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Blocking of p38 and Transforming Growth Factor β Receptor Pathways Impairs the Ability of Tolerogenic Dendritic Cells to Suppress Murine Arthritis

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Objective. **Dendritic cells (DCs) modulated with lipopolysaccharide (LPS) are able to reduce inflammation when therapeutically administered into mice with collagen-induced arthritis (CIA). The aim of this study was to uncover the mechanisms that define the tolerogenic effect of short-term LPS-modulated DCs on CIA.**

Methods. **Bone marrow–derived DCs were stimulated for 4 hours with LPS and characterized for their expression of maturation markers and their cytokine secretion profiles. Stimulated cells were treated with SB203580 or SB431542 to inhibit the p38 or transforming growth factor β (TGFβ) receptor pathway, respectively, or were left unmodified and, on day 35 after CIA induction, were used to inoculate mice. Disease severity was evaluated clinically. CD4+ T cell populations were counted in the spleen and lymph nodes from inoculated or untreated mice with CIA. CD4+ splenic T cells were transferred from mice with CIA treated with LPS-stimulated DCs or from untreated mice with CIA into other mice with CIA on day 35 of arthritis.**

Results. **Treatment with LPS-stimulated DCs increased the numbers of interleukin-10 (IL-10)–secreting and TGFβ-secreting CD4+ T cells, but decreased the numbers of Th17 cells. Adoptive transfer of CD4+ T cells from treated mice with CIA reproduced the inhibition of active CIA accomplished with LPS-stimulated DCs. The therapeutic effect of LPS-stimulated DCs and their influence on T cell populations were abolished when the p38 and the TGFβ receptor pathways were inhibited.**

Conclusion. **DCs modulated short-term (4 hours) with LPS are able to confer a sustained cure in mice with established arthritis by re-educating the CD4+ T cell populations. This effect is dependent on the p38 and the TGFβ receptor signaling pathways, which suggests the participation of IL-10 and TGFβ in the recovery of tolerance.**

Rheumatoid arthritis (RA) is a systemic autoimmune disease in which an uncontrolled immune response against self gives rise to severe joint inflammation, frequently involving other organs. In the pathogenesis of RA, the autoimmune process involves altered self-antigen presentation by dendritic cells (DCs), leading to the activation of autoreactive CD4+ T cells (1). Antigen-activated Th1 cells and Th17 cells in particular are the main mediators of inflammation and joint damage in RA and in animal models of this disease, through the secretion of interferon-γ (IFNγ) and interleukin-17 (IL-17), respectively, which activate the release of IL-1, IL-6, and tumor necrosis factor (TNF) by macrophages (2). In contrast, regulatory T cells have been described to be functionally impaired in RA patients and arthritic mice (3,4). Besides natural Treg cells, other types of regulatory T cells can protect against autoimmunity, mainly secreting the immunosuppressive cytokines transforming growth factor β (TGFβ) (Th3 cells) and IL-10 (Tr1 cells) (5,6).

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There is consensus that DCs are crucial to directing the immune response. This property resides largely in the maturation state of the DCs, which range from fully mature immunogenic DCs to tolerogenic immature DCs, spanning a wide variety of intermediate semimature states (7). When activated by pathogens or damage-associated molecular patterns, DCs up-regulate major histocompatibility complex (MHC) and costimulatory molecules, such as CD80 and CD86, to become immunogenic antigen-presenting cells. Unlike mature DCs, immature DCs, which express low levels of costimulatory molecules, render T cells anergic when presenting antigen in the absence of costimulation (8). In contrast, semimature DCs express intermediate levels of MHC and costimulatory molecules and have also been described as exhibiting regulatory capacities, based on the secretion of antiinflammatory cytokines such as IL-10 or TGFβ. Due to these features, immature DCs and semimature DCs have been designated as "tolerogenic DCs" (9).

Several studies have used in vitro–generated tolerogenic DCs to prevent or treat autoimmunity in animal models, including collagen-induced arthritis (CIA). The strategies used to endow DCs with tolerogenic properties suitable for CIA therapy include genetic modifications (10–12) or exposure to conditioning agents, such as TNF (13,14), NF-κB inhibitors (15,16), dexamethasone plus vitamin D_3 (17), and Toll-like receptor agonists (18), among others. In a previous study, we demonstrated that DCs treated with lipopolysaccharide (LPS) for a short period exhibit a semimature phenotype and are able to ameliorate established CIA in an antigen-specific manner (19).

The aim of the present study was to explore in depth the mechanisms underlying the therapeutic effect of short-term LPS-modulated DCs on the CIA model, focusing on pathogenic and regulatory CD4+ T cell populations and how they become re-educated by tolerogenic DCs.

MATERIALS AND METHODS

Mice. Male DBA/1LacJ mice ages 7–8 weeks old were obtained from The Jackson Laboratory. Mice were maintained according to international guidelines for animal care. Protocols were approved by the Bioethics Committee of the University of Chile.

Generation of DCs. DCs were generated from bone marrow precursors as described elsewhere (19). After 6 days of culture, DCs were stimulated with 1 μg/ml of LPS (from *Escherichia coli* serotype O26:B6; Sigma) for 4 hours or 24 hours or were left untreated. For DC inoculation experiments, cells were loaded on day 6 of culture with bovine type II collagen (Chondrex), and then stimulated with LPS. In some experiments, 1 hour before LPS stimulation, DCs were treated with 5 μM SB203580 (Calbiochem), 10 μM SB431542 (Sigma), or with both reagents together. For cell phenotyping, DCs were stained with the following antibodies: phycoerythrin (PE)–conjugated anti-CD11c, fluorescein isothiocyanate (FITC)–conjugated anti-CD86, FITC-conjugated anti-CD80, FITC-conjugated anti-CD40, or FITC-conjugated anti–class II MHC, or with their respective isotype controls (all from eBioscience). Cells were examined in a FACSCalibur flow cytometer (Becton Dickinson) and analyzed with the WinMDI 2.9 software.

Quantification of DC-secreted cytokines. Differentiated DCs were purified using anti-CD11c–coated magnetic beads (Miltenyi Biotec) and cultured for an additional 24 hours at 1×10^6 cells/ml under stimulation with LPS for 4 hours or 24 hours. Supernatants were collected and stored at –80°C. Levels of IL-12p70, IL-23p19, IL-10, and TGFβ were measured by enzyme-linked immunosorbent assay (ELISA) (Bender MedSystems).

CIA induction and evaluation. Induction of CIA, clinical evaluation, and determination of the joint score and the swollen joint severity score were performed as described previously (19). For the treatment protocol, 5×10^5 DCs generated as described above were injected intraperitoneally into the mice on day 35.

Histopathologic analysis. Five mice were euthanized on days 38, 42, 49, and 70 following type II collagen inoculation. The synovial membrane cellular infiltration index and articular cartilage destruction score were determined as described elsewhere (20).

T cell phenotyping and cytokine profile determination. Spleen cells and popliteal and inguinal lymph node cells obtained on day 47 after disease induction from treated or untreated mice with CIA were cultured overnight with 25 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml of ionomycin (Sigma). Brefeldin A (10 μg/ml; eBioscience) was added during the last 4 hours of incubation before harvest. Cells were then stained with FITC- or PE-conjugated anti-CD4 antibodies and stained intracellularly with the following antibodies: PE-conjugated anti–IL-17, PE-conjugated anti-IFNγ, PE-conjugated anti–IL-10, and FITC-conjugated anti–IL-10 (all from eBioscience), with Alexa Fluor 488–conjugated anti-TGFβ (Santa Cruz Biotechnology), or with their respective isotype controls. Alternatively, pools of CD4+ T cells isolated from spleens or lymph nodes by negative selection (Miltenyi Biotec) were cocultured at a 10:1 ratio with type II collagen–pulsed DCs that had been stimulated with LPS for 24 hours, and supernatants were collected after 72 hours for determination of cytokines by ELISA. Background levels of cytokines secreted by CD4+ T cells or DCs cultured alone were subtracted in each case. For regulatory T cell determination, spleen and lymph node cells were stained with FITC-conjugated anti-CD4 and allophycocyanin-conjugated anti-CD25 antibodies and then stained intracellularly with PE-conjugated anti-FoxP3 antibody (both from eBioscience). Cells were analyzed by flow cytometry.

Antigen-specific antibodies in serum. Microtitration plates that had been sensitized with bovine type II collagen protein were incubated with mouse serum. Antigen-specific

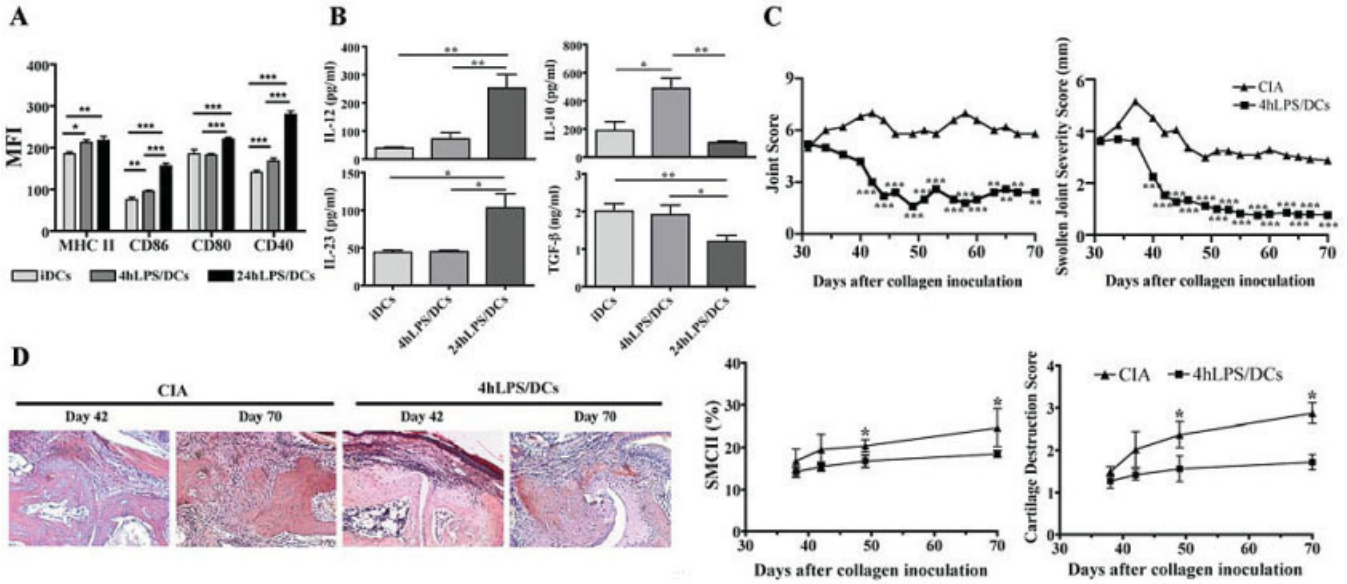

Figure 1. Characterization of short-term (4-hour) lipopolysaccharide (LPS)–modulated dendritic cell (DC) phenotype, cytokine profile, and tolerogenic function in collagen-induced arthritis (CIA). **A,** Expression of class II major histocompatibility complex (MHC), CD86, CD80, and CD40 on DCs stimulated with LPS for 4 hours (4hLPS/DCs) or for 24 hours (24hLPS/DCs) or on unstimulated immature DCs (iDCs). MFI = mean fluorescence intensity. **B,** Levels of interleukin-12 (IL-12), IL-10, IL-23, and transforming growth factor β (TGFβ) in supernatants from DCs isolated with magnetic beads and then cultured for a further 24 hours, as determined by enzyme-linked immunosorbent assay. **C,** Assessment of disease severity in untreated mice with CIA and in mice inoculated on day 35 after arthritis induction with 4-hour LPS-stimulated DCs, as determined by the joint score and the swollen joint severity score. Values are the mean of 5 mice per group. **D,** Histopathologic analysis of joint sections obtained on days 42 and 70 after inoculation with 4-hour LPS-stimulated DCs (left). Hematoxylin and eosin stained; original magnification $\times$ 10. Representative images are shown. The synovial membrane cellular infiltration index (SMCII) and cartilage destruction scores were also determined in joint sections (right). Values in **A**, **B**, and **D** are the mean $\pm$ SEM of 4 independent experiments. $* = P \leq 0.05$; $** = P \leq 0.005$; $*** = P \leq 0.001$.

antibodies were then revealed with anti-mouse subclass-specific antibodies.

Proliferation assays. On day 47 after disease induction, CD4+ T cells were isolated from lymph nodes by negative selection (Miltenyi Biotec) and then stimulated for 72 hours with 20 μg/ml of type II collagen, 20 μg/ml of ovalbumin or 5 μg/ml of concanavalin A in the presence of irradiated splenocytes. ^{3}H-thymidine incorporation was measured after 16 hours of incubation.

Adoptive transfer of T cells. Mice with CIA (both untreated and treated on day 35 post-CIA induction with DCs that had been stimulated for 4 hours) were euthanized 12 days after treatment, and their spleens were collected. Spleens from healthy mice were used as controls. Spleen cells were derived and incubated with FITC-conjugated anti-CD4 antibody and then isolated by fluorescence-activated cell sorting (FACSAria II; Becton Dickinson). CD4+ T cells (5×10^6) isolated from each of the 3 groups of mice were injected intravenously into groups of 4 mice with CIA on day 35. In addition, on day 35, one group of mice with CIA was injected with only phosphate buffered saline and another group was injected with LPS-stimulated DCs. Mice were clinically evaluated 3 times each week until day 70, when they were euthanized.

Statistical analysis. Comparisons between different groups of DCs or CD4+ T cells were performed by one-way analysis of variance (ANOVA) test for repeated measures, corrected with the Bonferroni post test or with a 1-tailed or 2-tailed paired *t*-test, as appropriate. A two-way ANOVA test for repeated measures, corrected with the Bonferroni post test, was applied for comparisons between clinical scores in the different groups of mice. *P* values less than or equal to 0.05 were considered significant. For statistical analyses, GraphPad Prism 5 software was used.

RESULTS

IL-10– and TGFβ-secreting LPS-stimulated DCs display a tolerogenic phenotype and exert a therapeutic effect on CIA. As previously described (19,21), DCs stimulated for 4 hours with LPS expressed similar levels of class II MHC, but significantly lower levels of CD80, CD86, and CD40 in comparison to fully mature DCs stimulated for 24 hours with LPS (Figure 1A). In addition to the previously described cytokine profile for 4-hour LPS-stimulated DCs (IL-10^{high} and IL-12^{low}), in the current study, we observed that the expression of TGFβ was greatly increased in comparison to that of 24-hour LPS-stimulated DCs (Figure 1B). In contrast, secretion of IL-23 was considerably lower in DCs stim-

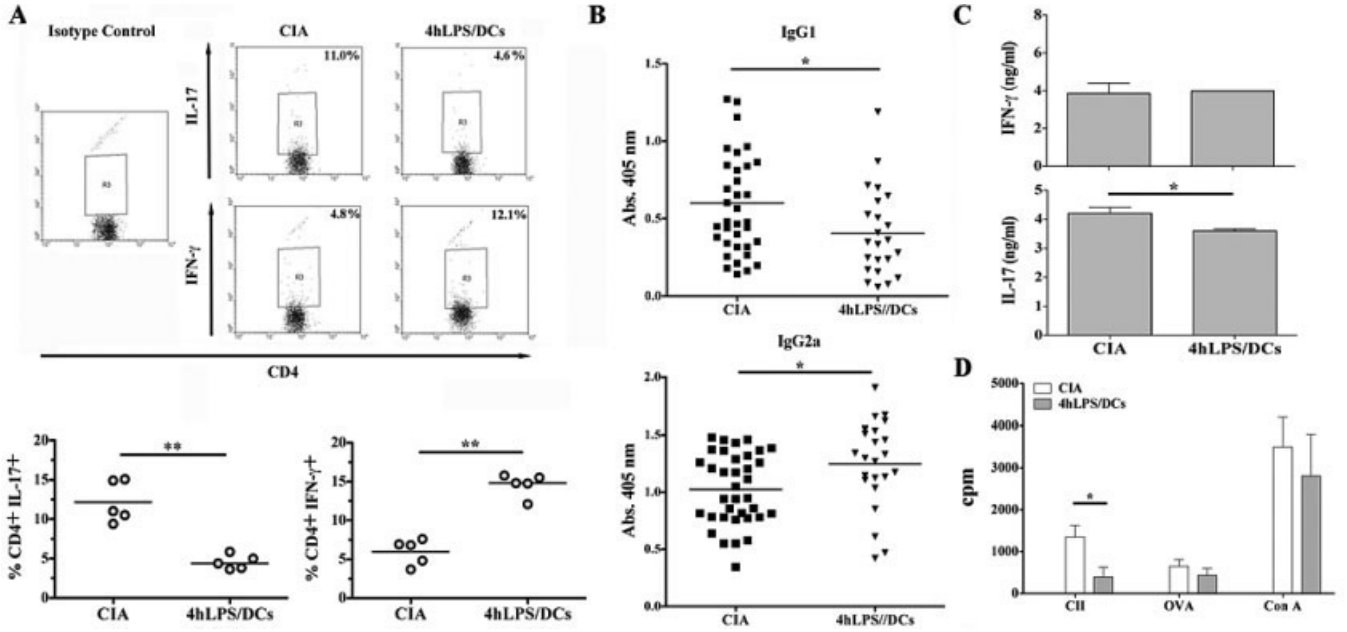

Figure 2. Effector T cells in mice with CIA treated with short-term (4-hour) LPS-stimulated DCs. **A,** Levels of IL-17– and interferon-γ (IFNγ)–expressing CD4+ T cells in splenocytes obtained on day 47 after disease induction from untreated mice with CIA and from mice with CIA treated with LPS-stimulated DCs. Cells were stimulated with phorbol 12-myristate 13-acetate/ionomycin and evaluated by flow cytometry. Isotype control is shown for comparison. Representative dot plots for each cytokine-secreting population are shown with the percentage of positive cells (top). Percentages of CD4+IL-17+ and CD4+IFNγ+ cell subsets in individual animals are also shown (bottom). Horizontal lines show the mean. **B,** Serum levels of type II collagen (CII)–specific IgG1 and IgG2a antibodies, as determined by enzyme-linked immunosorbent assay (ELISA). Each data point represents a single mouse; horizontal lines show the mean. Abs. = absorbance. **C,** Levels of IFNγ- and IL-17–expressing CD4+ T cells isolated from spleens obtained on day 47 after disease induction from untreated mice with CIA and from mice with CIA treated with LPS-stimulated DCs. Cells were cocultured with mature DCs pulsed with type II collagen, and supernatants were evaluated by ELISA. Values are the mean ± SEM of 2 independent experiments. **D,** ^{3}H-thymidine incorporation by CD4+ T cells isolated from lymph nodes obtained on day 47 after disease induction from untreated mice with CIA and from mice with CIA treated with LPS-stimulated DCs. Cells were stimulated for 72 hours with type II collagen, ovalbumin (OVA), or concanavalin A (Con A). Values are the mean ± SEM of 5–6 mice per group. * = $P \leq 0.05$; ** = $P \leq 0.005$. See Figure 1 for other definitions.

ulated for 4 hours than in those stimulated for 24 hours (Figure 1B). When mice with CIA were injected with 4-hour LPS-stimulated DCs in a single inoculation after disease onset, they showed a reduced severity of CIA (Figure 1C), as reported by Salazar et al (19). Furthermore, the clinical findings in treated mice correlated with decreased cellular infiltration and cartilage destruction of joints over time (Figure 1D).

CD4+ T cell populations become modulated after treatment with DCs stimulated for 4 hours with LPS. To better characterize the in vivo influence of DCs stimulated for 4 hours with LPS on different CD4+ T cell populations involved in the pathogenesis of CIA, spleen cells from mice treated with 4-hour LPS-stimulated DCs and euthanized on day 47 were stimulated for 16 hours with PMA/ionomycin and their cytokine expression pattern was evaluated by flow cytometry. The percentage of Th17 cells was greatly decreased in DC-treated versus untreated animals (Figure 2A). Instead, the proportion of Th1 cells was significantly increased after treatment (Figure 2A), which correlated with high serum titers of type II collagen–specific antibody for the IgG2a, but not the IgG1, isotype (Figure 2B). Furthermore, when CD4+ T cells isolated from the spleens of treated and untreated mice were cocultured with type II collagen–pulsed DCs stimulated for 24 hours with LPS, the CD4+ T cell cytokine secretion induced by antigenic activation confirmed the behavior observed after the polyclonal stimulus, but only for IL-17 secretion (Figure 2C).

We also assessed IL-17 and IFNγ secretion by CD4+ T cells derived from popliteal and inguinal lymph nodes. No significant differences between mice treated with 4-hour LPS-stimulated DCs and untreated mice were detected (data not shown). However, lymph node CD4+ T cells from treated mice exhibited a lower antigen-specific proliferative response to type II collagen than did cells from mice with CIA (Figure 2D).

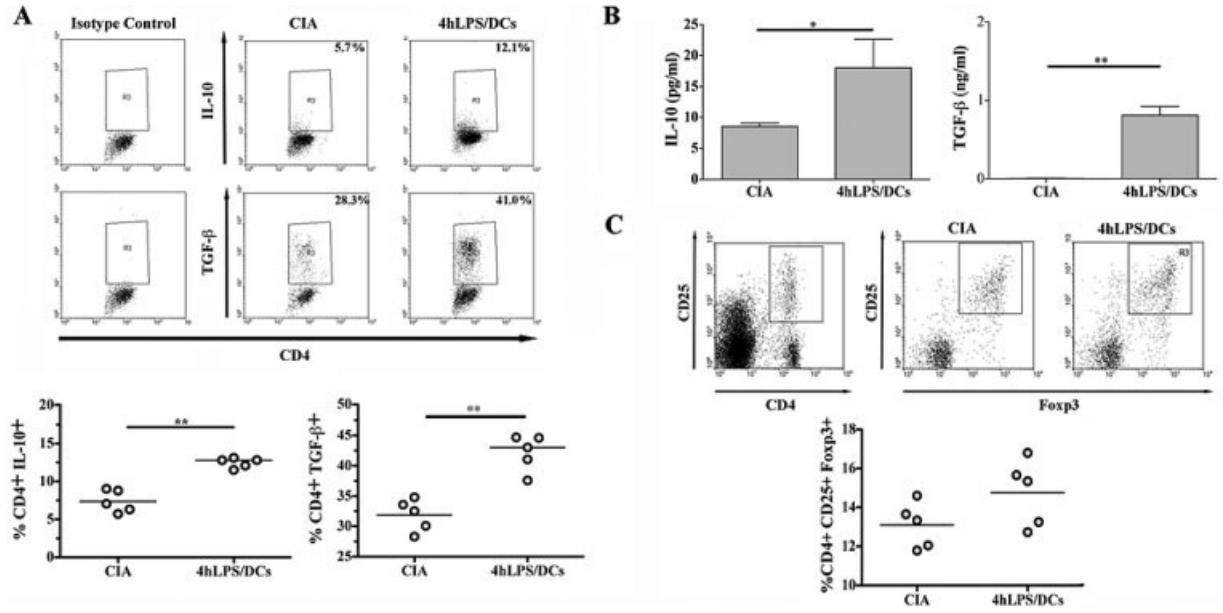

Figure 3. Induction of regulatory T helper cells in mice with CIA treated with short-term (4-hour) LPS-modulated DCs. **A,** Levels of IL-10– and TGFβ-expressing CD4+ T cells in splenocytes obtained on day 47 after disease induction from untreated mice with CIA and from mice with CIA treated with LPS-stimulated DCs. Cells were stimulated with phorbol 12-myristate 13-acetate/ionomycin and evaluated by flow cytometry. Isotype controls are shown for comparison. Representative dot plots for each cytokine-secreting population are shown with the percentage of positive cells (top). Percentages of CD4+IL-10+ and CD4+TGFβ+ cell subsets in individual animals are also shown (bottom). Horizontal lines show the mean. **B,** Levels of IL-10– and TGFβ-expressing CD4+ T cells isolated from spleens obtained on day 47 after disease induction from untreated mice with CIA and from mice with CIA treated with LPS-stimulated DCs. Cells were cocultured with mature DCs pulsed with type II collagen, and supernatants were evaluated by enzyme-linked immunosorbent assay. Values are the mean ± SEM of 2 independent experiments. **C,** CD4+CD25+FoxP3+ Treg cells in spleens obtained on day 47 after disease induction from untreated mice with CIA and from mice with CIA treated with LPS-stimulated DCs, as determined by flow cytometry. Representative dot plots show Treg cells within splenocyte populations (top). Percentages of Treg cells in individual animals are also shown (bottom). Horizontal lines show the mean. $* = P \leq 0.05$; $** = P \leq 0.005$. See Figure 1 for definitions.

In contrast, expression of the antiinflammatory cytokines IL-10 and TGFβ by splenic CD4+ T cells was markedly elevated in mice with CIA that had been inoculated with LPS-stimulated DCs, either after PMA/ionomycin stimulation (Figure 3A) or after antigen-specific stimulation (Figure 3B). The evaluation of these regulatory cytokines in lymph nodes did not show significant differences between the two groups (data not shown). In addition, we confirmed the absence of changes in the frequency of the splenic Treg cell population in treated mice (Figure 3C), as previously reported by Salazar et al (19).

Transfer of CD4+ T cells from mice with CIA treated with LPS-stimulated DCs displays a therapeutic effect. To investigate the mechanisms through which 4-hour LPS-stimulated DCs reduce the severity of CIA, we studied CD4+ T cells, which have been reported to be critical for the recovery of tolerance in this arthritis model (18,22,23). To discover whether LPS-stimulated DCs are somehow instructing CD4+ T cells to become tolerogenic, mice with CIA (day 35) were treated with at least 99% purified CD4+ T cells isolated from the spleens of healthy mice, LPS-stimulated DC–treated mice with CIA, or untreated mice with CIA, which were obtained on day 47 after arthritis induction, when treatment with LPS-stimulated DCs exhibited a steadily significant effect during the course of disease (Figure 1C). As expected, CD4+ T cells from mice treated with LPS-stimulated DCs reduced CIA severity, reaching similar clinical scores as those achieved with 4-hour LPS-stimulated DC inoculation. Mice that received CD4+ T cells from untreated mice with CIA showed a slight, but not significant, improvement compared with those given CD4+ T cells from healthy mice, although neither exhibited the same effect as that following administration of 4-hour LPS-stimulated DCs (Figure 4).

Signaling pathways involved in IL-10 and TGFβ secretion by LPS-stimulated DCs play a critical role in CIA improvement. Several studies have established that tolerogenic DCs are able to educate T cells through

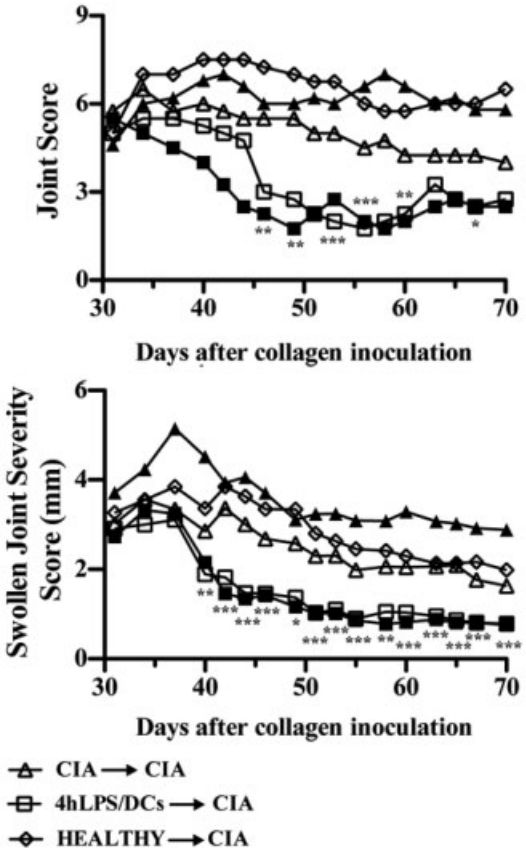

Figure 4. Adoptive transfer of CD4+ T cells obtained from mice treated with short-term (4-hour) LPS-stimulated DCs and interference with active CIA. CD4+ T cells were purified by fluorescence-activated cell sorting from spleens obtained on day 47 after disease induction. T cells (5×10^6) were isolated from mice with CIA, mice with CIA treated with LPS-stimulated DCs, and healthy mice and injected into groups of mice with CIA on day 35 after CIA induction. Disease severity was evaluated by the joint score and the swollen joint severity score. Phosphate buffered saline (PBS)–treated mice with CIA (▲) and mice with CIA treated with LPS-stimulated DCs (■) served as controls. Values are the mean of 4 mice per group. $* = P \leq 0.05$; $** = P \leq 0.005$; $*** = P \leq 0.001$ for mice inoculated with CD4+ T cells obtained from mice treated with short-term LPS-stimulated DCs versus mice injected with PBS. See Figure 1 for other definitions.

different means, including cell–cell interactions and secretion of antiinflammatory cytokines. For example, it has been demonstrated that cytokines such as IL-10 and TGFβ are essential to the induction of IL-10– and TGFβ-producing CD4+ T cells, respectively (24,25). To determine whether the secretion of these cytokines by LPS-stimulated DCs is necessary for their CD4+ T cell–mediated tolerogenic effect on CIA, a pharmacologic inhibition approach was attempted. Since IL-10 secretion by LPS-activated DCs is dependent on the p38 MAPK signaling pathway, we used SB203580, a specific inhibitor for this kinase (26). Similarly, it has been demonstrated that TGFβ signaling induces TGFβ secretion via an autocrine loop on murine DCs (27). Therefore, to achieve inhibition of TGFβ secretion by DCs we used SB431542, an inhibitor of TGFβ type I receptor–triggered Smad3 phosphorylation (28).

As expected, SB203580 inhibited IL-10 secretion, while it did not affect the secretion of TGFβ, IL-23, or IL-12 (Figure 5A). Likewise, SB431542 inhibited TGFβ secretion but not IL-10, IL-23, or IL-12 secretion (Figure 5A). Importantly, none of these reagents affected the expression of relevant surface molecules, such as class II MHC and costimulators (data not shown). The simultaneous use of both inhibitors maintained their effects without interfering with the secretion of other cytokines (Figure 5A) or with the expression of cellular markers (data not shown).

Next, we examined the effect of reduced secretion of IL-10 and/or TGFβ by pharmacologically modulated LPS-stimulated DCs during the course of CIA. As shown in Figure 5B, LPS-stimulated DCs treated with IL-10 inhibitor, TGFβ inhibitor, or both were unable to reproduce the therapeutic effect observed with unmodified LPS-stimulated DCs (Figure 5B).

Finally, we evaluated whether IL-10 and/or TGFβ inhibition of LPS-stimulated DCs had an impact on CD4+ T cell response modulation as described above. As shown in Figure 5C, mice receiving LPS-stimulated DCs treated with IL-10 inhibitor or with both IL-10 and TGFβ inhibitors became unable to induce the CD4+IL-10+ T cell population as compared to unmodified LPS-stimulated DCs. Likewise, the induction of CD4+TGFβ+ T cells was abrogated when mice with CIA were treated with LPS-stimulated DCs treated with TGFβ inhibitor or with both cytokine inhibitors. Interestingly, both the CD4+IL-10+ and the CD4+TGFβ+ T cell populations were reduced when mice with CIA were inoculated with LPS-stimulated DCs treated with TGFβ inhibitor and with IL-10 inhibitor, respectively, suggesting that IL-10 could induce the expression of TGFβ on CD4+ T cells and vice versa.

In contrast to the regulatory T cell populations, the decrease in Th17 cell frequency provided by treatment with LPS-stimulated DCs was partially reversed when mice were treated with LPS-stimulated DCs that had been modified with each inhibitor, but the percentages of Th17 cells were completely restored only when the expression of both cytokines was blocked. Moreover, the high frequency of Th1 cells observed after inoculation with LPS-stimulated DCs could not be reproduced

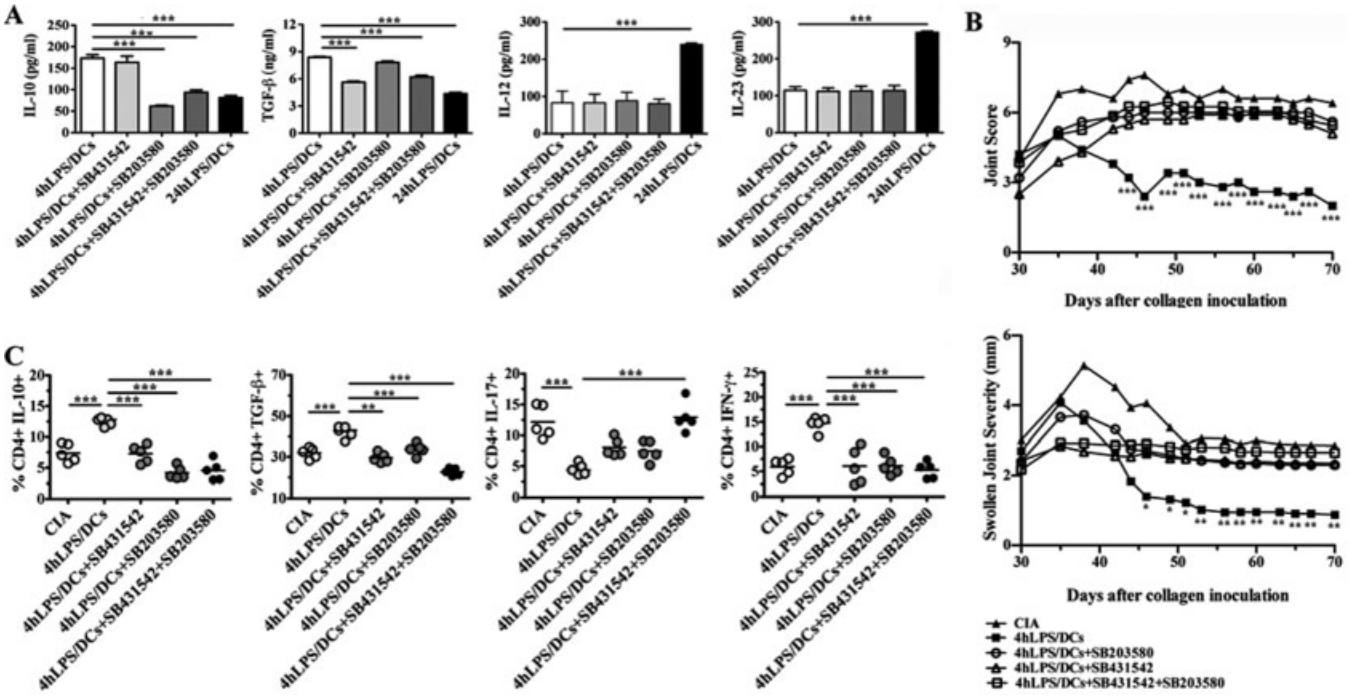

Figure 5. Inhibition of p38 and TGFβ receptor signaling pathways in short-term (4-hour) LPS-stimulated DCs and abrogation of their ability to ameliorate CIA. DCs stimulated with LPS for 4 hours were treated with SB203580 and/or SB431542, inhibitors of the p38 MAPK and TGFβ receptor signaling cascades, respectively. **A,** Production of IL-10, TGFβ, IL-12, and IL-23 by unmodified or pharmacologically inhibited LPS-stimulated DCs after 24 hours of culture. DCs stimulated with LPS for 24 hours were used as a mature DC control. Values are the mean ± SEM of 6 independent experiments. **B,** Disease severity on day 30 to day 70 of arthritis in untreated mice and in mice inoculated on day 35 after CIA induction with pharmacologically inhibited or unmodified LPS-stimulated DCs, as determined by the joint score and the swollen joint severity score. Values are the mean of 5 mice per group. **C,** Percentages of IL-10–, TGFβ-, IL-17–, and interferon-γ (IFNγ)–producing CD4+ T cells in splenocytes obtained on day 47 after disease induction from mice corresponding to the groups shown in **B**. Cells were stimulated with phorbol 12-myristate 13-acetate/ionomycin and evaluated by flow cytometry. Each data point represents a single mouse; horizontal lines show the mean. $* = P \leq 0.05$; $** = P \leq 0.005$; $*** = P \leq 0.001$ for the indicated comparisons in **A** and **C** and versus the CIA group in **B**. See Figure 1 for other definitions.

in mice treated with LPS-stimulated DCs that had been treated with IL-10 and/or TGFβ inhibitors (Figure 5C).

DISCUSSION

In this study, we developed an approach for defining the mechanisms through which LPS-stimulated DCs reestablish tolerance in mice with arthritis. Since CIA is mediated by pathogenic CD4+ T cells (29), we tested whether LPS-stimulated DCs could affect T cell responses in recipient mice with ongoing CIA. The adoptive transfer experiment clearly demonstrated that CD4+ T cells become educated in vivo under the influence of LPS-stimulated DCs and that the suppressive properties can be transferred into arthritic mice. Several studies have evaluated the ability of tolerogenic DCs to induce regulatory T cells in vivo through adoptive transfer experiments in models of autoimmunity; however, those studies used purified regulatory T cell populations from DC-treated mice to inoculate the host mice (18,22,30). In contrast, we transferred unselected CD4+ T cells, which best reflects the true composition of T cells in mice with the CIA immune status, and showed their ability to ameliorate disease in animals with an ongoing inflammatory process. These results demonstrated that LPS-stimulated DCs induce a shift in the balance of pathogenic and regulatory CD4+ T cell populations toward the reestablishment of tolerance.

The cytokine profile of CD4+ splenic T cells obtained from mice treated with LPS-stimulated DCs, both in a polyclonal manner and in an antigen-specific manner, revealed an increase in TGFβ- and IL-10–expressing cells (Figures 3A and B), similar to the findings in other studies that used tolerogenic DCs in an inflammatory setting (17,31). TGFβ secretion by CD4+ T cells has been attributed to the Th3 population, which was first described as being responsible for oral toler-

ance in models of autoimmunity (6). Likewise, multiple T helper cell populations have been reported to secrete IL-10, among which inducible Tr1 cells have been shown to play an important role in controlling autoimmunity (5,32). Along with the secretion of high levels of IL-10, Tr1 cells have been shown to secrete TGFβ or IFNγ, the latter in humans (33). Although we did find the coexpression of IFNγ in a minor percentage of IL-10–producing T cells (data not shown), it is difficult to determine whether the regulatory T cell populations induced by LPS-stimulated DCs are bona fide Th3 and/or Tr1 cells. Nevertheless, we confirm that this treatment did not increase the frequency of Treg cells, which is consistent with previous studies using tolerogenic DCs (19,34,35).

The importance of IL-10 and TGFβ secretion by DCs in the induction of tolerance has been largely demonstrated. For example, DCs genetically engineered to express IL-10 and/or TGFβ exhibit suppressive effects in models of alloreactivity (36,37) and autoimmunity (38). In addition, chemical inhibitors of signaling pathways that affect the secretion of these cytokines have been widely used to modulate DC functions (15,16,39,40). In this regard, Jarnicki et al (41) demonstrated that inhibition of p38 MAPK–dependent IL-10 secretion by DCs led to stronger Th1 responses and reduced Tr1 induction. Our data show that inhibition of the p38 and the TGFβ receptor pathways was sufficient to abolish the tolerogenic effect of LPS-stimulated DCs in CIA. Furthermore, this loss of efficacy was correlated with decreased induction of LPS-stimulated DC–mediated IL-10– and TGFβ-secreting T cells as well as with suppression of the Th17 response. Given that inhibition of p38 and TGFβ receptor signaling led to impaired secretion of IL-10 and TGFβ, we can attribute to these cytokines an important role in the tolerogenic phenotype of LPS-stimulated DCs; however, we cannot rule out the participation of other important molecules involved in regulatory DCs functions, which may be controlled by the above-mentioned signaling pathways.

Th17 has been assumed to be the most important T cell subset involved in the development of CIA (42,43). We found that treatment with LPS-stimulated DCs markedly reduced the Th17 population in mice with CIA. Our results are consistent with those reported by Stoop et al (17), who showed that DCs modified with dexamethasone, vitamin D_3, and LPS were able to induce a shift from Th17 cells to IL-10–producing T cells in mice with established CIA. The decrease in Th17 cells in mice with CIA following administration of LPS-stimulated DCs can be attributed to the combined effect of IL-10 and TGFβ secreted by the LPS-stimulated DCs, since the frequency of Th17 cells was completely restored when the pathways that led to the secretion of both cytokines were pharmacologically inhibited (Figure 5C).

While Th1 has been reported to be a relevant population in CIA development, its role remains a subject of controversy (44,45). Although we did not detect an increase in IFNγ-producing splenic CD4+ T cells after type II collagen stimulation in mice treated with LPS-stimulated DCs, we observed the induction of a polyclonal splenic Th1 population, which correlated with increased levels of anti–type II collagen antibodies of IgG2a isotype (Figures 2A–C). The expansion in Th1 cells is not likely to be induced directly by the LPS-stimulated DCs, since they express low levels of IL-12 and are poor stimulators of Th1 in vitro (19). Rather, an increase in IFNγ secretion by CD4+ T cells might be the consequence of a reduction in systemic levels of IL-17 following therapy, as observed in another experimental setting (46). Reciprocally, the Th17 response inhibition observed in mice treated with LPS-stimulated DCs could be also attributed to a protective role of IFNγ in the CIA model through direct suppression of Th17, as previously reported (47).

In conclusion, the findings of the present study contribute to the understanding of the mechanisms that mediate the therapeutic effects of short-term LPS-modulated DCs on mice with CIA. Our data attribute a central role to the p38 and the TGFβ receptor pathways in the induction of Tr1 and Th3-like cells and in the suppression of pathogenic Th17 responses in vivo by LPS-stimulated DCs. In addition, we proved that tolerance provided by LPS-stimulated DCs is transferable by CD4+ T cells. This knowledge constitutes an important step forward on the road to implementing new therapeutic trials involving tolerogenic DCs generated with clinically safe LPS analogs in RA patients.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Catalán and Aguillón had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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