Short-term lipopolysaccharide stimulation induces differentiation of murine bone marrow-derived dendritic cells into a tolerogenic phenotype

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ABSTRACT. Dendritic cells (DCs) are professional, antigen-presenting cells, which induce and regulate T cell reactivity. DCs are crucial in innate and adaptive immune responses, and are also involved in central and peripheral tolerance induction. Tolerance can be mediated by immature and semi-mature DCs expressing low levels of co-stimulator and major histocompatibility complex (MHC) molecules. The aim of this study was to investigate the ability of short-term lipopolysaccharide (LPS) stimulation to modulate the stage of differentiation of bone marrow-derived DCs. For this purpose, DCs obtained from DBA1/lacJ mice were stimulated for four (4hLPS/DCs) or 24 (24hLPS/DCs) hours with LPS, using DCs without stimulation (0hLPS/DCs) as a control. Flow cytometry analysis of 4hLPS/DCs showed intermediate CD40 and MHC class II expression, lower than that of 24hLPS/DCs (fully mature), and greater than that of 0hLPS/DCs (immature). A functional assay showed that 4hLPS/DCs displayed increased endocytotic ability compared to 24hLPS/DCs, indicating a semi-mature state. 4hLPS/DCs were greater producers of IL-10 protein and TGFβ1 mRNA than 24hLPS/DCs and immature DCs, displaying a cytokine production pattern that is characteristic of tolerogenic DCs. An assay for antigen-presenting capacity demonstrated that 4hLPS/DCs induced secretion of IL-2 from an OTH4 T cell hybridoma, indicating a functional presenting activity. Finally, the tolerogenic phenotype of 4hLPS/DCs was demonstrated by their ability to interfere with the progression of bovine type II collagen (bII)-induced arthritis (CIA) when they were loaded with bCII antigen and injected into mice with established CIA. We conclude that the stimulation of murine bone marrow-derived DCs with LPS for four hours generates semi-mature DCs with tolerogenic capability.

Keywords: dendritic cells, tolerance, lipopolysaccharide, autoimmunity

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) characterized by having great functional plasticity for antigens, allowing them to determine the type of immune responses that should be generated [1, 2]. Current data indicate that DC function is also related to their stage of maturation [3]. Thus, following an antigenic stimulus, CD40-mediated signalling causes immature DCs present in peripheral tissues become fully mature DCs, as shown by an up-regulation of expression of major histocompatibility complex (MHC) and co-stimulatory molecules, such as CD80 and CD86 [4-6]. At this point, mature DCs are equipped to deliver antigenic signals to T cells in an MHC-restricted manner, but do not uptake, transport, or process antigens as efficiently as immature DCs [7-10]. Unlike mature DCs, immature DCs are poor APCs, which have been shown to have antigen-specific regulatory properties [11]. This effect may be mediated by the induction of regulatory/suppressor CD4+ T cells that co-express CD25 and Foxp3 transcription factor, anergy, or alternatively, by the deletion of antigen-specific T cells [12-14]. Thus, DCs play a central role in orchestrating the immune response as the activation status of DCs imposes an important regulatory control in the induction and tolerization of immune responses against self and non-self antigens [15, 16]. To promote tolerance, DCs must capture, process, and present self antigens in the "steady state", which is an immature form of differentiation involved in the induction of peripheral T cell tolerance [17-20]. The novel finding that immunosuppressive and anti-inflammatory agents are able to generate tolerogenic DCs has revolutionized immunosuppressive therapy. Therefore, the generation of tolerogenic DCs in the laboratory has become the focus of new therapies for inhibiting the undesirable immune responses responsible for both graft-versus-host disease and autoimmunity [21, 22]. Several pharmacological strategies have been used to induce and

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maintain a regulatory phenotype of DCs, including agents such as IL-10 [23], vitamin D3 analogs [24], tumor necrosis factor (TNF) [25], vasoactive intestinal peptide [26], and chemically synthesized analogues of the immune suppressant 15-deoxyspergualin [27], among others. Tolerogenic DCs have been used to restore tolerance in experimental autoimmune encephalomyelitis (EAE) [25], type II collagen-induced arthritis (CIA) [28] and graft-versus-host disease [21]. Thus, repetitive injections of DCs matured with TNF have been shown to induce antigen-specific protection of mice from EAE and CIA [25, 26]. In this work, we investigated the capacity of LPS to modulate the stage of differentiation of DCs. We show that, depending on the duration of the LPS stimulus, it is possible to generate fully mature DCs or semi-immature DCs, with tolerogenic properties. In this way, short-term LPS stimulation induced the generation of tolerogenic DCs, characterized by high expression of costimulator and MHC molecules, intermediate endocytotic capacity, high immunoregulatory cytokine expression, and most importantly, the ability to interfere with the progression of CIA when loaded with type II collagen prior to injection.

METHODS AND MATERIALS

Mice

Seven to eight week-old DBA1/lacJ (H2q) and C57BL/6 mice, obtained from Jackson Laboratories (Bar Harbor, ME, USA), were maintained in accordance with international guidelines for animal care. All protocols were approved by the University of Chile Bioethics Committee.

Preparation of bone marrow (BM)-derived DCs

DCs were generated from BM obtained from mice in accordance with to the procedures described by Lutz [29]. Briefly, BM cells were cultured for seven days in medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF) [30]. After six days of culture, DCs were removed and used as immature DCs (**0hLPS/DCs**), or they were stimulated for four hours (**4hLPS/DCs**) or 24 hours (**24hLPS/DCs**) with 1 μg/ml LPS purified by gel filtration chromatography (*Escherichia coli*, serotype 026:B6 -Sigma, Chemical Co, USA). For CIA modulation experiments, after six days of culture, DCs were loaded for 24 hours with bovine type II collagen (bCII) (Chondrex, Redmond, WA, USA). The DCs were then removed and used as immature DCs (**0hLPS/bCII/DCs**), or they were stimulated with LPS for four hours (**4hLPS/bCII/DCs**) or 24 hours (**24hLPS/bCII/DCs**).

Antibodies and fluorescence-activated cell sorting (FACS) analysis

The following antibodies were purchased from eBioscience (San Diego, CA, USA): fluorescein isothiocyanate (FITC)-labeled anti-CD11c, anti-CD86, anti-MHC class II (AI/EI) and anti-CD40. Treated DCs were incubated with the appropriate antibodies for 30 minutes at 4°C in the dark. After washing, the cells were fixed in 4% paraformaldehyde. Flow cytometry was performed with a FACSCalibur machine (Becton Dickinson).

Endocytosis assays

200,000 DCs from the DBA1/lacJ mouse strain, previously subjected to the independent treatments mentioned above, were incubated with 1mg/ml of the endocytosis marker, FITC-conjugated DEXTRAN (40 kDa) (Molecular Probes, Leiden, The Netherlands) for 30 minutes at 37°C, or at 4°C as a control, and then analyzed by FACS. Mean fluorescence intensity values within the gate for the different endocytotically active stages were plotted [31].

IL-10 and IL-12p70 determination

Microtitration plates were sensitized overnight with specific anti-IL-10 or anti-IL-12 monoclonal antibodies (eBioscience). Supernatants and recombinant cytokine standards were incubated for two hours and then the plates were treated with biotinylated anti-cytokine antibodies. Detection was performed with LuminolTM (Pierce, PA, USA) using Luminoskan detector ASCENTR (Vantaa, Finland).

Determination of transforming growth factor β1 (TGFβ1) mRNA by RT-PCR

Total RNA from 4hLPS/DCs, 24hLPS/DCs and 0hLPS/DCs was isolated using a TRIZOLTM kit (Invitrogen Corporation, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA for TGFβ1 was obtained using the following primers: forward 5′-ACCGCAACAACGCCATCTAT-3′ and reverse 5′-GTAACGCCAGGAATTGTTGC-3′, designed with Primer Express version 2.0 software. For obtaining β-actin cDNA, the primers used were: forward 5′-ACTCTG GTGATGGTGT-3′ and reverse 5′- CGAGTAACC ACGCTCC-3′. DNA was amplified in 26 cycles at 95°C, 60°C, 72°C for 30 seconds, respectively, ending with a five-minute extension cycle at 72°C. The PCR products were separated in a 2% (p/v) agarose gel containing ethidium bromide. The intensity of ethidium bromide stained-DNA was determined with ImageJ 1.38e software. The intensity of the TGFβ1 cDNA signal was normalized to that of β-actin.

Evaluation of antigen-presenting capacity of 4hLPS/DCs

DCs obtained from C57BL/6 mice were generated as described above. DCs were pulsed for 24 hours with ovalbumin (ova) (Sigma, Chemical Co, USA) and then stimulated for four hours (4hLPS/ova/DCs) or 24 hours (24hLPS/ova/DCs) with LPS, and compared to immature DCs (0hLPS/ova/DCs). DCs not pulsed with ova (4hLPS/DCs, 24hLPS/DCs, 0hLPS/DCs) were used as controls. After washing, DCs were co-cultured with the ovalbumin-specific OTH4 T cell hybridoma in the following ratios (OTH4: DCs): 1:16, 1:8, 1:4, and 1:2. The OTH4 cells secrete IL-2 as a result of activation of the T cell antigen receptor. This cytokine was detected by ELISA in supernatants from the co-cultures.

Induction of CIA and clinical evaluation of arthritis

CIA was induced in DBA1/lacJ mice as described by van Duivenvoorde *et al*. [28]. bCII protein was dissolved in

0.1M acetic acid at a concentration of 2 mg/ml. The dissolved bCII was emulsified with an equal volume of complete Freund's adjuvant (CFA) (Chondrex), and 100 µl was injected subcutaneously into the base of the tail (100 µg bCII/mouse). This immunization was boosted three weeks later with a subcutaneous injection of 100 µg of bCII emulsified in Freund's incomplete adjuvant (Sigma). Three weeks after immunization, mice were examined three times per week for signs of arthritis. Arthritis severity in the paws was graded according to an established scoring system: 0 = normal joints, 1 = 1 or 2 swollen joints, 2 = 2-4 swollen joints, 3 = more than 4 swollen joints, and 4 = extreme swelling of the entire paw and/or ankylosis. An arthritis score for each mouse was calculated by adding the scores for each paw. The animals were euthanized at day 70 after starting the protocol.

DC inoculation

500,000 DCs, obtained from the DBA1/lacJ mouse strain according to the treatments described above, were suspended in 100 µl of phosphate-buffered saline and administrated intraperitoneally to four to six mice per group *via* a single inoculation at day 35 following the first inoculation with bCII (for CIA induction).

Statistical analysis

Differences in disease severity were analyzed by the Mann-Whitney U test. One-way ANOVA was used for other comparisons. A p value < 0.05 was considered statistically significant with a 95% confidence interval. The Graphpad Prism software was used for analysis of the relationships between groups.

RESULTS

Effect of LPS stimulation on DC maturation determined by phenotypic and functional properties

DCs were obtained from BM cells after seven days of culture in medium containing GM-CSF, with a yield of approximately 60%. *Figure 1*A shows that these cells expressed CD11c, a characteristic marker for DC populations. The cells also expressed CD11b, specific for myeloid lineage (data not shown). To study the response of DCs obtained from DBA1/lacJ mice to various maturation stimuli, DCs were exposed to LPS for four or 24 hours as mentioned previously, with non-exposed cells used as immature controls. After the different LPS-incubation times, the cells were harvested and analyzed by FACS for the expression of co-stimulatory (CD86 and CD40) and MHC class II molecules *(figure 1A)*. For all stimulatory treatments, the DCs showed equivalent levels of CD86 expression. Interestingly, 4hLPS/DCs displayed a lower expression level of CD40 ($p < 0.05$) and MHC class II molecules than 24hLPS/DCs. However, their levels were similar to those of untreated DCs (0hLPS/DCs), suggesting that 4hLPS/DCs correspond to a transition state between the immature and mature stages of phenotypic differentiation. Furthermore, DCs were analyzed for their ability to take up the endocytosis marker Dextran-FITC. Cells analyzed showed differing endocytotic abilities *(figure 1B)*. Thus,

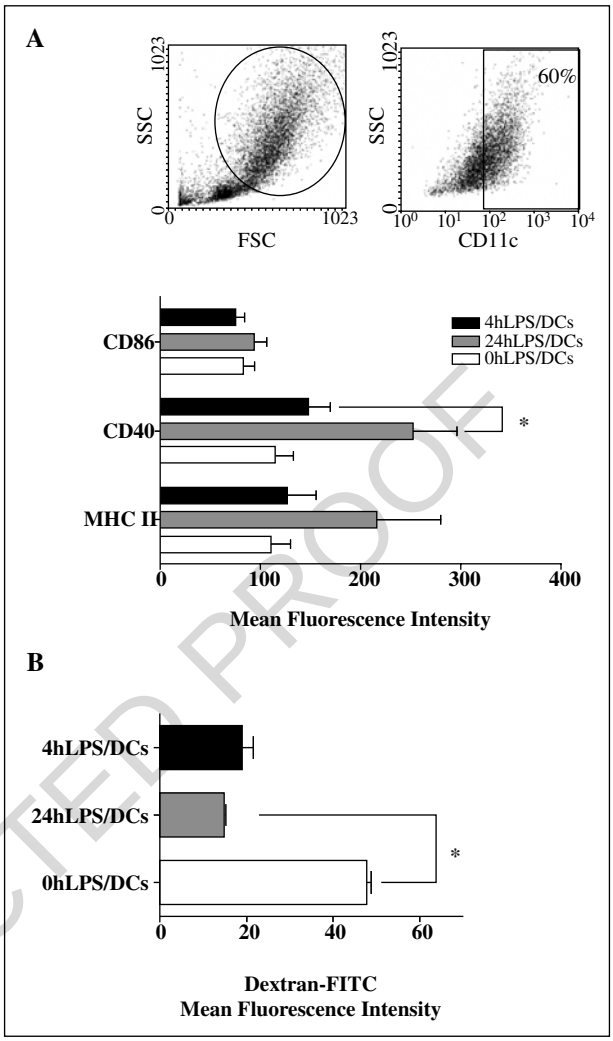

Figure 1
Surface phenotype and endocytosis assay of lipopolysaccharide (LPS)-treated dendritic cells (DCs) generated from bone marrow of DBA1/lacJ mice. **A,** After seven days of culture in medium containing granulocyte-macrophage colony-stimulating factor, the expression of CD11c was analyzed by fluorescence-activated cell sorting (FACS). FACS analysis showed the expression of co-stimulatory molecules (CD86 and CD40) and major histocompatibility complex (MHC) class II. DCs were stimulated for four hours (4hLPS/DCs) or 24 hours (24hLPS/DCs) with LPS and compared to immature DCs (0hLPS/DCs). Values are expressed as mean fluorescence intensity. * $p < 0.05$. **B,** Mean fluorescence intensity values for endocytotically active immature DCs cultured for seven days. DCs were differentially treated as mentioned in A. DCs were incubated with FITC-conjugated DEXTRAN -40 kDa-. * $p < 0.05$. Data from a representative experiment (of three experiments performed) are shown.

0hLPS/DCs displayed the highest levels of endocytosis, compared to 4hLPS/DCs which showed a diminished endocytosis level ($p < 0.05$), indicating a semi-mature transition state of these cells. 24hLPS/DCs lacked endocytotic ability, characteristic of functionally mature DCs.

Effect of LPS stimulation on DC maturation determined by cytokine expression pattern

Evaluation of the secretion pattern of IL-10 and IL-12 allows a most precise characterization of DCs, and provides important information about the possible mechanisms through which they can influence processes

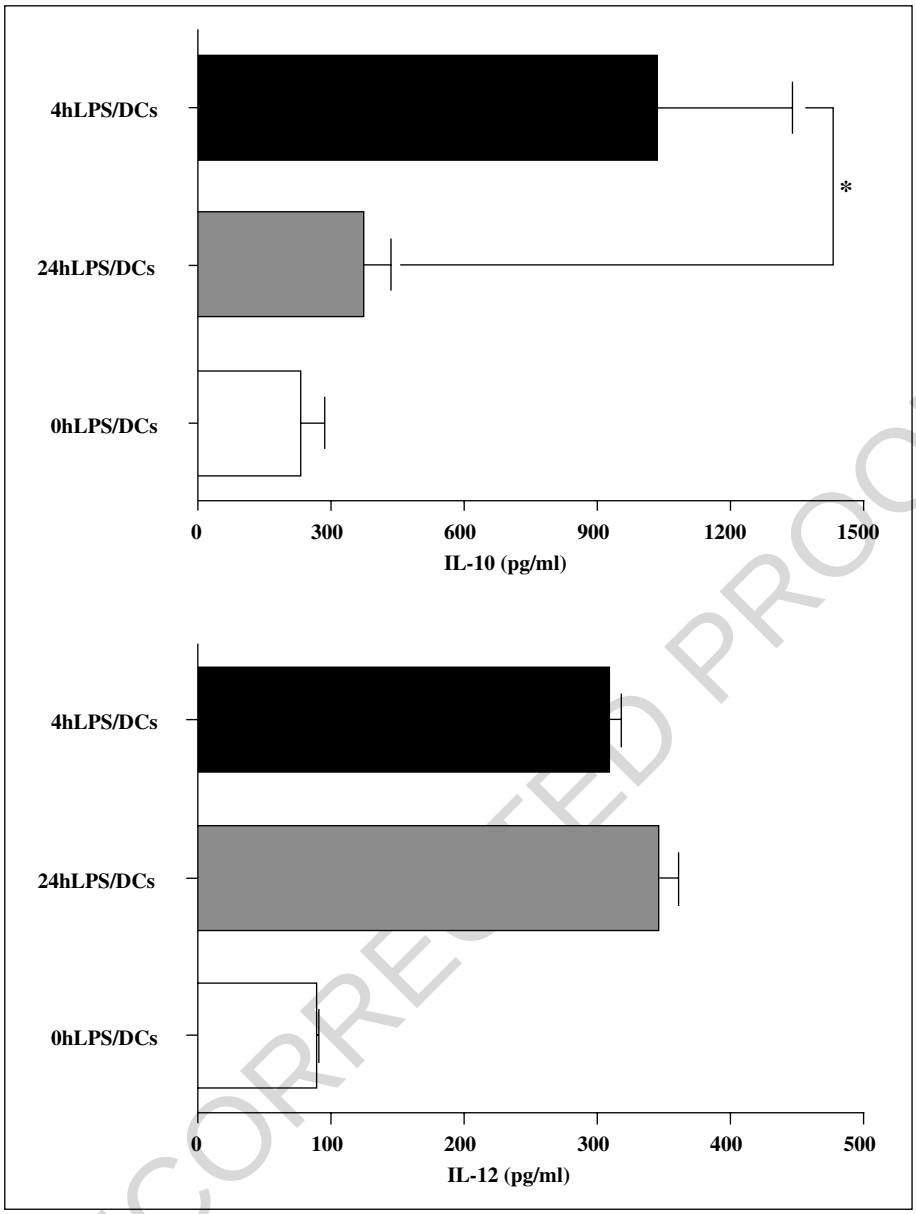

Figure 2

IL-10 and IL-12p70 detection in supernatants from bone marrow lipopolysaccharide (LPS)-treated DCs derived from DBA1/lacJ mice. DCs were stimulated for four hours (4hLPS/DCs) or 24 hours (24hLPS/DCs) with LPS and compared to immature DCs (0hLPS/DCs). Differentially stimulated DCs were washed and cultured for 24 hours and cytokine secretion was evaluated in supernatants. An ELISA test was used for cytokine quantification. *$p < 0.05$.

occurring *in vivo*. As depicted in *figure 2*, 4hLPS/DCs produced more IL-10 than 24hLPS/DCs ($p < 0.05$) and 0hLPS/DCs ($p < 0.01$). Although no significant differences in IL-12 secretion were detected between 4hLPS/DCs and 24hLPS/DCs, both groups produced significantly higher levels of IL-12 than 0hLPS/DCs ($p < 0.05$). The semi-quantitative evaluation of TGFβ1 mRNA showed that 4hLPS/DCs expressed significantly greater TGFβ1 mRNA levels ($p < 0.05$) than both 24hLPS/DCs and 0hLPS/DCs *(figure 3)*.

Evaluation of the antigen-presenting capacity of 4hLPS/DCs

The DC characterization data described so far suggest that 4hLPS/DCs represent an intermediate stage between immature and mature DCs. We next determined whether antigen-presenting ability is preserved in 4hLPS/DCs. To this end, we assessed the release of IL-2 by the ova-specific OTH4 T cell hybridoma in response to activation of its T cell antigen receptor by binding to the ova peptide-MHC complex, present on the surface of antigen-presenting DCs. When the different LPS-stimulated DCs were loaded with ova (4hLPS/ova/DCs, 24hLPS/ova/DCs and 0hLPS/ova/DCs) and co-cultured with OTH4 T cells, we observed that while all DCs induced the release of IL-2, 4hLPS/ova/DCs induced the greatest IL-2 secretion *(figure 4)*. In contrast, DCs not loaded with ova were unable to stimulate the IL-2 secretion *(figure 4)*. Thus, phenotypic and functional data indicate that 4hLPS/DCs represent a semi-mature state of DCs, with the ability to present antigenic peptides to naïve T lymphocytes.

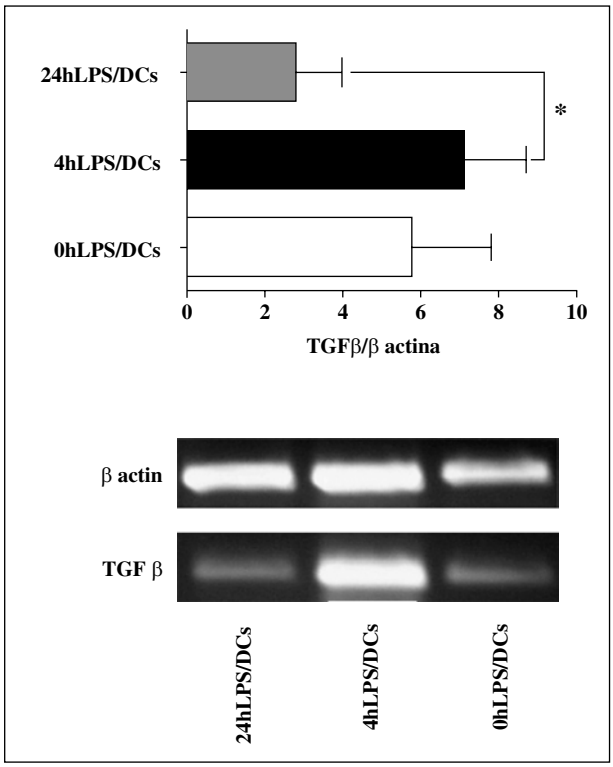

Figure 3
Determination of transforming growth factor β1 (TGFβ1) mRNA by RT-PCR in lipopolysaccharide (LPS)-treated dendritic cells (DCs), generated from bone marrow of DBA1/lacJ mice. Total RNA from 24hLPS/DCs, 4hLPS/DCs and 0hLPS/DCs was isolated using TRIZOLTM kit. cDNA for TGFβ1 and β-actin were obtained by PCR. The PCR products were separated in a 2% agarose gel containing ethidium bromide. The intensity of ethidium bromide stained-DNA was determined by the ImageJ 1.38e software. The intensity of TGFβ1 cDNA signal was normalized with respect to the β-actin and expressed as a ratio (TGFβ1 / β- actin). *$p < 0.05$.

Tolerogenic capability of 4hLPS/bCII/DCs to modulate CIA progression

The data described above indicate that 4hLPS/DCs are in a semi-mature state compared to non-activated DCs (immature), and 24hLPS/DCs (fully activated). To evaluate the ability of these differentially stimulated DCs to modulate CIA in an antigen-specific manner, they were loaded with bCII. After inducing active CIA, DBA1/lacJ mice were immunized intraperitoneally at day 35 (after the first bCII injection) with a single dose of 500,000 DCs subjected to different conditions. The progression and the onset of the disease in the groups treated with 0hLPS/bCII/DCs or 24hLPS/bCII/DCs were similar to those observed in the control group immunized with bCII/CFA alone *(figure 5)* ($p > 0.05$). In contrast, mice treated with 4hLPS/bCII/DCs displayed significantly less severe clinical disease as compared to the other groups, from day 50 through to day 70 ($p < 0.0001$). Additionally, injections of untreated DCs (without bCII loading) did not modify the natural progression of CIA, regardless of whether or not the DCs were stimulated with LPS (data not shown).

DISCUSSION

During the last five years, it has been demonstrated that DCs play a crucial role in immune responses against exogenous or self antigens and in mediating peripheral tolerance. Different approaches for modulating DCs in an antigen-specific manner have been successfully developed, including the establishment of human and murine modified DCs with potent immunoregulatory properties (designed as tolerogenic DCs). Unlike normal human DCs, which cause the activation of allogeneic CD4+ and CD8+ T cells, tolerogenic human DCs not only induce a state of anergy in these

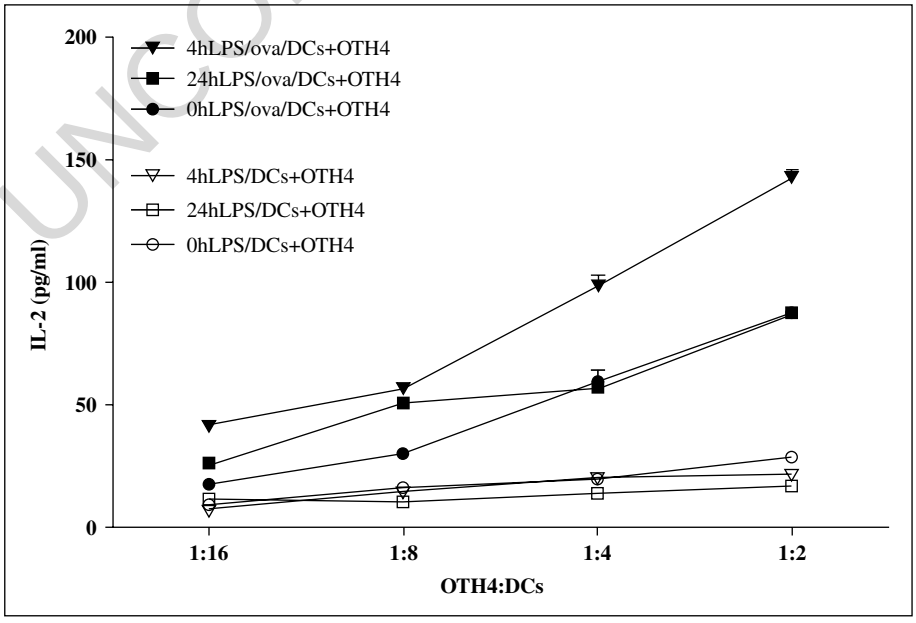

Figure 4
Evaluation of antigen-presenting capacity of four-hour lipopolysaccharide (LPS)- stimulated dendritic cells (DCs) generated from bone marrow of C57BL/6 mice. DCs were pulsed for 24 hours with ovalbumin (ova) and then stimulated for four hours (4hLPS/ova/DCs) or 24 hours (24hLPS/ova/DCs) with LPS and compared to immature DCs (0hLPS/ova/DCs). DCs not loaded with ova (4hLPS/DCs, 24hLPS/DCs, 0hLPS/DCs) were used as controls. After washing, DCs were co-cultured with the ova-specific OTH4 T cell hybridoma in the following ratios (OTH4: DCs): 1:16, 1:8, 1:4, and 1:2. IL-2 secreted by the OTH4 cells was detected by ELISA in supernatants from cell co-cultures.

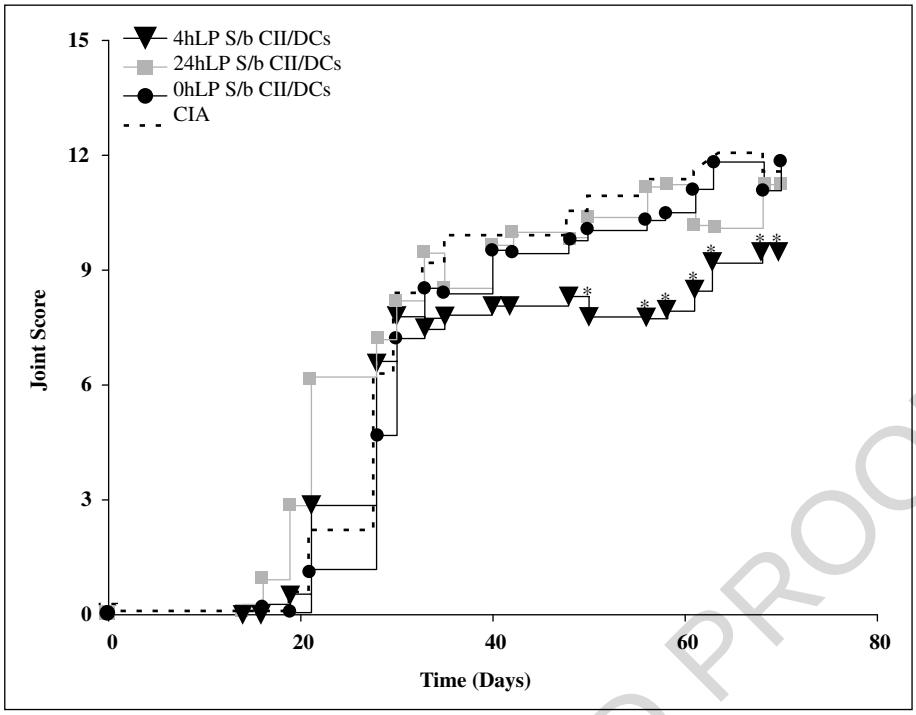

Figure 5

Modulation of active CIA in DBA1/lacJ mice by an injection of four hour lipopolysaccharide (LPS)-stimulated dendritic cells (DCs). CIA was induced with bovine type II collagen (bCII) added to complete Freund's adjuvant (CFA). Mice were inoculated intraperitoneally at day 35 following the first bCII administration, with 500,000 DCs as follows: **4hLPS/bCII/DCs**, four-hour LPS-stimulated DCs and loaded with bCII; **24hLPS/bCII/DCs**, 24 hour LPS-stimulated DCs loaded with bCII, and **0hLPS/bCII/DCs**, immature DCs loaded with bCII. **CIA**, corresponds to control mice that were immunized with bCII and CFA for CIA induction but did not receive a DC injection. The Joint Score, with a range of 0 to 16 for each animal, was determined in each group over time until day 70. Mice given 4hLPS/bCII/DCs had lower scores compared to all other groups (*$p < 0.0001$). Data are from a representative experiment (of six experiments performed; n = five to six mice per group).

T cells, but they also generate CD4+ or CD8+ regulatory T cells from their respective naïve subsets *in vitro* [23, 32-35]. In this study, we investigated the ability of LPS stimulation to modulate the stage of differentiation of DCs, demonstrating that, depending on the duration of the LPS stimulus, it is possible to generate fully mature or semi-mature DCs. Specifically, short-term LPS stimulation produced cells (4hLPS/DCs) characterized by an intermediate level of expression of co-stimulator and MHC molecules, a reduced endocytotic capacity, and the expression of high levels of immunoregulatory cytokines. In addition, 4hLPS/DCs displayed tolerogenic properties *in vivo*, interfering with the outcome of established CIA (as determined by joint score) in an antigenic-specific manner when they were administered to mice in a single injection. Although LPS has been identified as an activation and maturation agent for DCs, our results indicate that LPS also has the ability to induce an intermediate stage of DC maturation, depending on the length of LPS stimulation. The presence of intermediate CD40 and MHC class II expression levels, together with the reduced endocytotic ability detected in the 4hLPS/DCs, support the notion that they represent a semi-mature functional stage. In this sense, Lutz *et al*. [36] have reported that the duration to which BM-DC precursors are subjected to LPS treatment seems to be crucial for determining the final surface molecule expression and the functional properties of the cells. Thus, short-term LPS-treatment of BMDC cultures induced the generation of immature DCs only, which showed the ability to induce alloantigen-specific anergy of CD4+ T cells *in vitro* [36]. Additionally, several studies have identified LPS as a negative regulator of the differentiation of DCs from precursor cells. It has been demonstrated that, in contrast to the observed activating effect of LPS on differentiated DCs, LPS retards the generation of BM or monocyte-derived immature DCs, reduces DC yields, impairs their morphology and phenotype, and compromises their antigen presenting capacity [37, 38]. On the other hand, there are reports showing that, depending on the stimuli used to obtain terminally mature BM-DCs, the cells synthesize a different pattern of cytokines and exhibit distinct T helper cell-driving potential [39]. Thus, basal production of IL-6 and IL-10, which is initiated following stimulation of DCs with LPS, is modified in different ways by interaction with naïve CD4+ and CD4+CD25+ T cells. In particular, DC cytokine production is skewed toward IL-6 and away from IL-10 when the interaction is with the first subset, and the opposite occurs if the second subpopulation is involved [40]. Moreover, it has been demonstrated that after activation by LPS, DCs produced IL-12 only transiently and became refractory to further stimulation. Thus, soon after stimulation, DCs were able to prime strong Th1 responses, whereas at later time points the same cells preferentially primed Th2 and non-polarized T cells [41]. Very recently, it has been demonstrated that in response to specific self-antigens, DCs can secrete TGFβ1, IL-6 and IL-23, inducing a Th17 response [42]. This response is mediated by IL-17-producing T cells, responsible for the autoimmune damage observed in experimental models of rheumatoid arthritis and multiple sclerosis [43, 44]. However, DCs will be able to generate tolerance whether they have the ability to secrete a cytokine pattern dominated by IL-10 or TGFβ1, via the development of IL-10-producing Tr1 cells [45] or Treg [46] regulatory T lymphocyte subsets, respec-

tively. Considering the body of available information related to the functional significance of the pattern of cytokine secretion by DCs, and the results obtained in the present study demonstrating that 4LPS/DCs are both high IL-10 and TGFβ1 mRNA producers, we can infer that these DCs should induce tolerance *in vivo*. Consistent with our results, it has been demonstrated that the combination of low doses of LPS and cholera toxin is able to induce IL-10-producing, immature DCs, characterized by enhanced expression of CD80, CD86, and CD134, but inhibited LPS-driven induction of CD40, ICAM-I expression, and inflammatory cytokine/chemokine (IL-12, TNF, MIP-1α, MIP-1β) production [47]. Moreover, it has also been shown that *Bordetella pertussis*/TLR-4 signalling can activate innate IL-10 or may promote the induction of IL-10-secreting type 1 regulatory T cells, which could inhibit Th1 responses and limit inflammatory pathology in the lungs during bacterial infection [48]. In agreement with our previous observations but more clinically relevant, we found that injection of short-term LPS-modulated DCs loaded with bCII antigen interfered with CIA progression after the inflammatory process was already established. Furthermore, in unpublished results, we have demonstrated that 4hLPS/bCII/DCs dramatically inhibit IFNγ production by splenocytes from CIA mice, in a collagen-specific manner, when they are co-cultured. Taken together, our data that characterize the cytokine expression pattern of 4hLPS/DCs as pro-tolerogenic, and their *in vivo* capacity to modulate CIA, suggest that these DCs represent a tolerogenic stage, resulting from short-term LPS stimulation. From the point of view of *in vivo* immunoregulation, it has been reported that mice suffering from Gram negative bacterial infection develop a subset of tolerogenic DCs in response to bacterial LPS, which control the systemic inflammatory response. Unlike normal DCs, which produced proinflammatory cytokines in response to bacterial LPS, these DCs produced lower proinflammatory cytokine levels and instead preferentially produced IL-10. In addition, they suppressed LPS-induced production of proinflammatory cytokines in macrophages, reduced serum macrophage levels in mice, and protected mice against the lethality induced by experimental endotoxemia and bacterial peritonitis [49]. As mentioned above, tolerogenic DCs have great therapeutic potential, especially in those processes in which tolerance recovery is required, due to their specific immunological functions that are antigenically mediated. Thus, the use of injections of tolerogenic DCs has been successfully evaluated, and have been shown to promote tolerance recovery in experimental models of diseases such as multiple sclerosis [25], rheumatoid arthritis [28], Cröhn disease [50], and acute graft-versus-host disease [51]. However, the exact therapeutic efficacy of tolerogenic DCs remains to be demonstrated in patients suffering from diseases the experimental models of which, nevertheless, have shown auspicious results. Finally, four-hour LPS-treated DCs display a semi-activated phenotype, as determined by the expression of surface markers, endocytotic ability, high IL-10 protein and TGFβ1 mRNA production levels, and interference with CIA progression *in vivo*. Whether the semi-active status induced by LPS maturation is responsible for the effects observed, or whether specific, but as yet unidentified, signals are playing a dominant role is currently not known.

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