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Oral tolerance and OVA-induced tolerogenic dendritic cells reduce the severity of collagen/ovalbumin-induced arthritis in mice

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ABSTRACT

Dietary proteins play an important role in the regulation of systemic immune response, in a phenomenon known as oral tolerance (OT). To evaluate the effects of OT on a murine model of type II collagen (CII) plus ovalbumin (OVA)-induced arthritis (CIA), mice were fed with OVA either before or after CIA induction. OT significantly reduced the paw edema and synovial inflammation, as well as serum levels of anti-CII, the ex vivo proliferation and inflammatory cytokine production by spleen cells from CIA mice. The frequencies of Foxp3$^+$ and IL-10$^+$ cells were higher, whereas IFNγ^+ cells and IL-17$^+$ cells were lower, among gated CD4$^+$ spleen T cells from tolerized CIA mice than in those from non-tolerized CIA mice. Adoptive transfer of tolerogenic dendritic cells (DCs) before CIA induction mimics the effects observed in the OT. We demonstrate here that bystander suppression induced by OT can modify the course of CIA and tolerogenic DCs play a role this phenomenon.

1. Introduction

Human rheumatoid arthritis is a chronic inflammatory autoimmune disease characterized by pain, swelling and deformity of the joints. The immune response in arthritis involves the activation of antigen-specific lymphocytes secreting inflammatory cytokines that act on macrophages, osteoclasts and cells adjacent to inflamed synovial tissue [1]. Collagen-induced arthritis (CIA) is the most studied experimental model of rheumatoid arthritis, which involves the administration type II collagen (CII) plus complete Freund's adjuvant (CFA) in susceptible strains of mice and rats. In resistant BALB/c mice, however, CIA is only observed when a second protein, such as keyhole-limpet hemocyanin (KLH) or ovalbumin (OVA), is combined to the CII and CFA [2].

The use of knockout animals and blockage with monoclonal antibodies has shown that IL-17, IFN-γ and TNF-α play a fundamental role in the establishment of arthritis [3–5]. Monoclonal antibody based therapies have been proved to be an alternative to the classic use of immunosuppressive disease-modifying antirheumatic drugs [6], with rare adverse effects in humans [7,8].

Oral tolerance (OT) has been successfully used to alter the course of inflammatory responses in some experimental autoimmune diseases [9,10]. Joint inflammation, for example, has been significantly reduced in mice tolerized by repeated oral feeding of CII before CIA induction [9]. The tolerized mice also exhibited an increased serum IgG1, a reduction of serum IgG2a, a suppression of specific proliferative response of T cells from lymph nodes, and an increased frequency of cells producing IL-10 amongst T CD4$^+$CD25$^+$ splenic cells stimulated with CII.

Dendritic cells (DCs) play an important role in immune responses of T lymphocytes that occur in the microenvironment of digestive tract mucosa, directing them to either an effector profile or systemic tolerance to the antigen. The main effect induced by DCs in immune tolerance seems to be the expansion of TGF-β- and IL-10-secreting regulatory T lymphocytes (Tregs) that inhibit the development of IFN-γ-, TNF-α- and IL-17-secreting T cells [9–11]. CD103$^+$ DCs in small intestine exhibit an enhanced ability to metabolize vitamin A and generate its major active metabolite retinoic acid [12]. It has been suggested that retinoic acid underlies the CD103$^+$DC capacity to induce the gut homing receptors CC chemokine receptor (CCR)9 and α4β7 on responding T and B cells, to enhance transforming growth factor (TGF)β-dependent naïve T cell conversion to forkhead box (Fox)P3$^+$ T regulatory cells (Tregs) while suppressing TGFβ-dependent Th17 cell differentiation [13,14].

However, currently it is unknown whether OT to ovalbumin is capable of reducing clinical signs of CIA in BALB/c mice. In this

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study, we show that administration of OVA by oral route reduced the severity of CIA in BALB/c mice, and that this phenomenon occurred when OVA is administered either before or after the onset of the disease. Our data also suggested that bystander suppression induced by the ingestion of OVA leads to the generation of tolerogenic DCs with the consequent increase of the proportion of $CD25^{+}Foxp3^{+}$ Tregs and secretion of anti-inflammatory cytokines in arthritic mice.

2. Material and methods

2.1. Animals

Eight weeks old male BALB/c mice from the Multidisciplinary Center for Biological Research, University of Campinas (UNICAMP), were used in this study. The animals were kept in *specific-pathogen free* condition, in a controlled temperature and photoperiod environment, with autoclaved food and water *ad libitum* throughout the experiment. The protocols involving laboratory animals were approved by the Institutional Committee (Comissão de Ética no Uso de Animais – CEUA/UNICAMP; Protocol number 1759-1).

2.2. Oral tolerance

The induction of OT to OVA followed the protocol previously described [15]. Briefly, 4 mg/mL OVA (Sigma–Aldrich, St. Louis, MO, USA) were added to water supply, for a week. After this period, the liquid supply was replaced by protein-free water. Three animals in this group were challenged with two doses of 10 μg OVA plus 1 mg $AlOH_3$ at intervals of 1 week to evaluate the induction of OT. Low levels of anti-OVA detected in sera obtained from these animals indicated the success of the procedure adopted.

2.3. Type II collagen-induced arthritis

Arthritis was induced in male mice as described elsewhere [2]. Briefly, chicken type II collagen (kindly provided by Dr. Benedito dos Campos Vidal, UNICAMP) and OVA were emulsified in complete Freund's adjuvant (CFA). Animal of naïve group received a subcutaneous (s.c.) dose of antigens constituted by mixture of 100 μg of each protein emulsified in CFA (v:v) at the base of the tail. Two additional doses of the CII + OVA in CFA were applied on days 21 and 45. Half of this group of mice was fed with OVA to induce tolerance over the days 28 and 35 after the beginning of subcutaneous immunization. A third group consisted of male mice fed OVA for 7 consecutive days prior arthritis induction. On day 65, mice of all groups were euthanized for histological and immunological analysis.

2.4. Evaluation of inflammation and histological analysis

In order to evaluate the articular edema in CIA mice, paw thicknesses were measured weekly from day 21 to 65. On day 65 after CIA induction, the animals of each experimental group were sacrificed, its hind legs were dissected, fixed and decalcified in formic acid solution 15% and formaldehyde 5%, cut in microtome (cuts of 6 μm) and stained in Sirius Red for histopathological examination.

2.5. Detection of serum antibodies

Detection of anti-OVA serum antibodies was carried out in microtiter plates covered with 20 μg/mL OVA in sodium carbonate buffer, pH 9.0, as described elsewhere [15]. To detect anti-type II collagen serum antibodies, plates were covered with 20 μg/mL chicken type II collagen (Chondrex Inc, Redmond, WA, USA) in phosphate buffered saline (PBS) pH 7.2 as described elsewhere [16]. Absorbance was read at 492 nm in an ELISA reader (Labsystem MS, Finland). The results of ELISA were expressed as averages of sum ± standard error of mean (S.E.M.) of the optical densities obtained in each experimental group.

2.6. Co-culture experiments

Dendritic cells were isolated from spleens of naïve, immunized and OVA-tolerant mice employing anti-CD11c magnetic beads (Miltenyi Biotech, Auburn, CA, USA) following the manufacturer's recommendations. After purification procedures, about 80% of the cells were MHC II^{+} and only 3% were $CD3^{+}$. $CD11c^{+}$ DCs at concentration of 1×10^{6}cells/mL were pulsed for 18 h with 50 μg/ml of CII. Splenic cells from arthritic mice were enriched in T lymphocytes using nylon wool column, according to protocol previously described [15]. T lymphocytes were stained with carboxyfluorescein succinimidyl ester (CFSE) according to manufacturer's recommendations (Sigma), and cultured in 96-well plates at a concentration of 2×10^{5}/well in the presence of pulsed DCs at a ratio of 10:1 (T:DC) with 50 μg/ml CII for 96 h. Before the addition of antigen, an aliquot of stained cells was withdrawn for analysis by flow cytometer to define the maximum value of incorporation of the probe. Supernatants were collected for dosage of cytokines through ELISA. To measure proliferation, cells were fixed with PBS containing 1% formaldehyde, transferred to appropriate tubes and analyzed in flow cytometer. To examine expansion of subsets of T lymphocytes, aliquots were processed to flow cytometry.

2.7. Measurement of cytokines

At the end of the lymphocyte cultures, the supernatants were collected for dosage of cytokines through ELISA, using commercial kits according to manufacturer's recommendations. It was analyzed: IL-12, TNF-α (BD PharMingen), IL-17, IL-6, IFNγ, IL-10, IL-4 and TGF-β (e-Bioscience).

2.8. Adoptive transfer experiments

2.8.1. Dendritic cell purification and transfer

Splenic dendritic cells were isolated from naïve and OVA-tolerant mice employing anti-CD11c magnetic beads (Miltenyi Biotech), according to the manufacturer's recommendations. Cells were pulsed with OVA 50 μg/mL during 18 h and adoptively transferred to naïve mice via retro-orbital plexus, in three doses of 5×10^{5} cells per animal on days 6, 4 and 2 before the CIA induction.

2.8.2. Spleen cell proliferation

On day 65 after CIA induction, mice of all groups of the adoptive transfer experiments were killed and spleens were aseptically removed. Splenocytes were stained with CFSE, seeded at 2×10^{5} cells/well in the presence of 50 μg/mL CII, and then incubated for 96 h at 37 °C in humidified incubator with CO2 5%. Cells cultured in absence of stimuli were used as baseline control. Supernatants were collected for dosage of cytokines. The cells were fixed with PBS containing 1% formaldehyde and proliferation was assessed in $CD3^{+}CFSE^{low}$ by flow cytometry. The results were expressed as proliferation index (fold change), calculated by dividing the percentages found in the experimental groups by the percentage found in the control group.

2.9. Flow cytometry

Following cultivation or in vivo treatments, single cell suspensions were washed and suspended in staining buffer (PBS plus

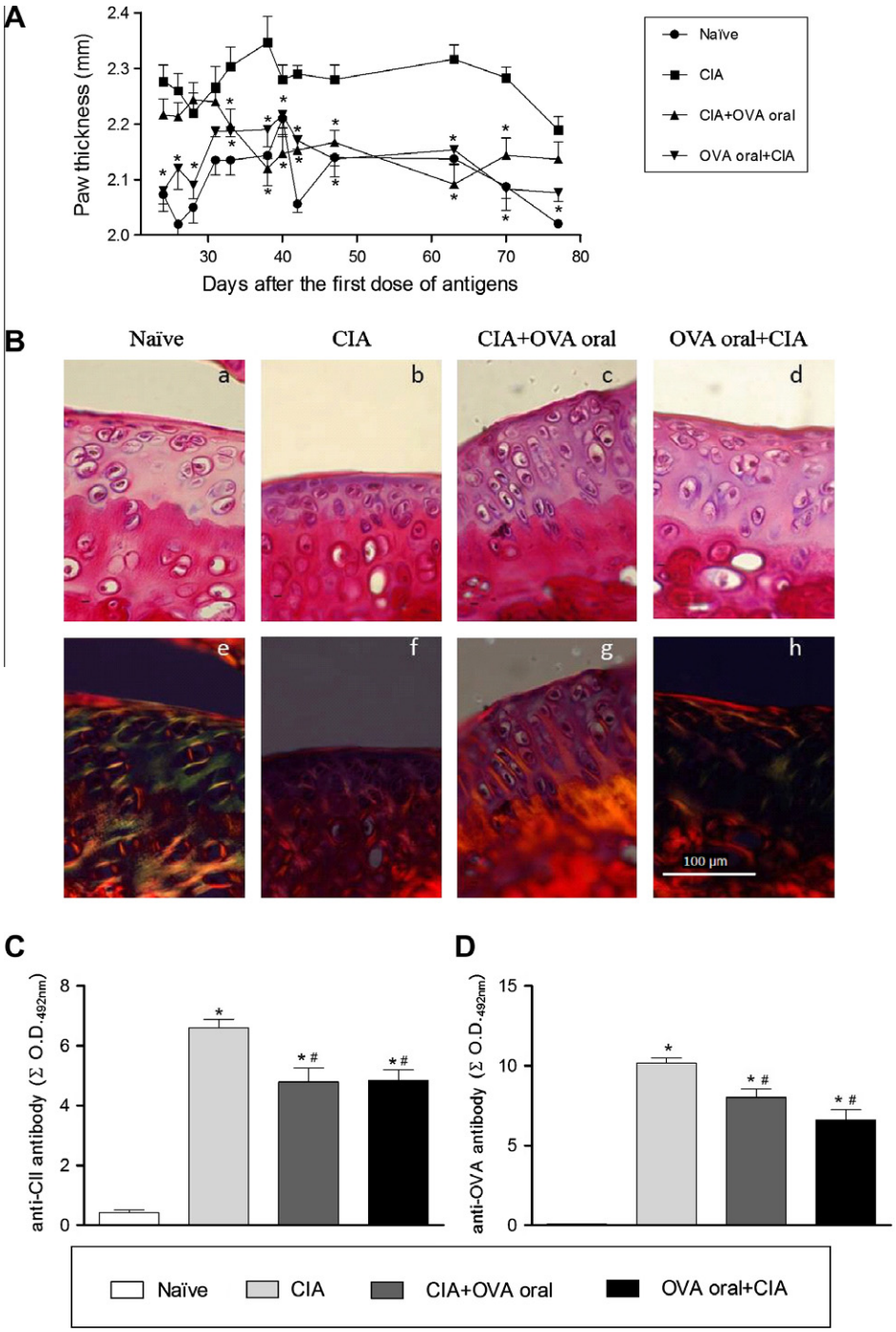

Fig. 1. Oral administration of OVA suppresses CIA in BALB/c mice. OVA solution (4 mg/mL) was offered in drink water to male BALB/c mice ($n = 5$) for 7 consecutive days, either before or after (at 28 days after the first dose of antigens) of the beginning of subcutaneous (sc) injections to induce CIA. Arthritis was induced by immunization with OVA + CII (100 µg of each antigen) emulsified with CFA, via s.c., administered at day 1, 21, and 45. (A) The thickness of the paws was taken every 3 days, since day 21 until the time of sacrifice. (B) Hind paws were dissected, embedded in paraffin and processed for histological analysis by Sirius red. (C, D) Sera were diluted from 1:100 to 1:1280 and tested in reactions of ELISA for detection of anti-CII and anti-OVA antibodies, respectively. Values are mean O.D. sums ± S.E.M. ($n = 6$). Data are representative of three independent experiments. Two-way ANOVA followed by Bonferroni's post-test were used to determine statistical significance in mean paw thickness in comparison to CIA group; $^{*}P \leqslant 0.05$. One-way ANOVA was used to determine statistical significance in mean O.D. sums; $^{*}P \leqslant 0.05$ in comparison to naïve mice and $^{\#}P \leqslant 0.05$ in comparison to CIA group. (For interpretation of color in Figs. 1 and 5, the reader is referred to the web version of this article.)

0.1% bovine serum albumin plus 0.09% sodium azide), and probed with anti-CD3-PerCP-Cy5.5, anti-CD4-PECy7, anti-CD25-PE and their respective isotype controls for 30 min at 4 °C. Next, cells were incubated with fixation/permeabilization buffer for 30 min and probed for intracellular cytokines using anti-Foxp3-APC, anti-IL-17-APC or -PE, anti-IFN-γ-APC or -PE and anti-IL-10-PE and their respective isotype controls. All FACS reagents were purchased from eBiosciense (San Diego, CA, USA) and used following manufacture's recommendation. Preparations were acquired in FACS Aria flow cytometer (BD Becton Dickinson, San Jose, CA,

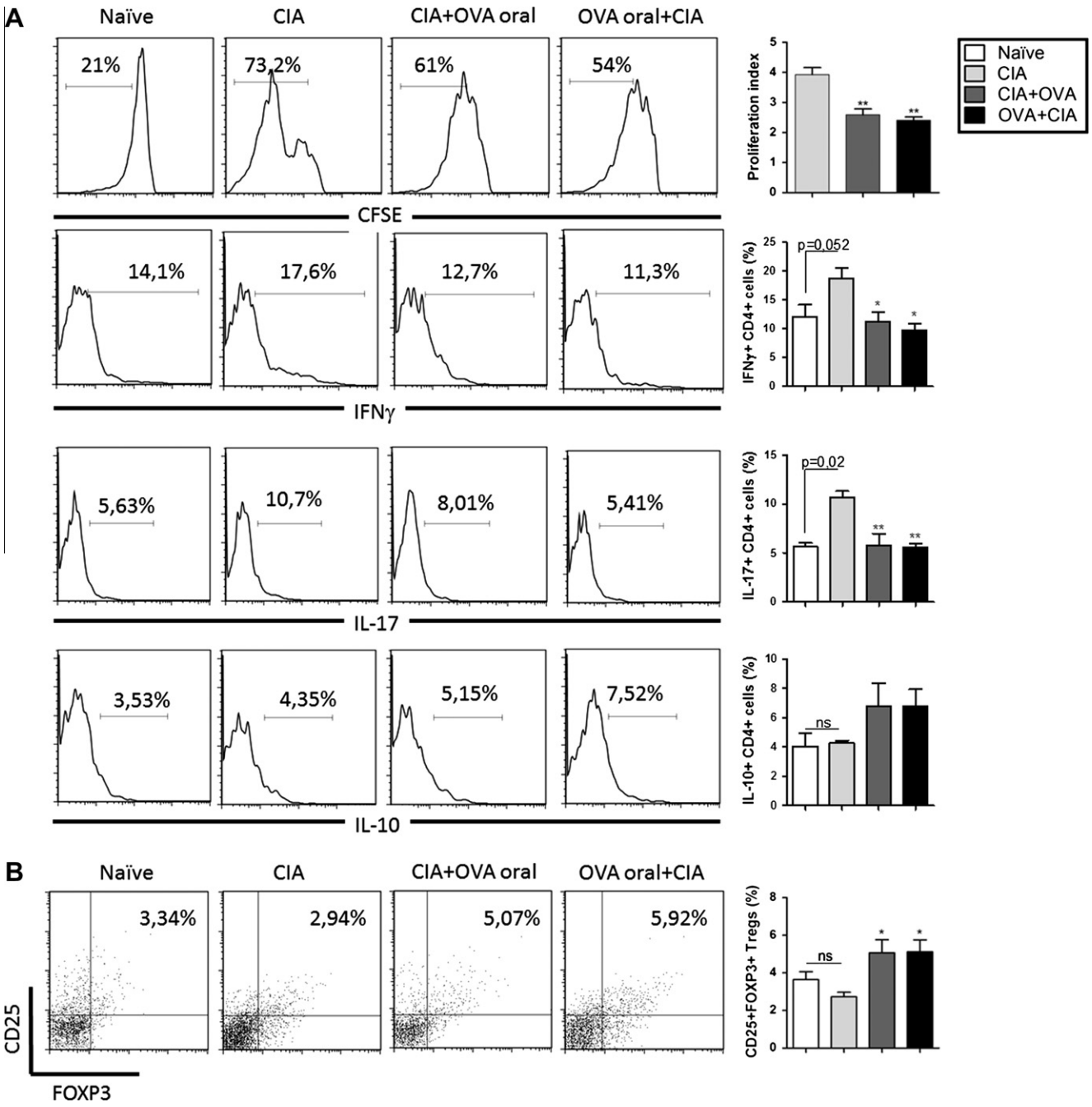

Fig. 2. Suppression of collagen-specific proliferation of T lymphocytes, increased proportion of $CD25^{+}Foxp3^{+}$ Tregs and IL-10-producing-T cells after oral administration of ovalbumin. BALB/c mice were fed with OVA, either before or after CIA induction by subcutaneous immunization with OVA + CII/CFA. Leukocytes were harvested from spleens 60 days after the primary immunization, and suspensions were stained with CFSE and cultured (2×10^5 cells/well) for 96 h in the presence of 50 μg/mL CII. (A) T cell proliferation was determined using flow cytometry and assessed by fluorescence decay of the probe in the gate of $CD3^{+}$ cells. IL-10-, IFNγ- and IL-17-producing cells were evaluated in the gate of $CD3^{+}CD4^{+}$ cells. (B) The frequency of $CD4^{+}CD25^{+}$ $Foxp3^{+}$ T lymphocytes was evaluated in the gate of $CD3^{+}CD4^{+}$ cells. Data in %bar graphs represent the mean ± S.E.M. ($n = 4$) of cell frequencies obtained in three independent experiments and histograms are derived from a representative experiment. One-way ANOVA followed by Bonferroni's post-test were used to determine statistical significances; $P \leqslant 0.05$ in comparison with naïve (*) and CIA (#) groups.

USA) and results were analyzed with Flow Jo 7.5 software (Tree Star Inc., Ashland, OR, USA).

2.10. Statistical analysis

The results were analyzed by one-way ANOVA or two-way ANOVA followed by Bonferroni's post-test, determining as statistically significant the differences with values of $P \leqslant 0.05$. For the purposes of drafting this analysis, we used the GraphPad Prism5 Software.

3. Results

3.1. Intake of ovalbumin modulates experimental arthritis

BALB/c mice immunized with CII + OVA/CFA developed arthritis characterized by inflammatory reaction in joints, with the increase of paw thickness and histological changes in synovial cartilages, as illustrated in Fig. 1. Intake of OVA either before or after CIA induction led to a significant decrease in paw edema and in the inflammatory reaction in joints (Fig. 1A). The most significant histological

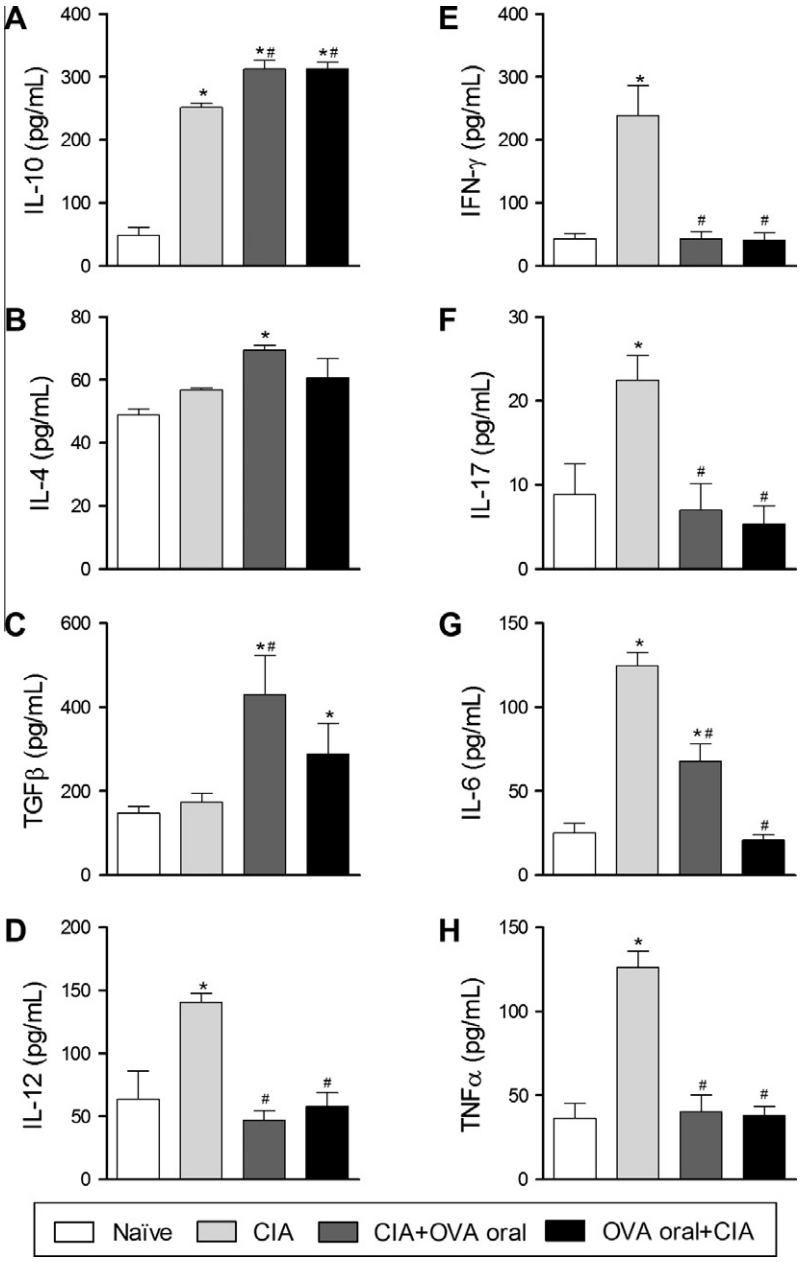

Fig. 3. Changes induced by OT to ovalbumin on cytokine production in cultures of splenic cells from CIA mice re-stimulated with CII. Leukocytes from spleens of CIA mice submitted or not to the protocol for OT with OVA were harvested 60 days after the primary immunization. Cell suspensions were cultured (2×10^5 cells/well) for 96 h in the presence of 50 μg/mL CII. Cells from naïve mice were used as a control. Culture supernatants were collected for dosage of IL-10, IL-4, TGFβ, IL-12p40, IFNγ, IL-17, IL-6 and TNFα (A–H, respectively) using commercial ELISA kits, according to manufacturer's indications (e-Bioscience). Data are representative of two independent experiments. One-way ANOVA followed by Bonferroni's post-test were used to determine statistical significances; $P \leqslant 0.05$ in comparison with naïve (*) and CIA (#) groups.

findings were inflammatory hyperplasia of cartilage and collagen deposition in the synovia of nontolerized CIA mice in comparison with tolerized group (Fig. 1B). Anti-CII and anti-OVA levels were also significantly more reduced in sera of tolerized group than in the nontolerized CIA mice (Fig. 1C and D, respectively).

We then investigated the proliferation of spleen cells under in vitro stimulation with CII. As depicted in Fig. 2, proliferation of T lymphocytes from CIA mice fed with OVA either before or after CIA induction was significantly lower than in CIA control group (Fig. 2A), while the frequency of $CD25^+Foxp3^+$ Tregs cells increased in those cultures (Fig. 2B). We also observed an increased frequency of $IL10^+CD4^+$ T cells in the cultures of spleen cells from mice fed with OVA, as well as a reduction of the frequencies of $IL17^+CD4^+$ and IFN-γ^+CD4^+ T cells, as compared with the CIA group (Fig. 2A). The levels of IL-10, IL-4 and TGF-β were higher in culture supernatants of spleen cells from tolerized mice than in CIA mice (Fig. 3A–C), whereas IL-12, IFN-γ, IL-6, IL-17 and TNF-α were more reduced in those cultures (Fig. 3C and E–H).

3.2. Tolerogenic dendritic cells favor immuneregulation

We next aimed to evaluate the effect of dendritic cells from tolerant mice on immune functions of lymphocytes from CIA mice. $CD11c^+$ DCs were purified from spleens of OVA-tolerant mice and co-cultured with spleen lymphocytes of CIA mice in the presence of CII. DCs isolated from naïve and OVA-immunized mice were used as controls. As depicted in Fig. 4, the antigen-specific proliferation of lymphocytes from CIA mice was significantly higher when

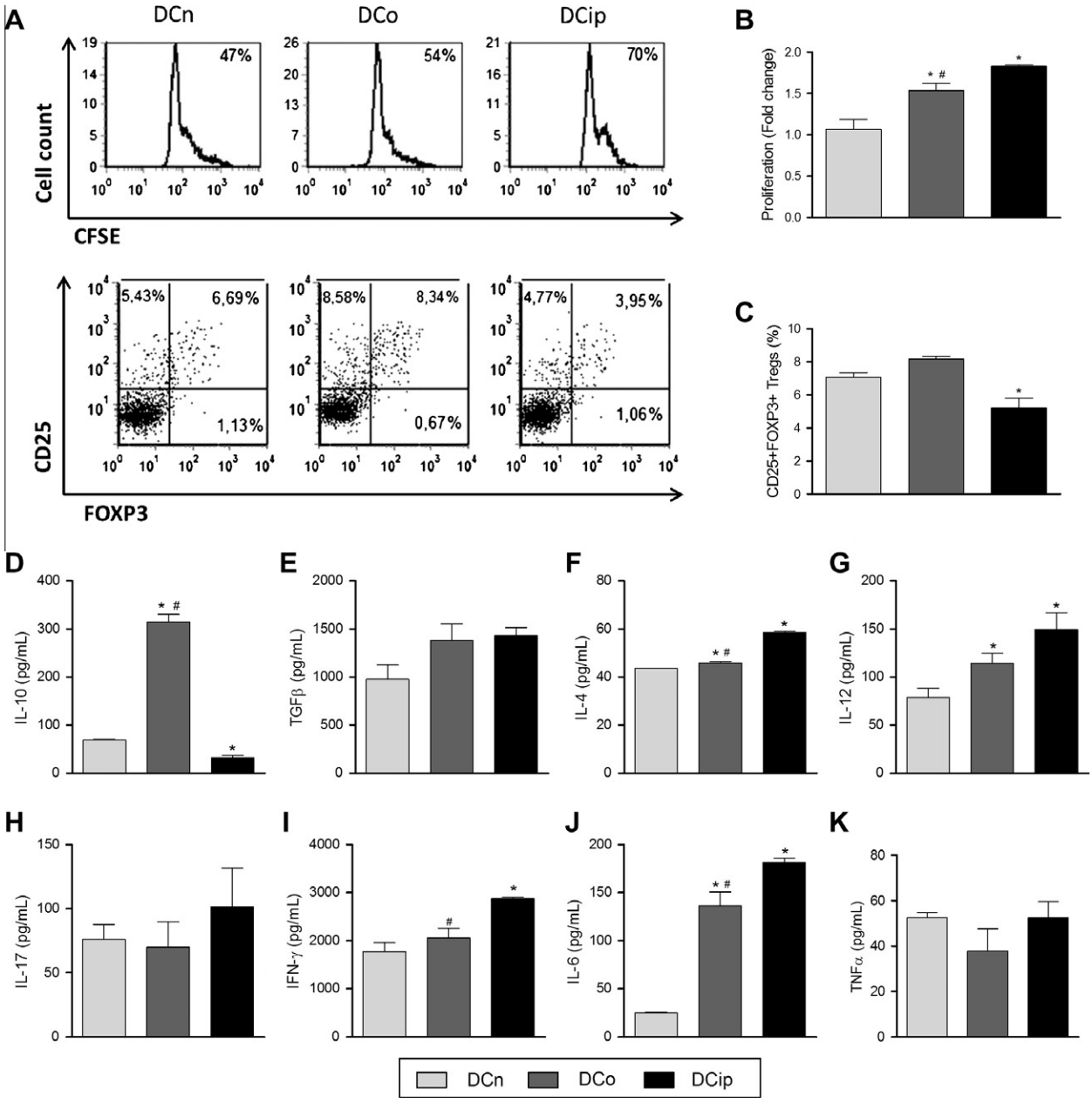

Fig. 4. Dendritic cells from tolerized mice suppress collagen-specific proliferation, increase proportion of $CD25^+Foxp3^+$ Tregs and change cytokine profile in co-cultures with spleen cells from CIA mice. $CD11c^+$ cells were isolated from spleens of naïve (DCn), immunized (DCip) and tolerant (DCo) mice using magnetic beads coated with a-CD11c antibodies, and then pulsed with CII (50 μg/mL) for 18 h. Leukocytes from spleens of CIA mice were harvested 60 days after the primary immunization, enriched in T lymphocytes by passage in nylon wool columns, and stained with CFSE. Spleen lymphocytes (2×10^5 cells/well) were cultured for 96 h in the presence of 2×10^4 pulsed DCs and 50 μg/mL CII. (A and B) T cell proliferation was determined using flow cytometry and assessed by fluorescence decay of the probe in the gate of $CD3^+$ cells. (A and C) The frequency of $CD25^+$ $Foxp3^+$ T lymphocytes was evaluated in the gate of $CD3^+CD4^+$ cells. (D–K) Cytokines IL-10, TGFβ, IL-4, IL-12, IL-17, IFNγ, IL-6 and TNF-α were detected in the culture supernatants by ELISA. Data in bar graphs represent the mean ± S.E.M. ($n = 6$) of cell frequencies and cytokine production obtained in three independent experiments. One-way ANOVA followed by Bonferroni's post-test were used to determine statistical significances; $P \leqslant 0.05$ in comparison with naïve (*) and CIA (#) groups.

DCs were derived from OVA-tolerant or -immunized mice (Fig. 4A and B). The analysis of lymphocyte subpopulations present in cultures shows that there was a significant increase in the proportion of $CD25^+Foxp3^+$Treg cells when T lymphocytes were grown in the presence of tolerogenic DCs (Fig. 4A and C). The secretion of IFN-γ and IL-12 was higher in co-cultures carried out in the presence of DCs from OVA-immunized mice than DCs from tolerant mice (Fig. 4I and G, respectively). On the other hand, the amounts of IL-10 and IL-4 were significantly higher in cultures conducted in the presence of DCs from tolerized mice (Fig. 4D and F, respectively). The secretion of IL-6 was significantly lower in cultures conducted in the presence of DCs from tolerant mice in comparison with those carried out with DCs from immunized mice (Fig. 4J). No significant differences were found in secretion of IL-17, TNF-α, and TGF-β in cultures carried out with naïve or arthritic mouse lymphocytes and DCs from naïve, tolerant or immune mice (Fig. 4H, K and E, respectively).

3.3. The adoptive transfer of tolerogenic dendritic cells modulates CIA

To examine the possibility of controlling the signs of the disease, we performed experiments of passive transfer of splenic DCs from either naïve or tolerant mice before inducing CIA in male mice. The passive transfer of tolerant $CD11c^+$DCs resulted in

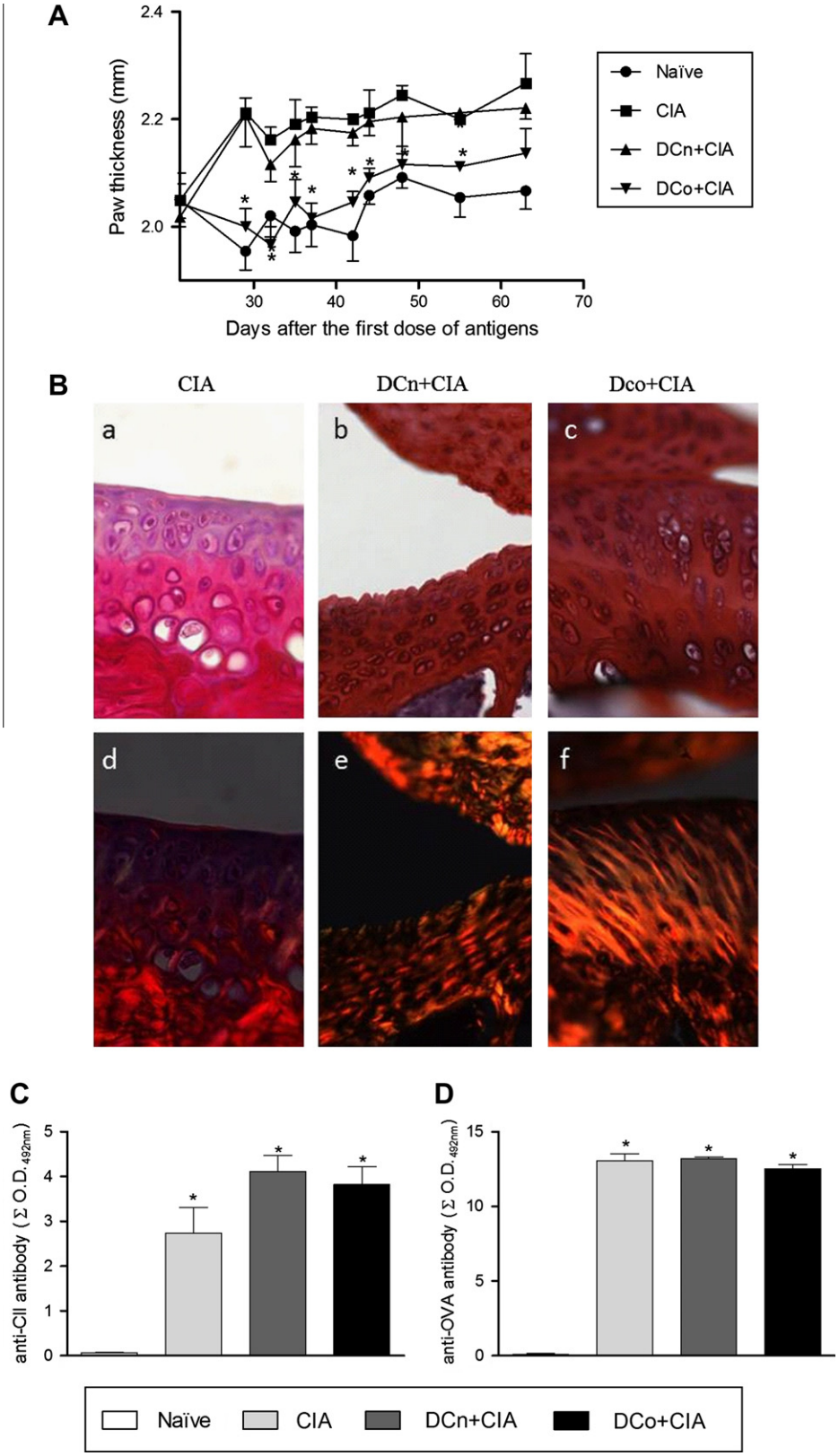

Fig. 5. Adoptive transfer of tolerogenic dendritic cells suppresses CIA in BALB/c mice. CD11c$^+$ DCs were isolated from spleens of naïve (DCn) and OVA-tolerized(DCo) mice and then adoptively transferred to male mice ($n = 5$, three injections of 5×10^5 DCs/mouse) before the administration of CII + OVA/CFA for induction of CIA. (A) Paw thickness was taken every 3 days after the first immunization until the end of the experiment. (B) At day 60, mice were euthanized and hind paws were dissected, embedded in paraffin and processed for histological analysis in Sirius red. (C and D) Sera were diluted from 1:100 to 1:1280 and tested in reactions of ELISA for detection of anti-CII and anti-OVA antibodies, respectively. Values are mean O.D. sums ± S.E.M. ($n = 6$). Data are representative of three independent experiments. Two-way ANOVA followed by Bonferroni's post-test were used to determine statistical significance in mean paw thickness in comparison to CIA group; $^*P \leqslant 0.05$. One-way ANOVA was used to determine statistical significance in mean O.D. sums; $^*P \leqslant 0.05$ in comparison to naïve mice and $^{\#}P \leqslant 0.05$ in comparison to CIA group.

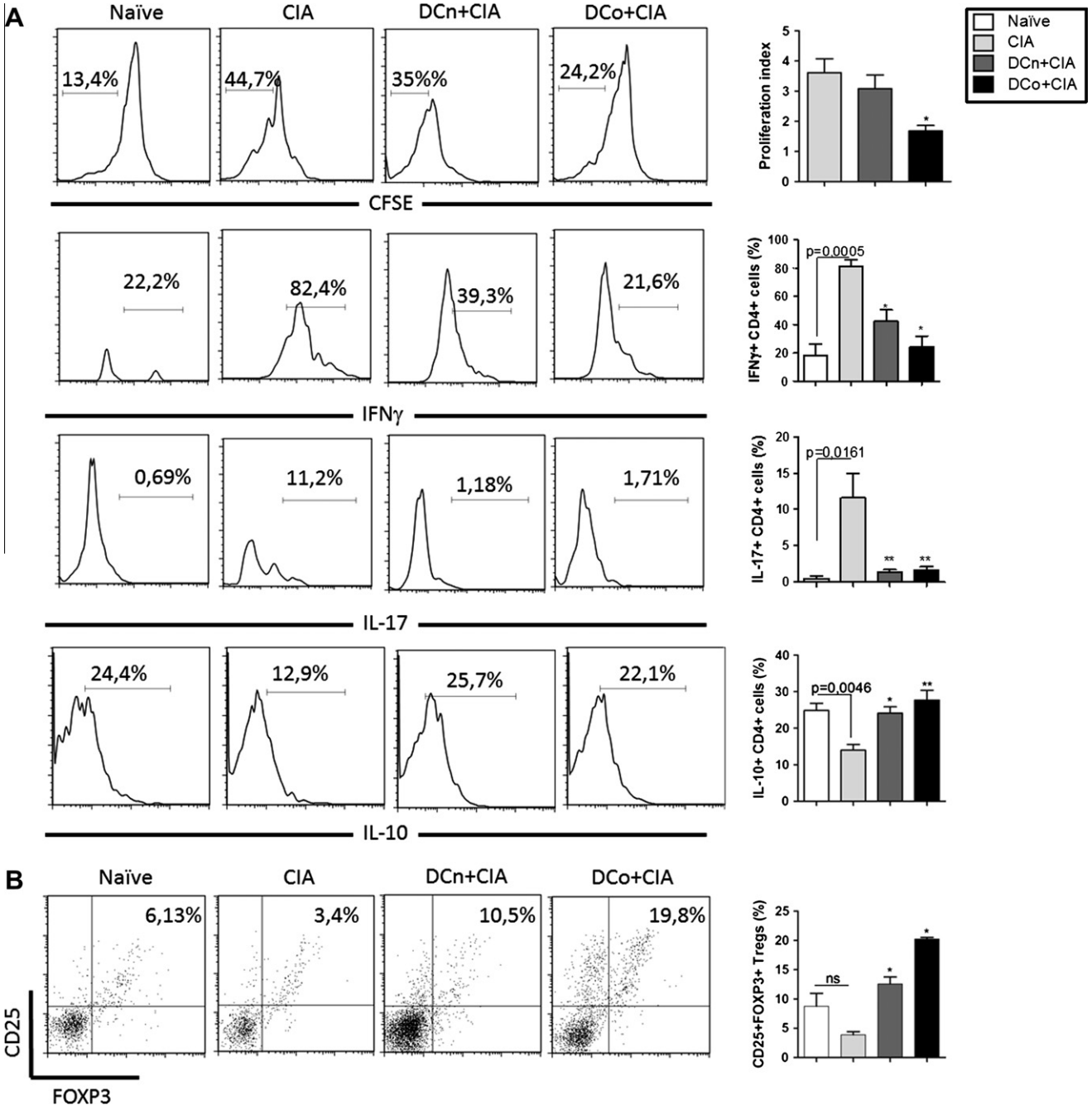

Fig. 6. Adoptive transfer of tolerogenic DCs reduces the collagen-specific proliferation of T lymphocytes, increase the proportion of $CD25^+Foxp3^+$Tregs and IL-10- producing-T cells. Sixty days after the first immunization to induce CIA, leukocytes were harvested from spleens of mice that previously received $CD11c^+$ DCs isolated from naïve (DCn) and tolerized (DCo) mice. Leukocytes from spleens of naïve and untreated CIA mice were used as controls. The spleen cells were stained with CFSE and cultured for 96 h in the presence of CII (50 μg/mL). (A) T cell proliferation was determined using flow cytometry and assessed by fluorescence decay of the probe in the gate of $CD3^+$ cells. IL-10-, IFNγ- and IL-17-producing cells were evaluated in the gate of $CD3^+CD4^+$ cells as well. (B) The frequency of $CD25^+$ $Foxp3^+$ Tregs in the different groups was evaluated in the gate of $CD3^+CD4^+$ cells. Data in %bar graphs represent the mean ± S.E.M. ($n = 4$) of cell frequencies obtained in two independent experiments and histograms are derived from a representative experiment. One-way ANOVA followed by Bonferroni's post-test were used to determine statistical significances; $P \leqslant 0.05$ in comparison with naïve (*) and CIA (#) groups.

significant reduction of both paw edema (Fig. 5A) and histologic synovial cartilage changes in CIA mice (Fig. 5B), but the levels of anti-CII and anti-OVA antibodies in sera of CIA mice were not modified by the adoptive transfer of any DCs (Fig. 5C and D, respectively). CII-induced proliferation was lower in T cell cultures from recipients of tolerogenic DCs than in cultures of the control groups (Fig. 6A). The frequency of IFN-γ- and IL-17-producing $CD4^+$ cells was significantly reduced in cultures of spleen cells from recipients of either naïve or tolerogenic DCs in comparison to control CIA group, whereas the frequency of IL-10-producing cells was significantly higher in those cultures (Fig. 6A). On the other hand, the frequency of $CD25^+Foxp3^+$ Tregs was higher in T cell cultures from recipients of either naïve or tolerogenic DCs than in the control groups (Fig. 6B). However, the levels of IL-17 and IFN-γ were significantly higher in supernatants of cultures of spleen cells from recipients of naïve DCs in comparison to those of the CIA control group (Fig. 7E and F, respectively), whereas the levels of TGF-β were significantly higher in cultures of spleen cells from recipients of tolerogenic DCs than in all other groups (Fig. 7C). The amounts of IL-6 and TNF-α were lower in the culture supernatants of spleen

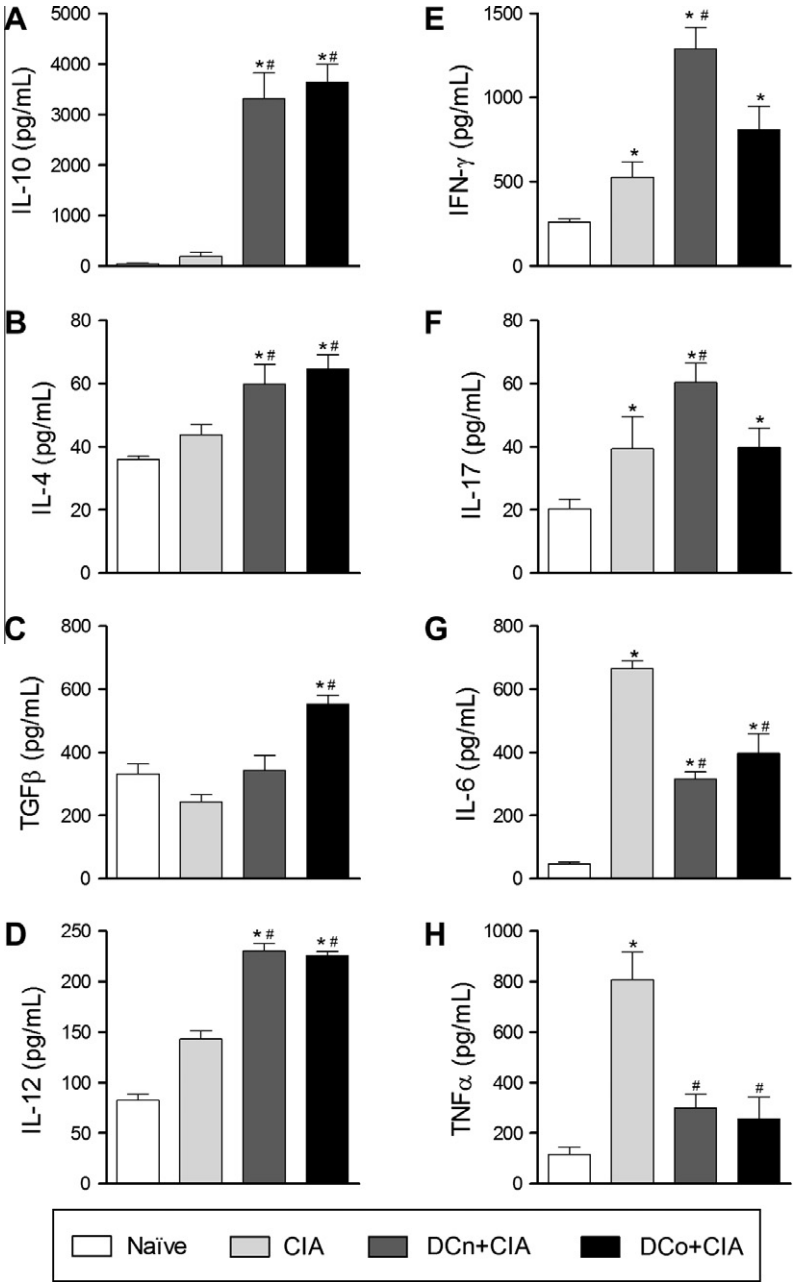

Fig. 7. Adoptive transfer of tolerogenic dendritic cells alters cytokine production in splenic cells from CIA mice re-stimulated in vitro. Sixty days after the first immunization to induce CIA, leukocytes were harvested from spleens of mice that previously received $CD11c^+$ DCs isolated from naïve (DCn) and tolerized (DCo) mice. Leukocytes from spleens of naïve and untreated CIA mice were used as controls. The spleen cells were cultured for 96 h in the presence of CII (50 μg/mL). (A–H) Cytokines IL-10, IL-4, TGFβ, IL-12p40, IFNγ, IL-17, IL-6 and TNF-α were detected in the culture supernatants by ELISA. Data in bar graphs represent the mean ± S.E.M. ($n = 6$) of cytokine production obtained in three independent experiments. One-way ANOVA followed by Bonferroni's post-test were used to determine statistical significances; $P \leqslant 0.05$ in comparison with naïve (*) and CIA (#) groups.

cells from recipients of either naïve or tolerogenic DCs than in CIA control group (Fig. 7G and H, respectively). On the other hand, the levels of IL-10, IL-4, and IL-12 were higher in the culture supernatants of spleen cells from recipients of any kind of DCs in comparison to those observed in cultures of spleen cells from either naïve or CIA mice (Fig. 7A, B and D, respectively).

4. Discussion

In this study, we show that CIA induced in BALB/c mice through the administration of OVA + CII in CFA can be both prevented and treated by OVA ingestion. We observed that OT to OVA led to an increase of $CD25^+Foxp3^+$Tregs and IL10-producing T lymphocytes in spleens of arthritic mice, with diminished production of IL-6, TNF-α and IFN-γ by spleen cells. We also found that transfer of splenic dendritic cells from OVA-tolerized mice was able to reduce the severity of arthritis through the induction of CII-specific Tregs. To our knowledge this is the first time that it is shown a reduction of the inflammatory response in experimental arthritis by transferring DCs pulsed with CII-unrelated antigen.

Arthritis induced by collagen is the most studied and characterized experimental model of rheumatoid arthritis, because the affected animals show similar clinical and immunological signs to

those observed in human disease [17]. The genetic background of animals as well as in humans exerts great influence on the development of the disease. Mice of H-2q and H-2r haplotypes, such as DBA 1 and B10q, are more susceptible to CIA [18]. It has been shown, however, that BALB/c mice (H-2d) develop CIA when an exogenous protein, such as KLH and OVA, is included in the antigen preparation together with bovine type II collagen [2]. Similarly to describe in other studies [19,20], we observed that BALB/c mice immunized with chicken type II collagen plus OVA in CFA showed clinical manifestations typical of arthritis, such as the cartilage erosion caused by chondrocyte hyperplasia and increased deposition of collagen in the zone of hyaline cartilage, leading to intensification of the calcification process in the more advanced stages of the disease. However, in contrast to that observed by Backstrom and Dahlgren [2], our data show an expressive proliferation of spleen T cell from CIA mice following in vitro re-stimulation with CII.

The manipulation of specific immune responses has been the subject of intense study, aiming at developing anti-inflammatory therapy alternatives to the use of nonspecific immunosuppressive drugs of broad-spectrum [21]. In this sense, the oral route of tolerance induction has been exploited in studies on the control of allergic [22] and autoimmune reactions [23], as well as experimental treatment of transplants [24]. In our study, we show that the consumption of OVA by a 7-day period, either before or after the parenteral immunization, significantly reduced the severity of CIA, as observed by reduction of paw edema in tolerized CIA animals. We also observed that the structures and arrangement of synovial cartilages were preserved in tolerized mice when compared to untreated CIA mice. The most relevant immunological parameters exhibited by OVA-tolerized CIA mice were the reduction of the levels of anti-CII antibodies, reduction of antigen-specific proliferation, increase of Treg and elevation of the anti-inflammatory cytokines TGF-β and IL10. Likewise, it was reported in the literature that the consumption of OVA reduced the immunological parameters of arthritis in DBA/1 mice, although the clinical signs were not significantly altered [25]. On the other hand, collagen-induced OT suppressed inflammation in arthritic DBA-1 mice trough the expansion of IL-10-producing $CD4^+CD25^+$ T cells. The same pattern of suppression was recently described in experimental lung transplantation preceded by oral administration of type V collagen [24]. In this case, the survival of transplant was also associated to immune responses involving expansion of Treg cells secreting IL-10 and TGFβ. Our data, however, show that OT to OVA can improve experimental arthritis in BALB/c mice as a consequence of the downregulation of both humoral and cellular immune responses to CII in a nonspecific fashion, through the increase of $CD25^+Foxp3^+$ and $CD4^+IL\text{-}10^+$ T cell subsets as demonstrated by in vitro re-stimulation with CII.

Dendritic cells play a fundamental role in the establishment of the peripheral tolerance [26,27]. Corroborating the literature data, our results show that DCs from tolerant mice are able to modulate the proliferation of T lymphocytes of arthritic mice and induce an increase in the frequency of CII-specific Treg amongst splenic cells stimulated in vitro with collagen. Based on these findings, we sought to evaluate whether adoptive transfer of DCs from OVA-tolerant mice could alter the course of CIA in BALB/c mice. Spleen cells from arthritic mice adoptively transferred with DCs from either naïve or tolerant mice showed increased levels of IL-10 and IL-4 and reduced levels of TNF-α and IL-6, following re-exposition to CII in vitro. On the other hand, IFN-γ and IL-17 were down-regulated in cells from mice adoptively transferred with either naïve or tolerogenic DCs, whereas TGF-β was up-regulated in cells from mice adoptively transferred with tolerogenic DCs. It was also observed a reduction of proliferative response to CII with an increase in the frequency of $CD25^+Foxp3^+$ Tregs in the cultures of spleen cells from recipients of tolerogenic DCs. These cytokine and cellular profiles seem to correlate with the severest cartilage alterations observed in animals that received naïve DCs in comparison to those treated with tolerogenic DCs. The literature provides some examples of improvements in experimental arthritis with tolerogenic DCs. In this sense, adoptive transfer of DCs modulated with CTLA-4-Ig, which make them tolerogenic cells, was capable of promoting the improvement of the clinical picture of CIA induced in DBA/1J by expansion of Treg cells [28], as observed in our work. Likewise, $CD11c^+CD11b^+$DCs isolated from Peyer patches of tolerized DBA-1 mice also confer protection against CIA [29].

5. Conclusions

Taken together, our results indicate that bystander suppression following OVA consumption is able to reduce the severity of clinical manifestations of experimental arthritis, an effect mimicked by adoptive transfer of DCs obtained from tolerized mice. Tolerogenic DCs, in turn, are able to induce the proliferation of antigen-specific regulatory T cells, with potential to suppress the response of pathogenic T lymphocytes, improving the clinical signs of experimental arthritis. Further studies, however, are necessary to evaluate the applicability of these procedures in other autoimmune manifestations and eventually in human patients.

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