISA kit as described by the manufacturer (R&D Systems, Minneapolis, MN).

2.10. In vitro induction of Tregs

Mononuclear cells from spleens of tolerized CIA mice were incubated with anti-mouse CD4-conjugated microbeads (Miltenyi Biotec, Auburn, CA) and then subjected to positive selection through magnetic-activated cell sorting. Sorted $CD4^+$ T cells (2×10^5 cells) from tolerized CIA mice were cultured in the presence or absence of CII for 3 days with $CD11b^+$ or $CD11b^-$ DCs (2×10^4 cells) isolated from splenic mononuclear cells from tolerized and untolerized CIA mice. Some DCs were pretreated with 1-MT (250 μM) for 2 h. After 3 days, the cultured cells were harvested and stained with mAbs to Treg-specific markers.

2.11. In vitro conversion of $CD4^+CD25^+$ Tregs and measurement of their suppression

To isolate $CD4^+CD25^-$ T cells, mononuclear cells from the spleens of tolerized CIA mice were stained with a mixture of anti-CD4 and -CD25 mAbs (BD Pharmingen) and sorted using the Vantage FACSorter (BD Biosciences). The purity of the sorted $CD4^+CD25^-$ cells was 95–99% as evaluated by flow cytometry. $CD11b^+$ DCs (2×10^4 cells) isolated from splenic mononuclear cells of tolerized or untolerized CIA mice were cultured in the presence or absence of CII for 4 days with $CD4^+CD25^-$ T cells (2×10^5 cells) obtained from the spleens of tolerized CIA mice. Some DCs were pretreated with 1–MT (250 μM) for 2 h. To measure the amount of intracellular Foxp3 and IL–17 in $CD4^+CD25^+$ T cells, $CD4^+$ T cells cultured with DCs were surface stained with anti-mouse CD4 and CD25 mAbs, and then with anti-mouse Foxp3 or IL–17 mAbs (eBioscience, San Diego, CA).

Converted $CD4^+CD25^+$ T cells (suppressors) were cultured with $CD4^+CD25^-$ T cells (responders), APCs (1×10^5) ,and 40 μg/ml of CII for 4 days. $CD4^+CD25^+$ T cells were added in varying numbers to generate suppressor-to-responder ratios of 1:1, ½:1, ¼:1, 1/s:1, and 0:1. In the last 18 h of culture, 0.5 μCi of [^{3}H] thymidine was added. The proliferative responses were measured by the amount of incorporated [^{3}H] thymidine.

2.12. Adoptive transfer

$CD11b^+$ DCs were isolated from splenic mononuclear cells of tolerized and untolerized CIA mice 5 weeks after tail injection of CII to induce CIA. $CD11b^+$ DCs (5×10^5 cells/mouse) were treated in vitro with 40 μg/ml of CII with or without 1-MT (250 μM) for 24 h. Cells were pretreated with 1–MT for 2 h. CII–pulsed $CD11b^+$ DCs were adoptively transferred into CIA mice via intravenous injection into a tail vein.

2.13. Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism, version 5.00 for Windows (GraphPad Software, San Diego, CA). The arthritis scores at different times were compared between groups using the nonparametric Mann–Whitney *U* test. Differences between groups were analyzed using an unpaired Student's paired or unpaired *t* test, assuming equal variances. A P value < 0.05 was considered significant.

3. Results

3.1. IDO was highly expressed in $CD11b^+$ DCs from PPs and spleens in orally tolerized CIA mice

The severity of arthritis was significantly lower in orally tolerized CIA mice than in untolerized CIA mice throughout the examined period (Fig. 1A). We demonstrated previously a greater number of IDO-expressing DCs in the PPs of orally tolerized CIA mice compared with untolerized controls [13]. To define further the association between IDO expression and immune suppression in orally tolerized mice, we examined IDO expression in DCs isolated from the spleens and PPs of orally tolerized (CII-fed) CIA mice, untolerized (saline-fed) CIA mice, and wild-type controls. In the total $CD11c^+$ DC population from spleens and PPs, IDO expression was higher in CIA mice than in wild-type mice. IDO expression was highest in $CD11c^+$ DCs from the spleens and PPs of tolerized CIA mice (Fig. 1B). The level of TGF-β in the culture supernatants was increased in CII-reactive cells cultured with DCs from PPs and spleens of tolerized mice. This increase in TGF-β production was supposed to be associated with high expression of IDO by $CD11c^+$ DCs under the inflammatory condition of CIA (Fig. 1C).

3.2. IDO expression was greatest in the splenic $CD11b^+$ DC subset from tolerized CIA mice

To identify the characteristics of IDO-expressing DCs in the spleen, we compared the IDO expression in $CD11b^+$ DCs, $CD8\alpha^+$ DCs, and pDCs from the three experimental groups of mice (Fig. 2A). In tolerized CIA mice, IDO expression was significantly greater in $CD11b^+$ DCs than in $CD8\alpha^+$ DCs. IDO expression was also higher in pDCs than in $CD8\alpha^+$ DCs but was lower than in $CD11b^+$ DCs. The relative difference in IDO expression between tolerized and untolerized groups was much larger in $CD11b^+$ DCs than in pDCs. These data indicate that the $CD11b^+$ subset was the major contributor of increased IDO expression in tolerized CIA mice. The mean fluorescence intensity (MFI) of IDO expression and the number of IDO positive cells were the highest in $CD11b^+$ cells among the DC subsets (Fig. 2B, 2C). IDO expression did not differ between $CD11b^-$ DCs and $CD8\alpha^+/^-$ DCs in the three groups. Cells sorted for $CD19^+$ DCs did not display a noticeable difference in IDO expression (data not shown). The increased IDO expression in the splenic $CD11b^+$ DC subset of tolerized CIA mice was also observed by confocal microscopy (Fig. 2D).

3.3. Splenic IDO^+CD11b^+ DCs from tolerized CIA mice exhibited reduced MHCII and CD80 expression

Next, we examined whether the IDO^+ DCs induced by oral tolerance are phenotypically distinct from IDO^- DCs. The expression of

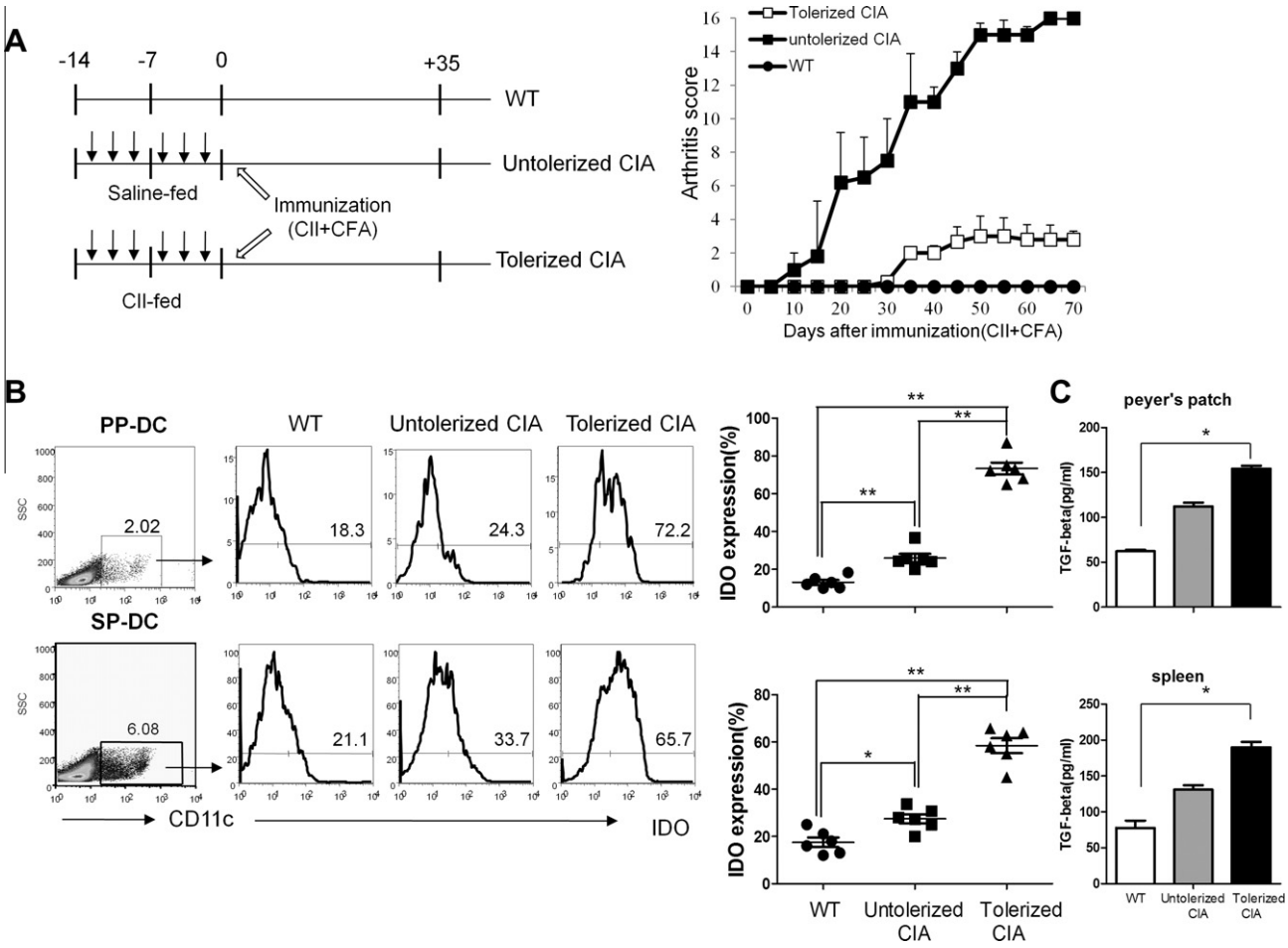

Fig. 1. Proportion of IDO-expressing DCs in Peyer's patches and the spleen. (A) Scheme for the induction of CIA and oral tolerance. To generate collagen-induced arthritis (CIA), DBA/1 J mice were immunized (open arrows) by injection at the tail base using type II collagen (CII) emulsified with complete Freund's adjuvant (CFA). To induce tolerance, mice were given six consecutive feedings of 100 μg CII before tail injection (filled arrows). These mice were designated the tolerized CIA group. Mice fed with saline instead of CII then injected for CIA were designated the untolerized CIA group. Normal DBA1/J mice were examined in parallel with the wild-type (WT) control group. The graph on the right shows the arthritis scores of the three groups. (B) Flow cytometric analysis of IDO-expressing cells among $CD11c^+$ DCs isolated from the Peyer's patches (PP–DC) and spleen (SP–DC). Cells were retrieved from the three groups, gated on $CD11c^+$ DCs, and probed for intracellular IDO. The proportion of IDO^+ cells is shown in the dot plot on the right ($^*P < 0.01$, $^{**}P < 0.001$). (C) The level of TGF-β in the supernatant of DCs cocultured with T cells was measured by ELISA ($^*P < 0.05$). The data are expressed as the mean ± SD from three independent experiments for six mice per group.

MHC II and CD80 was significantly lower in IDO^+CD11b^+ splenic DCs from tolerized CIA mice than from untolerized CIA mice (Fig. 3A, 3B). By contrast, the level of CD86 expression in IDO^+CD11b^+ DCs did not differ significantly between tolerized and untolerized mice. The levels of MHC II and CD80 were significantly lower in IDO^+CD11b^+ DCs than in IDO^-CD11b^+ DCs in tolerized CIA mice, but the CD86 level did not differ between these populations. Interestingly, the expression of PD–L1 and PD–L2 tended to be higher in the $CD11b^+$ DC subset from tolerized CIA mice, although the difference was not significant (data not shown). Together, these results indicate that the splenic DC subset of IDO^+CD11b^+ cells from tolerized CIA mice exhibits an immature phenotype.

3.4. IDO was required for the suppression of CII-reactive T cell proliferation by $CD11b^+$ DCs

To identify the immunological functions of IDO^+CD11b^+ DCs from tolerized CIA mice, we cultured $CD4^+$ T cells from CIA mice with $CD11b^+$ or $CD11b^-$ DCs from the spleens of tolerized and untolerized CIA mice in the presence of CII (Fig. 4A). The proliferation of CII-reactive T cells was assessed by measuring the incorporation of $[^3H]$ thymidine. $CD11b^+$ DCs from tolerized CIA mice significantly reduced the proliferation of T cells, whereas $CD11b^-$ DCs from tolerized CIA mice and/or DCs from untolerized mice did not exert such effect. This suppression was reversed by pretreatment of $CD11b^+$ DCs from tolerized CIA mice with 1–MT, a chemical inhibitor of IDO, suggesting that IDO was responsible for the suppression of T cell proliferation. Supporting this suggestion, the kynurenine level in the culture supernatant was highest when T cells were cultured with CII and $CD11b^+$ DCs from tolerized CIA mice (Fig. 4B). The increment in kynurenine level was abrogated by pretreatment of DCs with 1–MT. Taken together, these data show that $CD11b^+$ DCs from tolerized CIA mice suppressed the CII-induced proliferation of T cells through an IDO-dependent mechanism.

3.5. Splenic IDO^+CD11b^+ DCs from tolerized CIA mice promoted in vitro induction of Tregs in a TGF-β-dependent manner

Many investigators have reported that IDO-expressing DCs can induce and stimulate the differentiation of Tregs [13,16,23]. To examine whether the splenic $CD11b^+$ DCs from tolerized CIA mice could increase the Treg population, we measured the proportion of

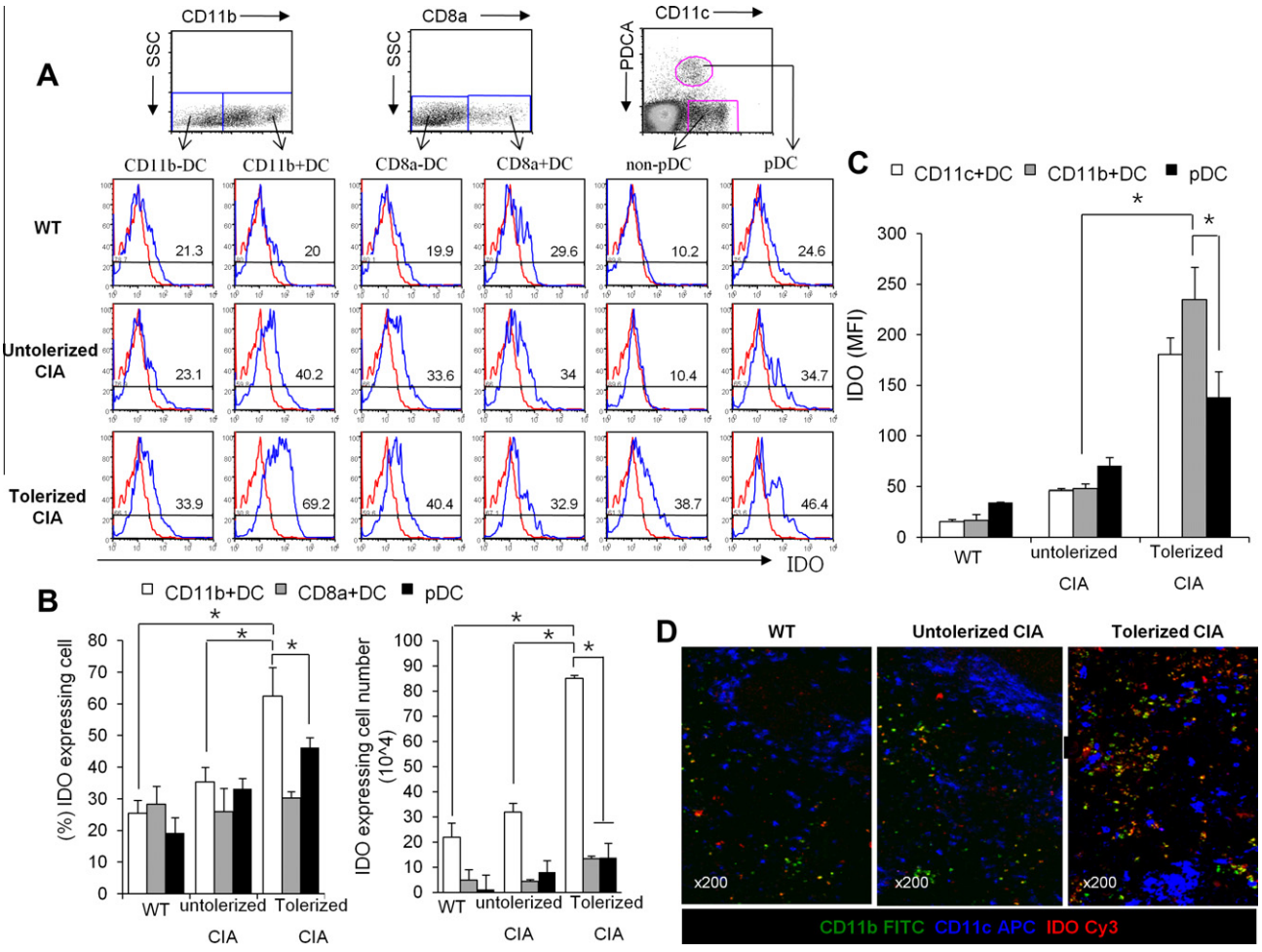

Fig. 2. Assessment of IDO expression in each subset of splenic DCs. (A) $CD11c^{high}$ DCs from the spleens of WT, untolerized CIA, and tolerized CIA mice were gated by the surface markers CD11b and CD8α, and to distinguish pDCs. IDO expression was analyzed by probing the gated cells with anti–IDO mAb. The blue histogram lines represent the cells stained with anti–IDO antibody, and the red histogram lines show staining with the isotype control antibody. The data shown are representative of three independent experiments. (B) The percentage (left) and absolute number (right) of IDO-expressing cells compared between $CD11b^+$, $CD8\alpha^+$, and pDC subsets isolated from the spleens of the three groups of mice. (C) Mean fluorescence intensity (MFI) of IDO expression in splenic $CD11c^+$ DCs, $CD11b^+$ DCs, and $CD11b^-$ DCs. Bars represent the SD of six mice per group (*$P < 0.05$). (D) Confocal microscopy of spleen sections from each group of mice. Cells were stained with fluorescence-tagged antibodies to identify CD11c (blue), CD11b (green), and IDO (red). Merged green and red ($CD11b^+IDO^+$ cells) is shown as yellow, and merged blue, green, and red is shown as orange ($CD11c^+CD11b^+$ IDO^+ cells). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$CD25^+$ $Foxp3^+$ Tregs among $CD4^+$ cells in a coculture with $CD11b^+$ or $CD11b^-$ DCs from tolerized and untolerized CIA mice. The proportion of $CD25^+Foxp3^+$ Tregs increased in cocultures with all four types of DCs when CII was added (compared with no CII stimulation), but the increment was greatest when $CD11b^+$ DCs from tolerized CIA mice were added (Fig. 5A). The Treg-inducing effect of $CD11b^+$ DCs was almost abolished completely when DCs were pretreated with 1-MT. Notably, anti-TGF-β antibody prevented the increase in the $Foxp3^+$ Treg population (Fig. 5B). The level of TGF-β was significantly elevated in the culture supernatant when CII-specific Tregs were induced by tolerized $CD11b^+$ DCs (Fig. 5D); this effect was eliminated by pretreatment of DCs with 1-MT. Taken together, these results indicate that TGF-β is closely associated with the induction of Tregs by IDO-producing $CD11b^+$ DCs.

We also analyzed the expression of costimulatory molecules on the surface of induced Tregs. Interestingly, Tregs induced with $CD11b^+$ DCs from tolerized CIA mice displayed increased expression of CTLA–4 and PD–1, whereas the expression of other Treg markers such as GITR and ICOS remained unchanged (Fig. 5C). Pretreatment of DCs with 1–MT prevented the increased expression of these markers. This result suggests that the IDO signal pathway initiates Treg induction by tolerized $CD11b^+$ DCs.

3.6. Splenic $CD11b^+$ DCs promoted the in vitro conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ $Foxp3^+$ T cells

Theoretically, the Treg population can be increased either by expansion of the population of preexisting Tregs or by conversion of $CD4^+CD25^-$ cells to $CD4^+CD25^+$ cells. To test the possible contribution of the latter, we isolated $CD4^+CD25^-$ T cells from tolerized CIA mice and cultured them with $CD11b^+$ DCs from tolerized or untolerized CIA mice in the presence of CII for 4 days. $CD11b^+$ DCs from tolerized CIA mice converted more $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ cells than did $CD11b^+$ DCs from untolerized CIA mice, especially in the presence of CII (Fig. 6A). This effect was abrogated by pretreatment of DCs with 1-MT, indicating that IDO produced by the $CD11b^+$ DCs was responsible, at least partly, for the conversion.

Next, we examined the immune-regulatory properties of converted Tregs in the cocultures. Tregs converted in the culture with tolerized $CD11b^+$ DCs suppressed the CII-induced proliferation of CII-specific T cells more efficiently than did Tregs cultured with untolerized $CD11b^+$ DCs (Fig. 6B). We also analyzed the cytokine production profile of these cells. The levels of IL-10 and TGF-β were higher and the level of IL–12 was lower in the culture containing

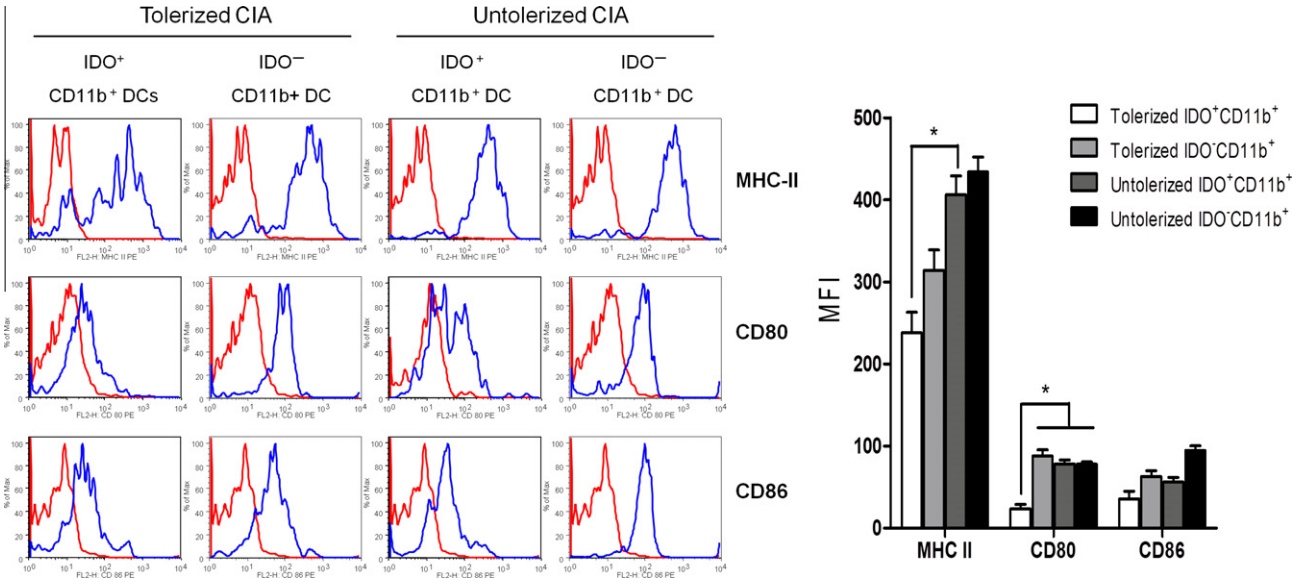

Fig. 3. Characterization of IDO-expressing splenic CD11b$^+$ DCs from tolerized and untolerized CIA mice. (A) Comparison of MHC II, CD80, and CD86 expression on the IDO$^+$ vs. IDO$^-$ subsets of CD11b$^+$ DCs isolated from the spleens of tolerized CIA and untolerized CIA mice. Cells probed with each mAb are represented by blue histogram lines. The histograms were gated on IDO$^+$ CD11b$^+$ DCs or IDO$^-$ CD11b$^+$ DCs and the red line shows isotype control. The data shown are representative of three independent experiments. (B) The bar graph on the right shows the mean fluorescence intensity (MFI) of the three tested markers. The data are expressed as mean ± SD for six mice per group ($^*P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

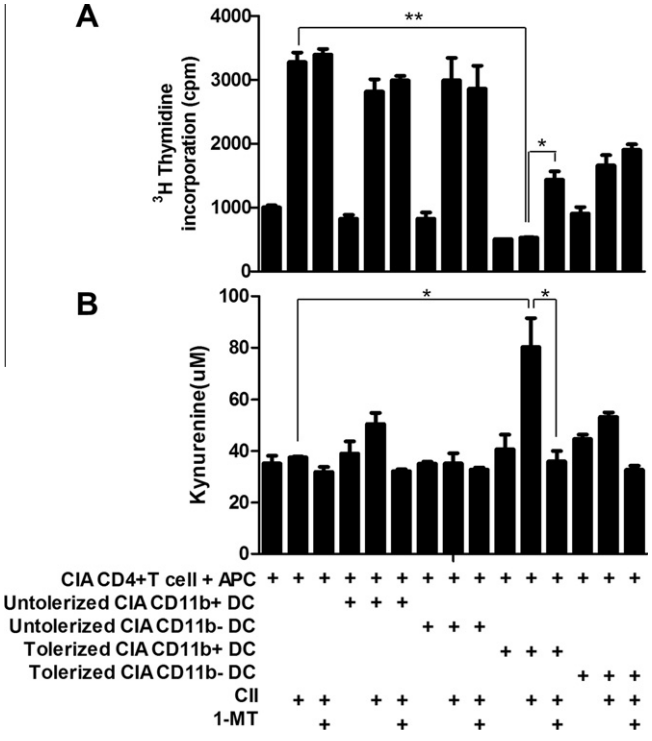

Fig. 4. Suppression of the CII-reactive T cell proliferative response by IDO-expressing CD11b$^+$ DCs. (A) CD4$^+$ T cells from the spleens of CIA mice were cultured with irradiated APCs, CD11b$^+$ DCs (1×10^4 cells/well), from untolerized CIA or tolerized CIA mice in the presence or absence of CII for 3 days. Some of the cells were pretreated with 1–MT, a chemical inhibitor of IDO. In the last 18 h of culture, [^{3}H] thymidine was added to each well, and the incorporated radioactivity was counted. Data are presented as the mean cpm of triplicate cultures ($^*P < 0.01$, $^{**}P < 0.001$). (B) IDO activity was assessed in culture conditions described in (A) by measuring kynurenine concentrations at 492 nm. Bars represent mean ± SD of three independent experiments ($^*P < 0.05$).

CD11b$^+$ DCs from tolerized CIA mice compared with that of untolerized CIA mice (Fig. 6C). Pretreatment of DCs with 1-MT abrogated the increase in the production of IL–10 and TGF-β. Antigen presentation by CD11b$^+$ DCs from tolerized CIA mice induced Foxp3$^+$ cells but did not affect the induction of Th17 cells (Fig. 6D, bottom panels). By contrast, CD11b$^+$ DCs from untolerized CIA mice increased induction of IL-17-secreting cells upon CII stimulation (Fig. 6D, top panels). The ratio of Foxp3$^+$ cells to IL-17$^+$ cells gives further information about T cell differentiation. This ratio increased markedly in cultures pulsed with CII (Fig. 6E); this effect was abolished in DCs pretreated with 1–MT. Even without CII stimulation, slightly more Foxp3$^+$ cells were induced when tolerized CD11b$^+$ DCs were used as the APCs. This result indicates that CD11b$^+$ DCs shifted the Treg/Th17 balance toward Tregs and that IDO appears to be a key factor in this shift.

3.7. Adoptive transfer of splenic IDO$^+$CD11b$^+$ DCs from tolerized mice alleviated the severity of arthritis in recipient CIA mice

Finally, we examined whether splenic CD11b$^+$ DCs from tolerized CIA mice could exert an immune-regulatory effect *in vivo*. CD11b$^+$ DCs were isolated from the spleens of tolerized and untolerized CIA mice, and pulsed with CII in vitro with or without 1–MT. Treated DCs were then adoptively transferred into CIA-induced mice, and the severity of joint inflammation was monitored (Fig. 7A). CIA mice given CD11b$^+$ DCs from tolerized mice showed significantly less joint inflammation compared with the control mice that did not receive these cells. Interestingly, the suppressive effect was reduced significantly when the donor DCs were pretreated with 1–MT, indicating that IDO is associated with the induction of immune suppression after adoptive transfer. By contrast, severe arthritis developed in the mice that received CD11b$^+$ DCs from untolerized CIA mice regardless of whether the cells had been pretreated or not treated with 1-MT.

Serum levels of TNF-α, IL-1β, IL-6, and IL-21 were significantly lower in the mice that received tolerized CD11b$^+$ DCs than in the untransferred controls (Fig. 7B). Pretreating DCs with 1–MT before

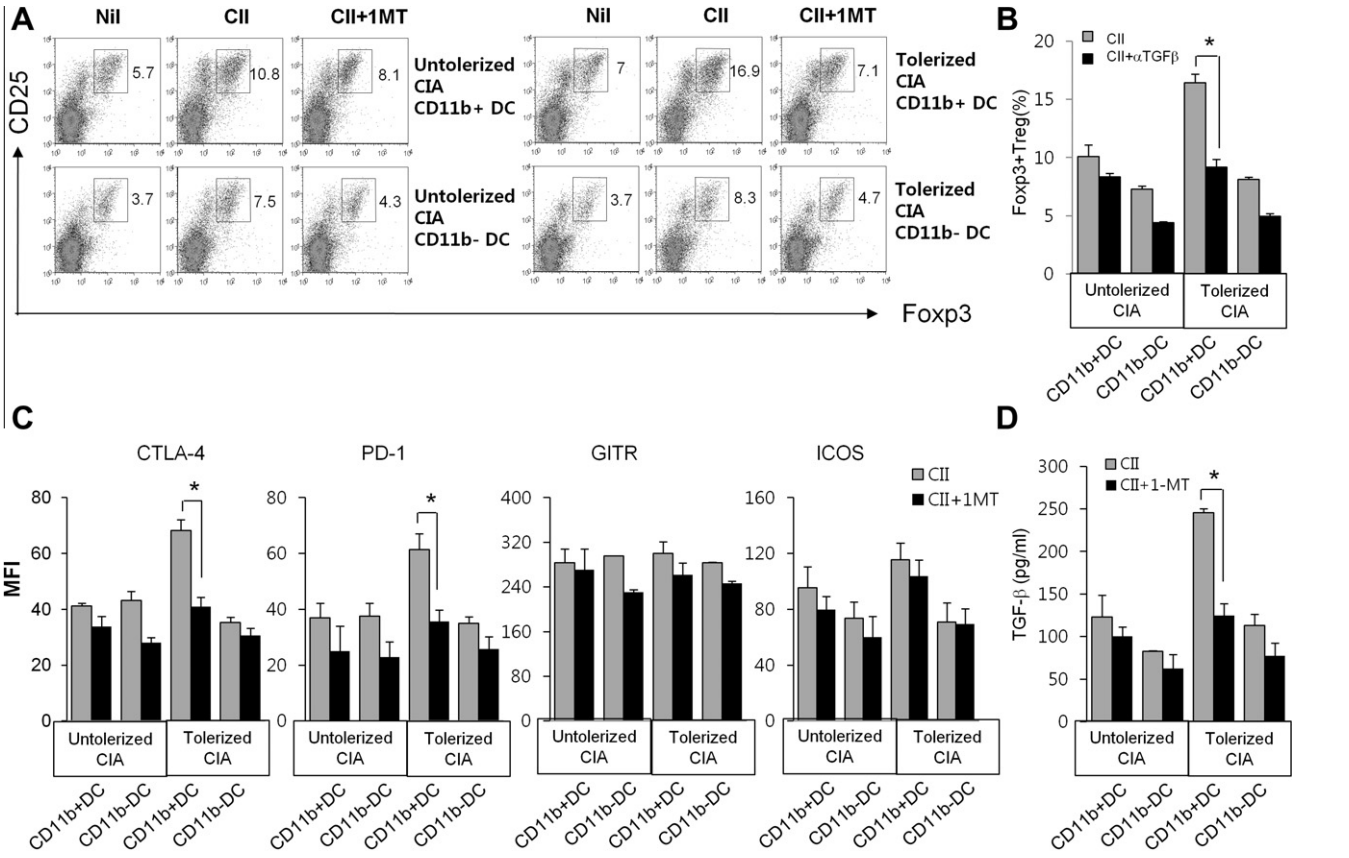

Fig. 5. Induction of Foxp3$^+$ Tregs by IDO-expressing CD11b$^+$ DCs. (A, B) Freshly isolated CD4$^+$ T cells from tolerized CIA mice were cultured with and without CII and 1-MT in the presence or absence of 10 μg/ml anti-TGF-β blocking antibody. CD11b$^+$ or CD11b$^-$ DCs isolated from the spleen of tolerized CIA mice were added to the culture, and the proportion of CD25$^+$Foxp3$^+$ cells among the CD4$^+$T cells was assessed (Nil: cultured without CII and 1–MT). (C) CD4$^+$CD25$^+$Foxp3$^+$ Tregs were pulsed with CII and the expression of CTLA–4, PD–1, GITR, and ICOS were analyzed by flow cytometry. A representative of three independent experiments is presented as mean fluorescence index (MFI) (*$P < 0.05$). (D) TGF-β level in the supernatant was measured by ELISA. The data are expressed as means ± SD from three independent experiments (*$P < 0.05$).

transfer partially restored the production of proinflammatory cytokines.

Changes in the populations of Tregs and Th17 cells in the spleen were investigated after adoptive transfer (Fig. 7C). Transfer of tolerized CD11b$^+$ DCs increased the proportion of Foxp3$^+$ Tregs compared with untransferred controls, but the opposite effect was observed for Th17 cells. Pretreatment of DCs with 1-MT reduced the proportion of Th17 cells in the mice that received DCs, suggesting that IDO was involved.

4. Discussion

Repeated oral administration of antigen induces unresponsiveness in the GALT and then systemic tolerance [3]. Antigen-specific T cells in peripheral lymphoid organs can obtain tolerogenic properties within hours after feeding, suggesting that APCs outside the GALT also participate in oral tolerance [24,25]. DCs in the spleen are a probable candidate for such APCs because the spleen is one of the lymphoid organs responsible for inducing Tregs in the periphery. However, the mechanism responsible for the process by which splenic DCs contribute to the establishment of oral tolerance remains elusive.

We have demonstrated previously that the IDO-producing DCs in PPs play an essential role in the induction of oral tolerance [13]. Another group reported that pDCs from tumor-draining lymph nodes directly activate mature Tregs via IDO [17]. Splenic DCs are distinct to the DCs of PPs and pDCs from draining lymph nodes in many aspects, but they may share the effects of IDO in the induction of tolerance. Although DCs in PPs mainly prime naive CD4$^+$ T cells to secrete IL–10 and TGF-β in an antigen-specific manner, splenic DCs prime T cells predominantly to secrete IFN-γ [10]. It is probable that IFN-γ secreted by spleen DCs acts as an IDO inducer [26,27]. The CD11b$^+$ subset of DCs is another noteworthy candidate as the major player in tolerance induction. Our group reported that CD11b$^+$ DCs in PPs are crucial for the establishment of oral tolerance [8]. Ehirchiou et al. reported that CD11b-deficient mice were more resistant to oral tolerance because of greater secretion of IL-6, which consequently leads to the production of IL–17 [28]. We observed a significant reduction in the CD11b$^+$ DC population in IDO-knockout mice compared with wild-type mice, whereas the proportion of other DC subsets remained unaffected (unpublished observation). These findings suggest that the IDO-producing CD11b$^+$ subset of splenic DCs is important for induction of oral tolerance.

In this study, we investigated the immune-regulatory capability of distinct splenic DC subsets by focusing on their IDO production and ability to induce Tregs under the inflammatory condition of CIA. In CIA mice, oral feeding with CII elevated IDO expression in the splenic DCs and in PP DCs. Notably, the increase in IDO expression was prominent in the CD11b$^+$ and pDC subsets, and CD11b$^+$ DCs exhibited much stronger expression of IDO. We also found that IDO-expressing CD11b$^+$ DCs of tolerized CIA mice exhibited immunological properties distinct from those of untolerized CIA mice. For example, CD11b$^+$ DCs from tolerized CIA mice displayed low levels of MHC II and CD80. This difference may explain why only IDO$^+$CD11b$^+$ DCs from tolerized CIA mice could suppress CII-specific T cell proliferation. Taken together, our results indicate that

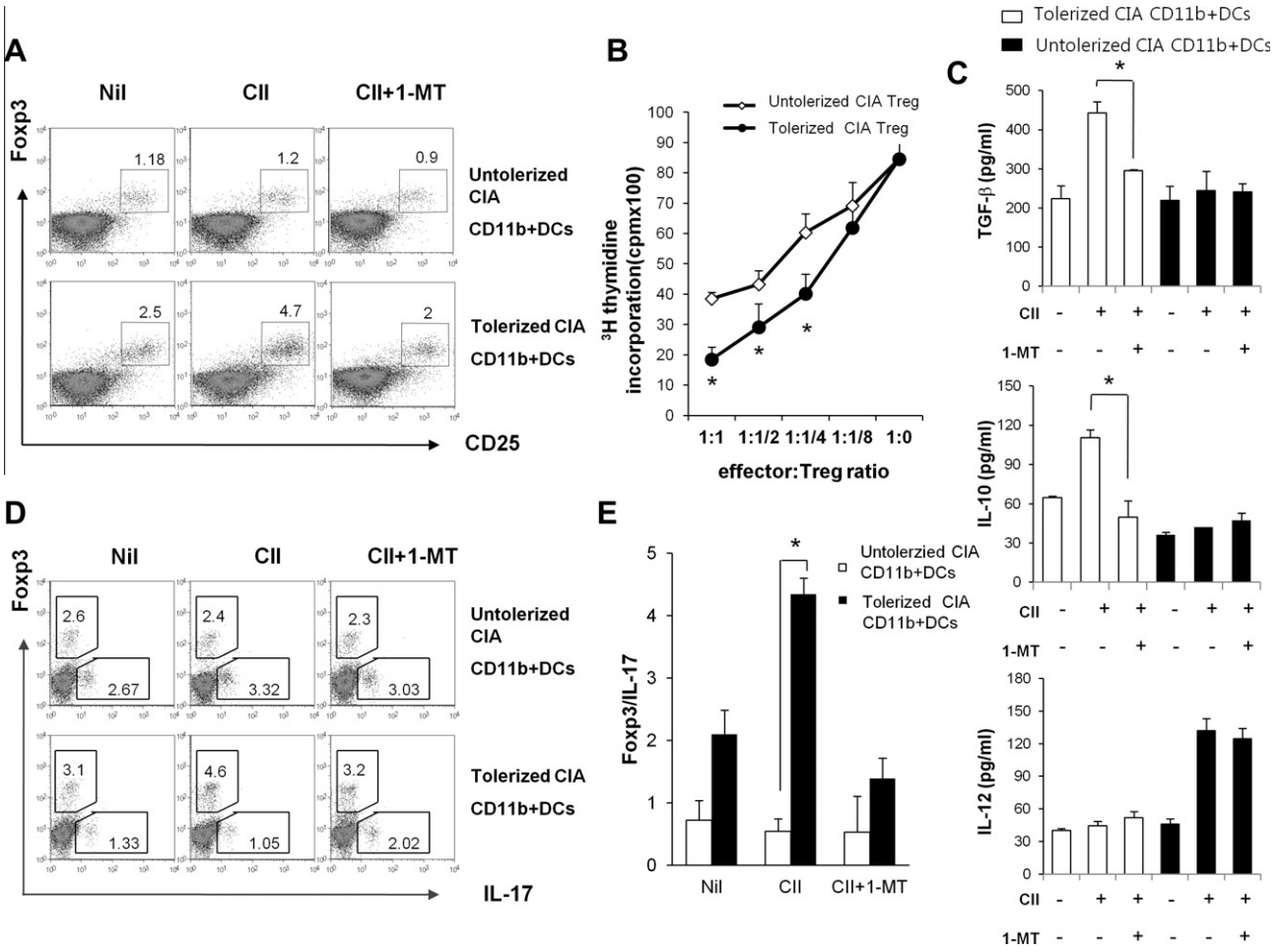

Fig. 6. Conversion of $CD4^+CD25^+$ Tregs by IDO-expressing $CD11b^+$ DCs. (A) Splenic $CD4^+CD25^-$ T cells from tolerized CIA mice were cultured with $CD11b^+$ DCs from tolerized or untolerized CIA mice in the presence or absence of CII and 1–MT. After 4 days, $CD4^+$ cells were stained for CD25 and intracellular Foxp3. The numbers in each plot indicate the percentages of $CD25^+Foxp3^+$ cells among $CD4^+$ T cells. (B) $CD4^+CD25^-$ cells were converted into $CD4^+CD25^+$ Tregs by CII stimulation in the presence of $CD11b^+$ DCs from tolerized CIA mice. Varying numbers of $CD4^+CD25^+$ cells were cultured for 3 days with CII-reactive effector ($CD4^+$) T cells from CIA mice, irradiated APCs (1×10^5), and CII. Cell proliferation was assessed by the incorporation of [^{3}H] thymidine. The data are expressed as mean ± SD from three independent experiments ($^*P < 0.01$). (C) On day 4 of culture, cytokine levels in the supernatants were measured by ELISA. The data are expressed as mean ± SD from three independent experiments ($^*P < 0.05$) (D) As described in (A), $CD4^+CD25^-$ T cells were cultured with CII in the presence of $CD11b^+$ DCs from tolerized CIA or untolerized CIA mice. After 4 days, $CD4^+$ cells were stained for Foxp3 and IL–17. The numbers in each plot indicate the percentages of $Foxp3^+$ or IL–17-producing cells among $CD4^+$ T cells. (E) The relative proportion of $Foxp3^+$ and $IL–17^+$ cells was analyzed by flow cytometry. The data represent the ratio of the percentage of $CD4^+Foxp3^+$ cells divided by the percentage of $CD4^+IL–17^+$ cells. The data are expressed as the mean ± SD from three independent experiments ($^*P < 0.05$).

the IDO-producing $CD11b^+$ DC subset in the spleen of tolerized CIA mice is the major player in the establishment of oral tolerance.

Among the array of helper T cell subtypes, Th17 cells have been designated as the main participants in inflammatory arthritis [29,30]. The widely accepted T cell plasticity concept states that conversion of Tregs into Th17 cells also contributes to inflammatory damage [31,32]. Interestingly, IDO has been reported to activate Tregs and block their conversion into Th17-like T cells [23,33]. This raises the question whether the IDO^+CD11b^+ subpopulation of DCs from tolerized CIA mice can regulate the Tregs/Th17 cell balance. The results from our study suggest that $CD11b^+$ DCs from tolerized CIA mice effectively suppressed CII-specific T cell proliferation by shifting the Tregs/Th17 cell balance toward Tregs. Functionally, the Tregs induced by IDO-expressing $CD11b^+$ DCs showed increased expression of CTLA–4 and PD–1, costimulatory molecules known to suppress stimulatory responses. It would be intriguing to investigate whether the expression of these molecules defines the characteristics of Tregs induced by IDO^+CD11b^+ DCs.

The reason for this difference in the immunological properties between $CD11b^+$ DCs from tolerized and untolerized CIA mice is not known. We presume this difference might reflect the cytokine environment in each animal. The levels of TGF-β and IL–10 were higher in the medium from T cells cocultured with $CD11b^+$ DCs from tolerized CIA mice. Pallota et al. demonstrated recently that stimulating pDCs with TGF-β induced IDO production, which in turn acts as a signaling molecule to stimulate the expression of multiple genes including TGF-β, a Treg-inducing cytokine [34]. In our study, the increased expression of IDO in the tolerized $CD11b^+$ DCs appeared to have induced both TGF-β and IL–10. It is possible that, in our study, both an inflammatory state (via CIA induction and CFA stimulation) and a tolerogenic state (via oral feeding of CII) induced IDO to trigger TGF-β signaling through an alternative, nonenzymatic pathway. More detailed analysis of the mechanism involved in this TGF-β–IDO axis would provide additional insights into the signaling pathways that prime $CD11b^+$ DCs for their immune-suppressive function in oral tolerance.

The immune-regulatory activity of $CD11b^+$ DCs from tolerized CIA mice was also demonstrated *in vivo*. Adoptive transfer of these cells into CIA-induced mice suppressed the development of arthritis, increased the IDO^+ cell population in the spleen and decreased the serum levels of inflammatory cytokines. These anti-arthritogenic

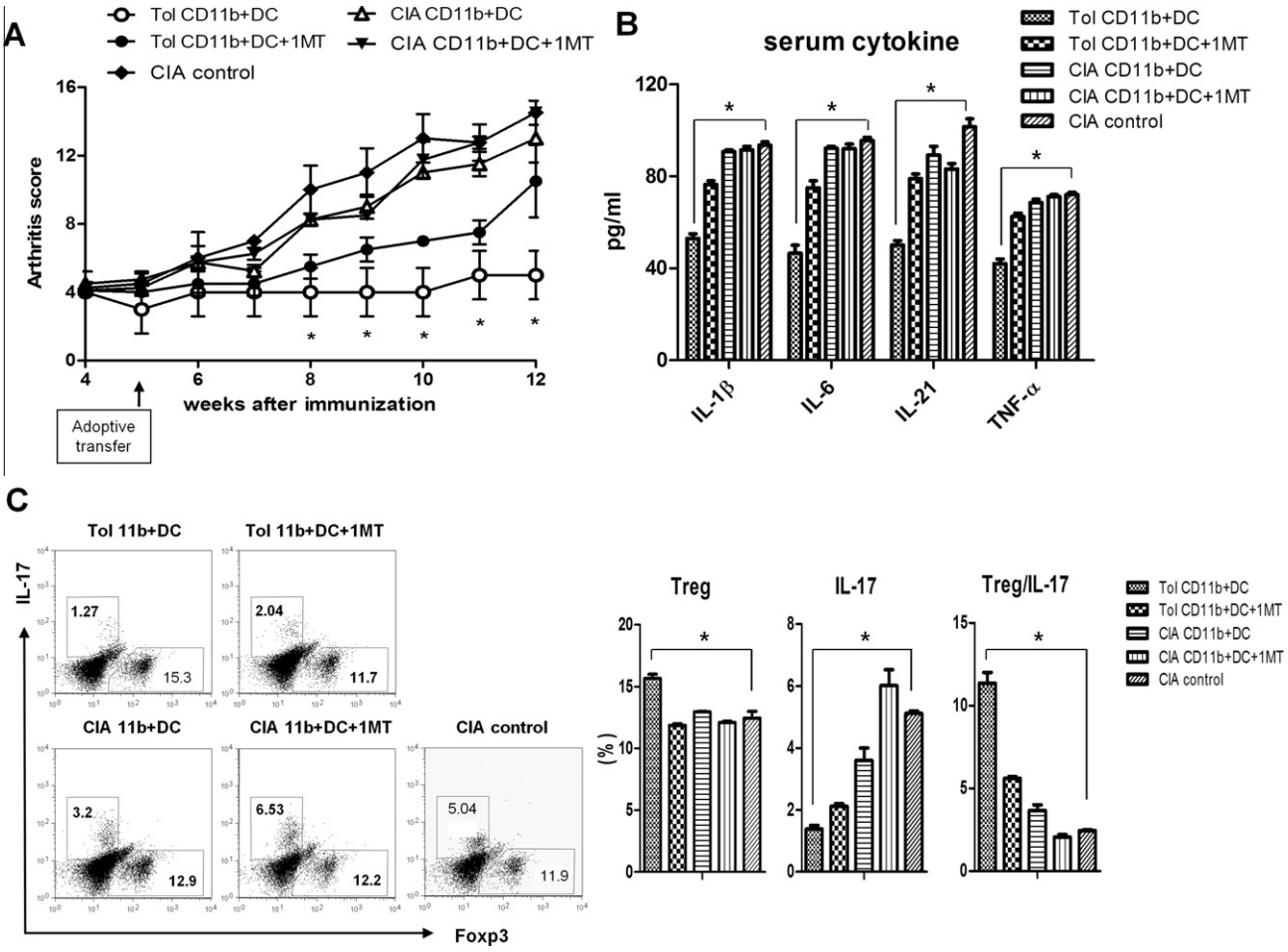

Fig. 7. Adoptive transfer of CD11b$^+$ DCs from tolerized CIA mice. (A) These DCs were used for adoptive transfer into untolerized CIA mice 5 weeks after primary immunization. The severity of arthritis was compared between untransferred CIA control mice (triangle) and mice transferred with tolerized CD11b$^+$ DCs that had been cultured with (closed circle) or without 1–MT (open circle). Data are shown as mean ± SD arthritis score at each indicated time ($n = 4$ mice per group, $^*P < 0.05$ vs. untransferred CIA mice). (B) Serum levels of inflammatory cytokines were measured by ELISA 3 weeks after the transfer of tolerized DC. Bars show mean ± SD ($^*P < 0.05$). (C) The proportions of CD4$^+$CD25$^+$ Foxp3 T cells and CD4$^+$IL–17$^+$ T cells in the spleen of recipient mice were measured 3 weeks after transfer. The dot plot is a representative of three independent experiments. The bar graph represents the ratio of Foxp3$^+$ to IL-17$^+$ cells among CD4$^+$ T cells; the data are expressed as the mean ± SD of three independent experiments ($^*P < 0.05$). (E) Intracellular expression of IDO in the total CD11c$^+$ DC population from the spleens of the three groups. The gray histogram designates the isotype control. Representative data from three independent experiments are presented. On the right, the bars show mean MFI ± SD of IDO expression in each group ($^*P < 0.05$).

effects of CD11b$^+$ DCs from tolerized CIA mice were associated with an increase in the population of CII-specific CD4$^+$Foxp3$^+$ regulatory T cells and inhibition of Th17 cells, which were dependent on IDO. Consistent with our findings, IDO deficiency and systemic inhibition of IDO by administration of 1-MT increased both the severity of CIA and production of the proinflammatory cytokines IFN-γ and IL-17 by lymph node T cells [20]. In the study by Criado et al., increased infiltration of Th1 and Th17 cells was also observed in the inflamed joints when the activity of IDO was challenged.

The mechanism by which splenic CD11b$^+$ DCs are primed to produce IDO and become tolerogenic after oral administration of antigen is not clear. It is known that Tregs induce the immune-regulatory enzyme IDO in DCs through CTLA4/B7 ligation to make these cells tolerogenic [35]. This suggests that Tregs induced in the GALT may move to the spleen where they induce splenic DCs to express IDO and become tolerogenic. To understand better the mechanism responsible for oral tolerance, it would be interesting to study the systemic trafficking of the Tregs induced in the GALT and their effect on the immunological properties of various DC subsets in extraintestinal lymphoid organs.

Although our data supports the major role of IDO-producing CD11b$^+$ DCs in the activation of Tregs and induction of oral tolerance in CIA-induced mice, this does not necessarily rule out the contribution of other subsets of tolerogenic DCs in oral tolerance. Plasmacytoid DCs were also reported to mediate oral tolerance [36] and CD103$^+$ CDs isolated from the mLNs have been shown to drive preferentially the development of CD4$^+$Foxp3$^+$ Tregs via a TGF-β and retinoic acid dependent mechanism [37]. Furthermore, it was found that Gut CD103$^+$ DCs express IDO and influence Treg/Teff balance and oral tolerance induction [16].

In summary, we have demonstrated that the establishment of systemic tolerance against CIA by oral administration of CII accompanies increase of IDO-expressing CD11b$^+$ DCs not only in the PPs but also in the spleen. Enhanced expression of IDO on splenic IDO$^+$ CD11b$^+$ DCs is associated with both subdued proliferation of CII-reactive T cells in this organ and the CII-activated increase in converted Tregs and Tregs/Th17 ratio. When adoptively transferred into CIA-induced mice, splenic CD11b$^+$ DCs from tolerized CIA mice suppressed the development of arthritis along with increase in the Tregs/Th17 ratio among splenic CD4$^+$ T cells and reduced the production of inflammatory cytokines. Our data demonstrated that IDO-expressing CD11b$^+$ DCs play a pivotal role in the propagation of immune tolerance out to peripheral lymphoid organs under inflammatory state, and may represent a novel candidate for therapeutic modality in the treatment of autoimmune arthritis.

Acknowledgments

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