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ORIGINAL ARTICLE

Dendritic cells treated with chloroquine modulate experimental autoimmune encephalomyelitis

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Chloroquine (CQ), an antimalarial drug, has been shown to modulate the immune system and reduce the severity of experimental autoimmune encephalomyelitis (EAE). The mechanisms of disease suppression are dependent on regulatory T cell induction, although Tregs-independent mechanisms exist. We aimed to evaluate whether CQ is capable to modulate bone marrow-derived dendritic cells (DCs) both phenotypically and functionally as well as whether transfer of CQ-modulated DCs reduces EAE course. Our results show that CQ-treated DCs presented altered ultrastructure morphology and lower expression of molecules involved in antigen presentation. Consequently, T cell proliferation was diminished in coculture experiments. When transferred into EAE mice, DC-CQ was able to reduce the clinical manifestation of the disease through the modulation of the immune response against neuroantigens. The data presented herein indicate that chloroquine-mediated modulation of the immune system is achieved by a direct effect on DCs and that DC-CQ adoptive transfer may be a promising approach for avoiding drug toxicity.
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Dendritic cells (DCs) are antigen-presenting cells that have an important role in activation and modulation of naïve T lymphocytes.[1–3] The modulation of T cell functions occurs during antigen presentation, where DCs provide three signals: first, epitope–major histocompatibility complexes are recognized by the T cell receptor; second, co-stimulatory signals sent by the interaction of DCs' CD80 and CD86 with CD-28 expressed by T cells; third, the cytokine milieu created by DCs and other cells in the microenvironment.[4,5] The combination of these signals leads to T cell differentiation into Th1, Th2, Th17 and Th9 effector $CD4^+$ T cells.[4,6–8] The functional and phenotypical plasticity of DCs is dependent on the activation/maturation status of these cells turning them an interesting target for therapy studies.[2,9–11] Indeed, the adoptive transfer of *in vitro*-modulated DCs was able to reduce the severity of collagen-induced arthritis, diabetes and experimental autoimmune encephalomyelitis(EAE).[12–16]

Chloroquine (CQ), an antimalarial drug, has been used as an anti-inflammatory agent in the control of rheumatic conditions.[17–19] Although the mechanisms are not fully elucidated, CQ acts as a weak base and passively diffuses through the cytoplasm toward the lysosomes where it is retained by ionic trapping.[20] It was previously demonstrated that treatment of DCs with CQ inhibits antigen processing and presentation *in vitro* in a pH-dependent manner.[21–23] An increasing amount of evidence suggests that CQ also exerts its effects through pH-independent mechanisms.[24–27] Recently, we showed that CQ administration ameliorates the clinical and immunological parameters of EAE, a model for the human multiple sclerosis.[28]

EAE is established after neuroantigen-specific effector T cells breaks in the central nervous system (CNS) of mice.[29] In the CNS, effector T cells secrete a myriad of molecules that activates resident microglia and recruits inflammatory leukocytes ultimately leading to demyelination.[30] The clinical sign of the EAE start as paw weakness and progressively aggravate as the damage to the myelin sheath increases, resulting in death.[31] Therefore, the cellular immune response in EAE must be restrained. In this context, DCs may be modulated toward an anti-inflammatory profile. Our previous results with CQ administration in EAE have indicated that both Treg-dependent and -independent mechanisms are responsible for disease suppression.[28] We hypothesize that CQ also interferes with dendritic cell maturation thus reducing the cellular response against neuroantigens.

In this study, we evaluated the effect of CQ administration to DCs. Our results showed that CQ treatment promotes alterations in the expression of molecules related to antigen presentation as well as morphological and functional changes after *in vitro* antigen stimulation. We also observed that adoptive transfer of CQ-modulated DCs ameliorated the clinical course of EAE by the downregulation

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of the immune response toward neuroantigens and reduction in the glial reactivity and local inflammation. Collectively, our data suggest that CQ exerts its suppressive effects by interfering with the function of DCs and that adoptive transfer of CQ-treated DCs may be an interesting approach for the modulation of inflammatory conditions, such as EAE.

RESULTS

CQ treatment modulates bone marrow-derived DCs *in vitro*

The expression of major histocompatibility complex class II molecules was significantly reduced in cultures of DCs treated with CQ (Figure 1a). Similarly, the expression of CD80 and CD86 molecules on DCs was reduced after the treatment with the drug at higher concentrations—ranging from 25 to 100 μM (Figure 1a). The morphology of DCs after microbial challenge was altered in cells treated with the drug compared with PBS-treated controls, such as reduction on absolute size and dendropoiesis (Figure 1b). High CQ concentrations exert toxic effects *in vivo*; in order to evaluate the possible cytotoxic action of the drug, DCs were cultivated in the presence of CQ at several doses. As shown in Figure 1c, the treatment with CQ did not reduce the viability of DCs.

For functional analysis, DCs were treated with CQ, dexamethasone or PBS (DC-CQ, DC-Dexa and DC-PBS, respectively) and cultivated with $CD4^+$ T cells in the presence of $MOG_{35–55}$ peptide. The results showed that T lymphocytes cultivated in the presence of DC-CQ proliferated significantly less compared with cells cultivated with other DCs (Figure 2a). It was previously observed that CQ administration promotes an increase in the expression of nitric oxide synthase (NOS) in several cell types, interfering with renal function and other NO-dependent mechanisms.[32–34] However, in macrophages, CQ treatment reduced the NO synthesis.[35,36] There is lack of evidences about NO production by CQ-treated DCs. In order to solve this question, DCs were cultivated in the presence of increasing doses of CQ and NO secretion was measured later. Surprisingly, the results showed that there was a dose-dependent augmentation in NO production by DCs (Figure 2b). The next goal was to determine whether the higher production in NO was directly influencing the T cell proliferation. Thus, DCs were treated with CQ and cultivated with $CD4^+$T cells in the presence of a NOS inhibitor, L-NAME. The data obtained show that T cells cultured with DC-CQ proliferated significantly less than cells cultivated with DC-PBS; however, the treatment with L-NAME restored the proliferative response of T cells from DC-CQ mixed cultures (Figure 2c).

Transfer of CQ-modulated DCs ameliorates EAE

The data presented here show that DCs are modulated by CQ *in vitro*. The therapeutic usage of CQ-modulated DCs was investigated as well. For that purpose, PBS- and CQ-treated DCs were adoptively

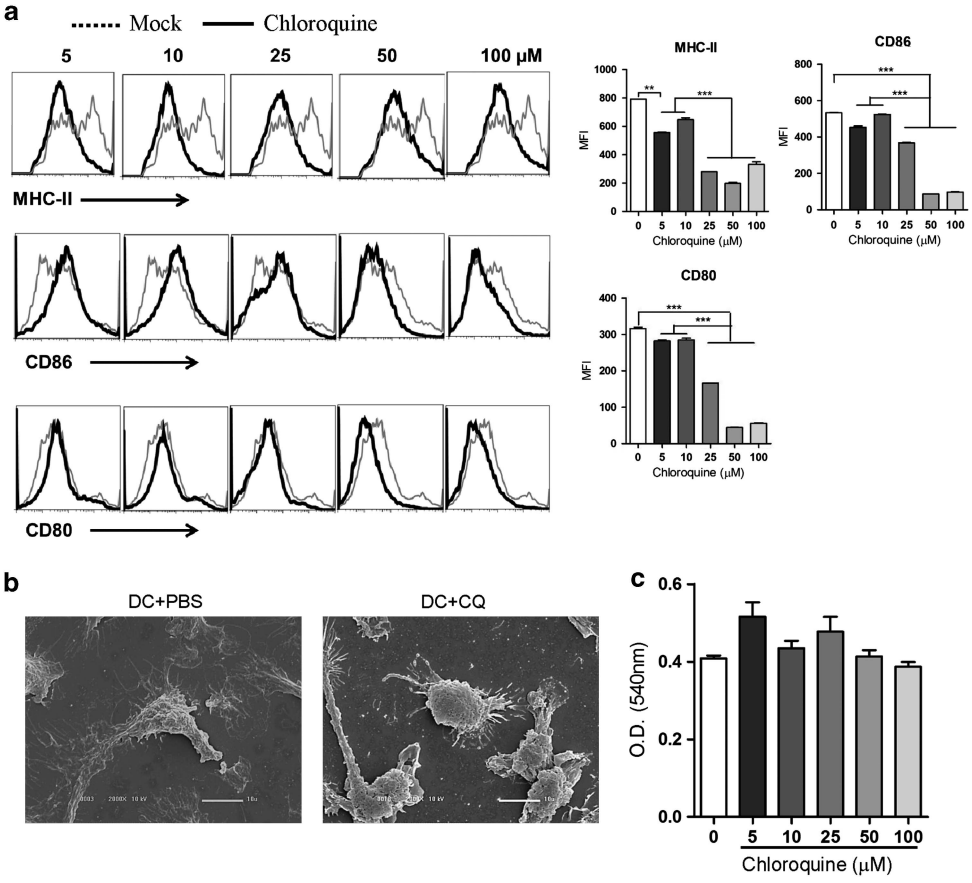

Figure 1 CQ treatment provokes phenotypical alterations in DCs. Bone marrow-derived DCs were treated with distinct CQ doses and stimulated with LPS (1 ηg ml^{-1}) for 18 h. (**a**) The mean fluorescence index (MFI) of MHC-II, CD86 and CD80 was reduced in CQ-treated cells (solid line) compared with the PBS-treated ones (dotted line); $^{**}P<0.01$ and $^{***}P<0.001$. (**b**) Scanning electron microscopy analysis showed that the treatment with CQ promoted alterations in the structure of DCs. The figures are representative of three independent experiments. (**c**) The toxicity of CQ was also evaluated by MTT assay.

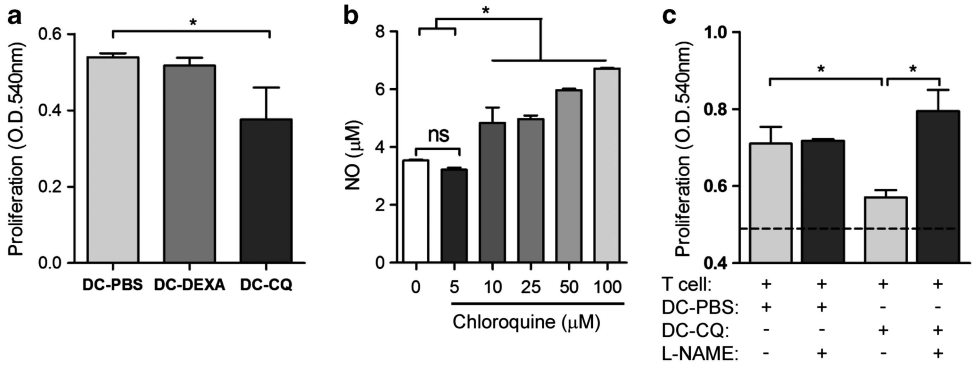

Figure 2 CQ treatment provokes functional alterations in DCs. Bone marrow-derived DCs were treated with CQ and stimulated with LPS (1 ηg ml^{-1}) for 18 h. (**a**) DCs were treated either with dexamethasone (DC-Dexa, 50 μM) or CQ (DC-CQ, 50 μM) and stimulated with LPS (1 ηg ml^{-1}) and then pulsed with MOG_{35-55} peptide (10 μg ml^{-1}) for 18 h. CD4$^+$ T lymphocytes were isolated from spleens of naïve mice using magnetic beads and cocultured with DCs (1:1) for 96 h in the presence of the antigen. The proliferation was evaluated by MTT. (**b**) The production of nitric oxide was determined by adding Griess reagent on culture supernatants from DCs treated with CQ. (**c**) DCs were treated with CQ (DC-CQ, 50 μM) and stimulated with LPS (1 ηg ml^{-1}) and then pulsed with MOG_{35-55} peptide (10 μg ml^{-1}) for 18 h. CD4$^+$ T lymphocytes were isolated from spleens of naïve mice using magnetic beads and cocultured with BMDCs (1:1) for 96 h in the presence of the antigen and an NOS inhibitor (L-NAME, 1 mM). The proliferation was evaluated by MTT. Data are representative of three independent experiments. *$P<0.05$.

transferred to naïve mice and the development of EAE was monitored. EAE was induced by immunization of mice with MOG_{35-55} peptide in an inflammatory context. Mice recipient of DC-PBS presented reduction of body weight and development of severe EAE, whereas the transfer of DC-CQ was capable to reduce the clinical signs of the disease (Figures 3a and b, respectively). In all experiments, DC-CQ-recipient mice presented lower cellular infiltration in the CNS in comparison with DC-PBS transferred and untreated EAE-inflicted mice (Figure 3c). To assess the presence of MOG-reactive cells in the periphery, EAE mice were killed and spleen cells were cultivated in the presence of MOG_{35-55} peptide and whole myelin basic protein. The cells were cultivated in the presence of ovalbumin as a control of an unrelated antigen. The results showed that spleen cells from DC-CQ-recipient EAE mice proliferated significantly less compared with the DC-PBS transferred and untreated EAE-inflicted mice in the presence of neuroantigens; however, there were no statistical differences between groups regarding the proliferation against ovalbumin (Figure 3d).

Transfer of CQ-modulated DCs reduces the glial reactivity in the CNS

The transfer of CQ-modulated DCs reduced the clinical course of EAE and was correlated with the lower T cell responsiveness in the spleen. Inflammation in the CNS results in neurodegeneration. The infiltration of inflammatory cells in the CNS of DC-CQ + EAE mice was found reduced compared with the control groups, DC-PBS transferred and untreated EAE mice (Figure 3c). Gliosis is the result of the activation of the glial cells, comprising astrocytes, oligodendrocytes and microglia (Iba-1$^+$ cells) after microenvironmental disturbances. The activation of astrocytes during inflammatory conditions favors the tissue microglial response, which ultimately leads to neuronal death.[37] Astrocytes express the glial fibrillary acidic protein (GFAP) upon stress. Due to its importance as a marker for glial reactivity, the presence of GFAP$^+$ cells was analyzed in the CNS of EAE mice. The results showed that untreated and DC-PBS-transferred EAE-inflicted mice had significantly higher frequency of GFAP$^+$ cells compared with the naïve group, whereas the transfer of DC-CQ was able to reduce the overall activation of astrocytes (Figure 4). Interestingly, the frequency of Iba-1 + cells was found significantly upregulated in both treatment groups compared with the naïve and untreated EAE, although the Iba-1 expression in the CNS from DC-CQ-treated mice was significantly reduced compared with the DC-PBS group.

Transfer of CQ-modulated DCs alters the gene expression in the CNS of EAE mice

As adoptive transfer of CQ-modulated DCs was capable of reducing the clinical manifestation of EAE and as mice recipient of DC-CQ presented lower cellular infiltration in the CNS as well as reduced activation of glial cells, the gene expression of inflammatory mediators was investigated. The results indicated that although the expression of TGF-β and IFN-γ was not altered between groups, the levels of IL-6 and IL-17 were found significantly reduced in CNS tissue from mice recipient of CQ-modulated DCs compared with EAE groups, both untreated and DC-PBS transferred. There was also an increase in the expression of IL-10 in the spinal cords of DC-CQ-recipient mice compared with all other groups (Figure 5). The expression of FOXP3 in CNS from DC-CQ-transferred EAE mice was found significantly elevated compared with healthy mice. Collectively, the data presented here show that the reduction in clinical outcome of EAE correlated with the downregulation of inflammatory mediators in the CNS and in the periphery of the immune system.

DISCUSSION

The development of new approaches in the treatment of autoimmune diseases is a common feature in today's research. It is known that autoimmune diseases arise in uncontrolled inflammatory responses, and that loss of self-tolerance is a key step in this process. Therefore, therapies that restore either the control of inflammation or the tolerance to self are desired. In this context, DCs are an interesting target, because they can stimulate the generation of Tregs and control inflammation.[3,38] The present study shows that transfer of CQ-modulated DCs reduces the clinical signs of EAE.

CQ, an antimalarial drug, has been used in the control of rheumatic diseases as well.[39,40] The mechanism of action resides in the fact that CQ is a weak base that passively diffuses toward acidic organelles, like the lysosomes.[41,42] The rise of pH interferes with the function of several enzymes and metabolic processes within the cell.[43,44] However, pH-independent mechanisms also occur, as

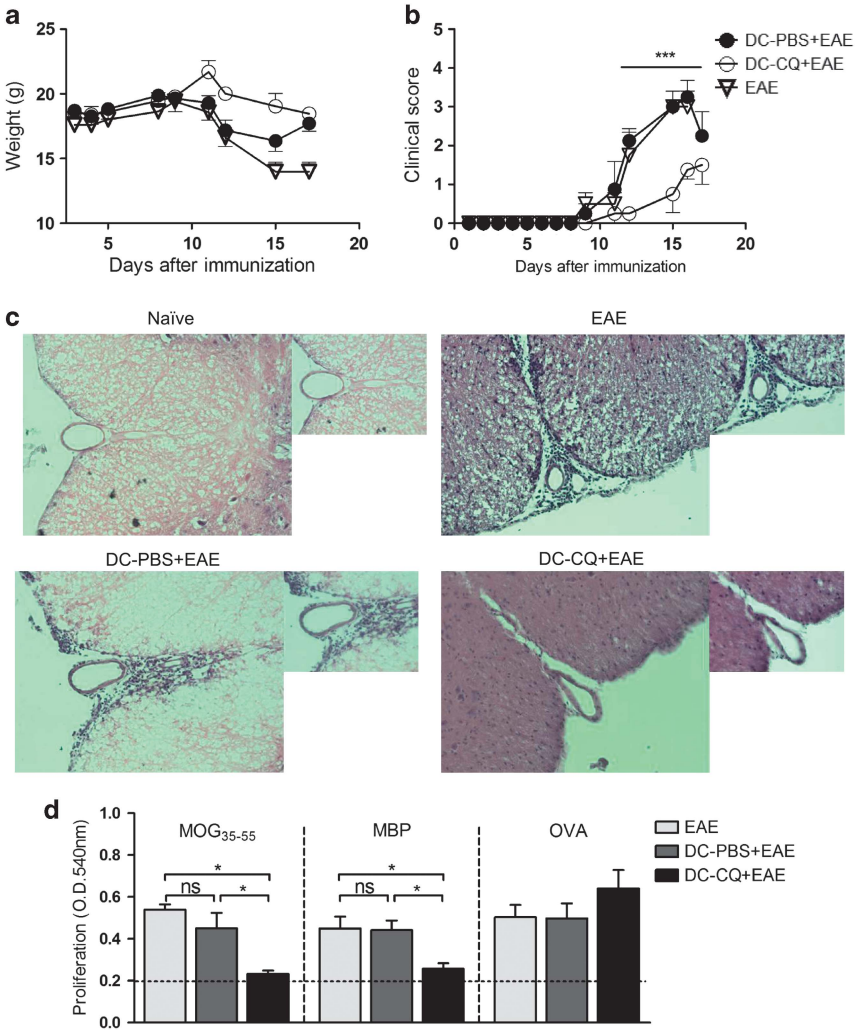

Figure 3 Adoptive transfer of CQ-treated DCs ameliorates EAE. DCs were treated with CQ (50 μM) and LPS (1 ηg ml^{-1}), and then pulsed with MOG_{35-55} peptide (10 μg ml^{-1}) for 18 h and adoptively transferred (1.5×10^6 cells per mouse) into C57BL/6 mice ($n=6$ per group) before the induction of EAE. (**a**) The weight variations were evaluated daily. (**b**) The clinical score of DCs-transferred and untreated EAE-inflicted mice was evaluated as well; ***$P<0.001$. (**c**) The histological analysis of the spinal cords from DC-CQ-recipient mice showed mild infiltration of inflammatory cells than in DC-PBS recipient and untreated EAE mice. (**d**) The spleens of naïve, EAE and DC-transferred EAE (both DC-PBS and DC-CQ) mice were collected and total leukocytes (5×10^5 per well) were cultured in the presence of MOG_{35-55} peptide, Myelin basic protein and Ovalbumin (10 μg ml^{-1}, 50 μg ml^{-1} and 50 μg ml^{-1}, respectively) for 96 h. The proliferation was analyzed using the MTT method; *$P<0.05$. Data are representative of three independent experiments. Magnification of histological sections: ×200 (main) and ×400 (details).

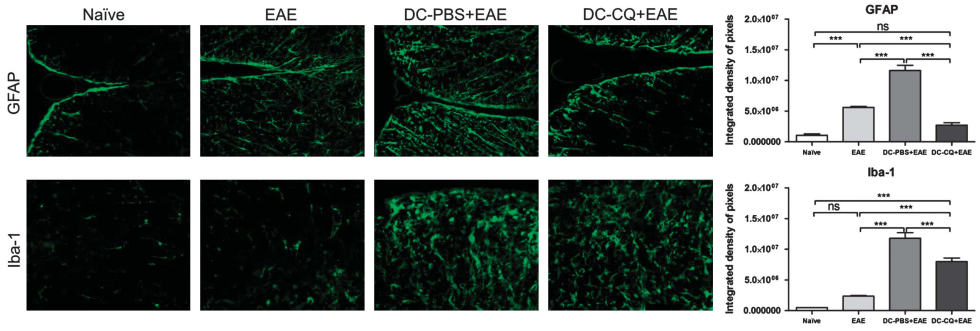

Figure 4 Microglia and astroglial response in EAE mice after DC transfer. DCs were treated with CQ (50 μM) and LPS (1 ηg ml^{-1}), and then pulsed with MOG peptide (10 μg ml^{-1}) for 18 h and adoptively transferred (1.5×10^6 cells per mouse) into C57BL/6 mice ($n=6$ per group) before the induction of EAE. The spinal cords of EAE mice were collected 14 days after immunization. Frozen thin sections (12 μm) were made, fixed in formalin and the reactivity sites were blocked with PBS-BSA 3%. GFAP and Iba-1 primary antibodies were added and treated with secondary antibodies conjugated with Cy2. The sections were analyzed in epifluorescence microscope. The images were analyzed by ImageJ software and the integrated density of pixels was determined. ***$P<0.001$. Figures are representative of three independent experiments. Magnification: ×200.

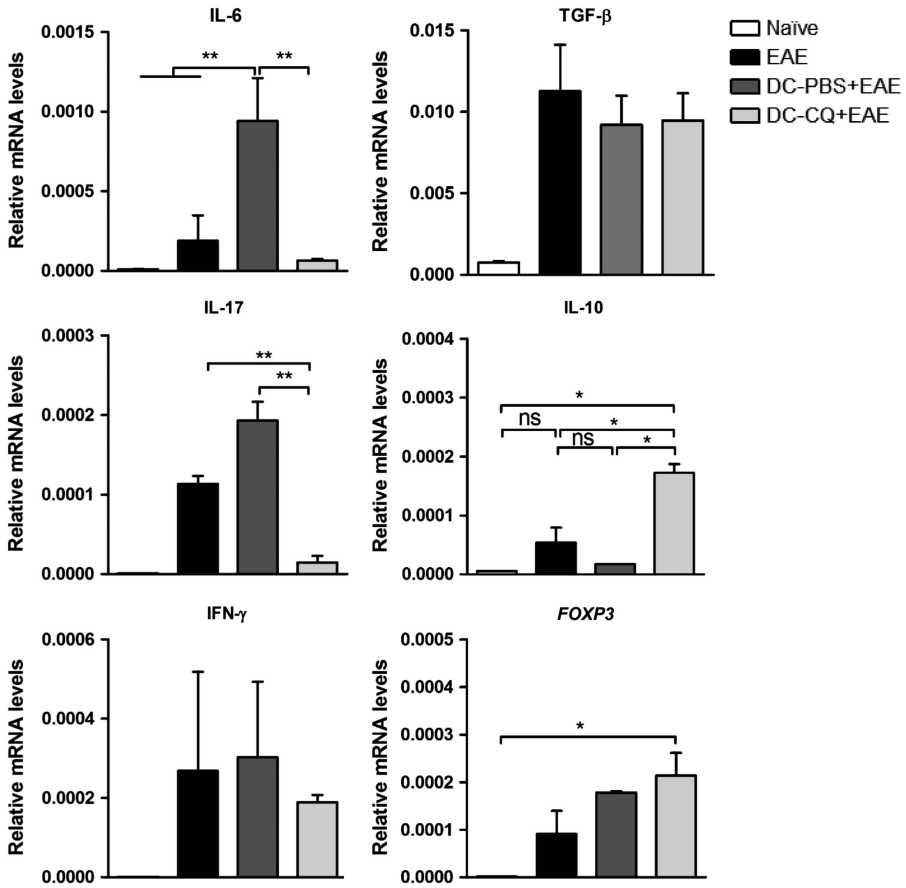

Figure 5 Altered gene expression of inflammatory mediators in CNS tissue of DC-transferred mice. Mice received PBS- and CQ-treated DCs (1.5×10^6 cells per mouse) 3 days before EAE induction. The CNS tissue (brain and spinal cord) was removed at the peak of the disease. RNA was extracted, converted to cDNA and the relative messenger RNA expression of IL-6, TGF-β, IL-17, IL-10, IFN-γ and FOXP3 was determined by reverse transcriptase-PCR reactions in naïve, EAE and DC-transferred EAE (both DC-PBS and DC-CQ) mice. $^*P<0.05$ and $^{**}P<0.01$. Results are expressed as mean ± standard error mean (s.e.m.) for at least four animals from each group from two independent experiments.

the treatment with CQ interferes with the iron metabolism in macrophages.[27] The treatment of DCs with CQ was shown to inhibit processing of antigens, which is pH-dependent.[45] Interestingly, CQ administration favors the cross-presentation by DCs and improves the development of adaptive immune responses toward ovalbumin.[22] We observed that the treatment of DCs with CQ reduced the expression of molecules related to antigen presentation. Likely, the treatment promoted ultrastructural changes in the morphology of the cells. These observations suggest that the drug has an anti-inflammatory effect. The lysosome dysfunction promoted by CQ facilitates accumulation of cell debris and leads to cell death, especially cells of the retinal pigment epithelium.[41,46] We found that CQ, in the doses tested in this study, has no toxic effect on DCs.

The maturation status of DCs defines the profile of activation of T cells.[47] Mature DCs express higher levels of class II major histocompatibility complex and co-stimulatory B7 molecules, whereas the immature/semi-mature phenotype produces intermediate expression of these molecules.[11] CQ does change the profile of DC maturation phenotypically. Functionally, mature DCs stimulate the differentiation of Th1, Th17 and Th2 T cells.[48] Semi-mature DCs induce the differentiation of naïve T cells in regulatory T cells by differentiated co-stimulation.[47] Failure in antigen presentation leads to improper activation of T cells. We observed that CQ-modulated DCs failed to induce proliferation of $CD4^+$ T cells *in vitro*. One could argue that DCs, under the effect of CQ, do not process antigens. However, in these set of experiments, DCs were pulsed with protein peptide, which overcomes the need for lysosome-dependent antigen degradation.

Previous reports showed that CQ stimulates the expression of NOS isoforms.[32–34] Although NO is believed to aggravate neuroinflammation,[49] its role as an immunomodulator has gained attention. It was previously demonstrated that NO blockade favors the development of antigen-specific response toward *Listeria monocytogenes* and mycobacterial infection.[50,51] It was also shown that EAE is exacerbated in the absence of NO.[52] Conversely, nitric oxide stimulates the generation of Tregs while suppressing IL-17-producing cells.[52,53] In this context, the existence of TNF/iNOS-producing 'Tip'-DCs has been demonstrated.[54] These DCs are responsible for determinant spread in EAE, and are believed to have an important role in neuroinflammation.[55] Nevertheless, Tip-DCs seem to limit the expansion of autoreactive T cells in experimental autoimmune myocarditis.[56] Thus, the dual role of Tip-DCs must be better characterized in autoimmune conditions. Our results showed that CQ stimulated NO production by DCs in a dose-dependent manner and produced high amounts of TNF-α after lipopolysaccharide stimulation (data not shown), which may classify these cells as Tip-DCs. NO production, in our analysis, seemed to have a role in the stimulation of T cell proliferation. We

observed that the suppressive effect of CQ-modulated DCs was abolished after L-NAME, an NOS inhibitor, treatment. These observations suggest that the suppressive effects exerted by CQ-modulated DCs are NO dependent.

The transfer of tolerogenic DCs is an interesting approach for the control of chronic inflammation.[13,57,58] However, it is unclear which signals turn DCs into fully mature and which turn them immunosuppressive.[11] For instance, it was previously demonstrated that vitamin D is an important regulator of DC function.[59] Transfer of vitamin D-modulated DCs was shown to reduce the clinical signs of EAE through the expansion of regulatory T cells.[16] Also, treatment with apoptotic cells drives these cells into a permissive state, and that transfer of apoptotic cell-induced tolerogenic DCs ameliorates the course of EAE through the downmodulation of inflammatory T cells.[60] Several studies also showed that pathogen-derived molecules interfere with DC maturation status, prompting them for the use in the control of inflammatory conditions.[58,61,62] We observed that CQ reduces the expression of antigen-presentation molecules and the functional profile of DCs *in vitro*. Thus, CQ administration induces a semi-mature phenotype in DCs. In addition, the transfer of CQ-modulated DCs reduced the clinical signs of EAE, a T cell-mediated disease. The peripheral cellular response toward neuroantigens (both MOG and myelin basic protein) was diminished as well as the infiltration of inflammatory cells in the CNS. Interestingly, the immune response toward ovalbumin, an unspecific antigen, was maintained, which strongly suggests that the reduced response generated after DC-CQ transfer is antigen/tissue specific.

The infiltration of leukocytes in the CNS promotes local inflammation and may lead to neurodegeneration, in which the glial reactivity is an important hallmark.[63] Astrocytes, upon environmental disturbances, express higher levels of the intermediary filament GFAP in sites of nerve injury.[64] Uncontrolled astrogliosis leads to the formation of glia scars that are irreversible, also called anisomorphic gliosis.[65–67] We found an upregulation in the expression of GFAP in EAE-inflicted mice both untreated and treated with DC-PBS. Interestingly, mice that received CQ-modulated DCs showed lower glial reactivity. The numbers of Iba-1^+ cells were similar between the treatment groups, which may indicate: (1) the frequency of microglia does not dictate the course of EAE, but their activation profile does; (2) the transferred DCs (which may express Iba-1[68]) migrated to the CNS; (3) both these phenomena occur concurrently. In this context, we observed that the expression of cytokines within the CNS was found altered in EAE-inflicted mice, with an increase in regulatory IL-10 and reduced inflammatory IL-17 and IL-6 after transfer of DC-CQ. Our data support the hypothesis that a change in the inflammatory status of the CNS toward an anti-inflammatory profile is responsible for the amelioration observed in the clinical course of DC-CQ-treated mice.

It is not yet clear the mechanism by which CQ exerts its immunomodulatory effects on DCs. Although pH-dependent interference exists, recently it has been demonstrated that CQ interacts with the mTOR pathway in MCF7 cancer cells.[69] In DCs, it was shown that manipulation of the mTOR signaling cascade facilitates the antigen presentation in a Treg-inducing milieu while inhibiting Th1/Th17 differentiation.[70] In this context, more studies must be conducted in order to solve whether CQ and mTOR pathway are related in DCs modulation.

The chronic CQ usage has been shown to provoke collateral side effects, including retinal toxicity.[71–73] We demonstrated that low-dose CQ administration is capable of reducing the clinical sign of EAE through the stimulation of regulatory T cells.[28] Although the treatment period was brief and that the dose used was low, the chances of retinal toxicity still exists in a chronic treatment regimen. We propose that an alternative for the chronic consumption of the drug is the modulation of DCs *in vitro* and its adoptive transfer. This approach not only prevented toxicity of the drug but also reduced the EAE severity in an antigen-specific manner. Other treatments currently in use such as those with anti-inflammatory drugs and monoclonal antibodies, although still important and necessary, act promoting a general nonspecific suppression. In this sense, the administration of CQ or CQ-modulated DCs together with classical FDA-approved anti-inflammatory drugs/monoclonal antibodies may reduce the doses of the medicines consumed by the patients, thus decreasing the elevated costs of the multiple sclerosis treatment. In addition, transfer of mitomycin C-treated DCs was able to reduce the cellular response toward neuroantigens *in vivo*.[12] In this study, the authors showed that the *in vitro* modulation of DCs with a clinically approved drug and its posterior adoptive transfer is an interesting approach for the treatment of multiple sclerosis and EAE. Indeed, in coculture experiments with patient's mitomycin C-treated DCs and effector T cells, the proliferation of the latter was inhibited, whereas the dendritic cell immature phenotype was not reverted.

Collectively, our data indicate that CQ modulates DCs both phenotypically and functionally. The adoptive transfer of CQ-modulated DCs was able to reduce the clinical signs of EAE by the downregulation of cellular immune response toward neuroantigens. Consequently, the glial reactivity was diminished in the CNS of mouse that received CQ-treated DCs and may have contributed to the amelioration of disease course. Taken together, our data indicate that adoptive transfer of CQ-modulated DCs may become an alternative for the chronic consumption of the drug, thus avoiding toxicity.

METHODS

Mice

Six-to-eight week-old female C57BL/6 mice from the Multidisciplinary Center for Biological Research, University of Campinas, were used in this study. Mice 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 Institutional Committee (Comissão de Ética no Uso de Animais–CEUA/UNICAMP; Protocol number 2687-1) approved the protocols involving laboratory animals.

Generation of DCs, treatment and adoptive transfer

Bone marrow-derived precursors were used in the generation of DCs, according to a previous report.[74] Briefly, femurs were collected and the bone marrow cells were flushed out with RPMI medium supplemented with 2-mercaptoethanol (2 mM), fetal bovine serum (10% v-v) and gentamicin (50 $\mu g\,ml^{-1}$)–referred to as complete medium. 5×10^6 cells were seeded in 24-well culture plate containing complete medium supplemented with GM-CSF (10 $\eta g\,ml^{-1}$). Fresh medium was added at days 3 and 6 of culture. This culture method results in DC generation of 85–95% purity, assessed by flow cytometry. DCs were used in transfer or coculture experiments. For adoptive transfer experiments, DCs were treated with CQ (50 μM, Sigma-Aldrich, St Louis, MO, USA) or dexamethasone (50 μM) for 18 h in the presence of lipopolysaccharide from *Escherichia coli* O111:B4 (1 $\eta g\,ml^{-1}$, lipopolysaccharide, Sigma-Aldrich) and pulsed overnight with 10 $\mu g\,mL^{-1}$ of MOG_{35-55} peptide (Rheabiotec, Campinas, Brazil). PBS was added as a control reagent. 1.5×10^6 cells were adoptive transferred via intravenously by the retroorbital route 3 days before EAE induction.

Coculture experiments

For the coculture experiments, 5×10^5 per well DCs treated as above were seeded in flat-bottom 96-well plates. Splenocytes were enriched in lymphocytes by centrifugation in Percoll gradient, following a previously published

protocol.[75] CD4$^+$ T lymphocytes were isolated using Dynabeads following manufacturer's instructions (CD4$^+$T cell isolation kit, Applied Biosystems, Life Technologies, Austin, TX, USA). This protocol resulted in a purity of 90–95% CD4$^+$ T cell, confirmed through analysis by flow cytometry. 5×10^5 per well CD4$^+$ T cells were seeded together with DCs at a ratio of 1:1 (DC:T) in 200 µl of complete RMPI medium containing $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK, 10 µg ml^{-1}, Rheabiotec). As controls, CD4$^+$ T cells were grown in the absence of DCs. The plates were incubated at 37 °C for 96 h. For the evaluation of NO blockade, some cultures were conducted in the presence of the NOS inhibitor, *N* (G)-nitro-L-arginine methyl ester (L-NAME, 1 mM, Sigma-Aldrich). After the incubation period, the cells were treated with 10 µl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg ml^{-1}, Sigma-Aldrich) for 4 h and the formazan crystals dissolved with 100 µl of isopropanol. Absorbances were read at 540 nm by spectrophotometer (VersaMax, Molecular Devices, Sunnyvale, CA, USA).

NO dosage

Nitrite production was determined using the Griess reagent following a well-established protocol.[76,77]

EAE induction and evaluation

EAE was induced and evaluated in mice according to a previously published paper.[28] Briefly, each mouse was injected with 100 µg MOG35–55 (RheaBiotec) emulsified with complete Freunds adjuvant (CFA, Sigma). Pertussis toxin (Ptx, Sigma) 200 ηg was administrated via i.p. at 0 and 48 h after antigen challenge. Clinical signs were followed and graded daily according to a score method, were 0: no sign, 1: flaccid tail, 2: hind limbs weakness, 3: hind limbs paralysis, 4: hind paralysis and fore limbs weakness, 5: full paralysis/dead. At the indicated time points after antigen challenge, mice were killed, spinal cords were removed and snap frozen in OCT Tissue Tek; 12-µm thin slices were made and stained with haematoxylin and eosin.

Immunofluorescence

Fourteen days after EAE induction, mice were killed and the spinal cords were collected, snap frozen as mentioned above and 12-µm thin slices were made. The reactive sites were blocked with phosphate buffer (0.1 M, pH7.2)-BSA 3%. The cells were stained with purified rabbit anti-glial fibrillary acid protein (GFAP, at a 1:1200 dilution, Dako, Glostrup, Denmark) and anti-Iba-1 antibodies (at a 1:1200 dilution, Dako). The reaction was treated with Cy2-conjugated goat anti-rabbit immunoglobulin G (diluted 1:250, Vector) and analyzed under epifluorescence microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA).

Scanning electron microscopy

1×10^6 DCs treated both with CQ or PBS were stimulated with lipopolysaccharide (1 ηg mL^{-1}) and cultivated over glass coverslips for 18 h at 37 °C. Cells were fixed (1 ml of a solution consisting of glutaraldehyde 2% and tannic acid 1%) for 1 h. The post fixation was made in aqueous 1% osmium tetroxide for 2 h. The material was dehydrated with series of ethanol and then brought to the critical point with liquid CO_2 (Balzers CPD 030) and covered with gold bath (Sputter Coater Balzers SCD 050, Capovani Brothers Inc., Scotia, NY, USA). Cells were viewed in a JEOL 5800LV scanning electron microscope.

Lymphoproliferative analysis

For the evaluation of antigen-specific proliferation, leukocytes were collected from spleens of control and EAE-inflicted mice. Briefly, spleen were collected aseptically and macerated with the aid of piston and sieve to prepare cellular suspensions. Cells were treated with ACK lysis buffer (ammonium-chloride-potassium) for 3 min and washed with complete RPMI medium. 5×10^5 cells per well were seeded in 96 flat-well plates with 200 µl of complete RPMI medium containing 10 µg ml^{-1} of $MOG_{35–55}$ (Rheabiotec) and incubated at 37 °C for 96 h. As control, cells were grown in the absence of antigen. After the incubation period, the cultures were treated with 10 µl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg ml^{-1}, Sigma-Aldrich) for 4 h and the formazan crystals dissolved with 100 µl isopropanol. Absorbances were read at 540 nm by spectrophotometer.

Reverse transcriptase PCR

The RNA was extracted from the samples with TRIzol reagent (Life Technologies, Austin, TX, USA). RNA concentration was calculated by reading the absorbance at 280 nm and the purity guaranteed by the ratio of 280/260 nm (ratios ranging from 1.8 to 2.0 were used). One microgram of RNA was converted to cDNA using High Capacity cDNA Reverse Transcription kit following the manufactures recommendations (Life Technologies). Expression of IL-6 (Mm00446190_m1), IL-10 (Mm00439614_m1), IL-17 (Mm00439618_m1), IFN-γ (Mm01168134_m1), TGF-β (Mm01178820_m1) and FOXP3 (Mm00475162_m1) was analyzed in comparison with GAPDH (Mm99999915_g1, housekeeping gene). Reverse transcriptase PCR reactions were performed using Taqman reagents according to manufacturer's recommendations (Applied Biosystems).

Flow cytometry

For flow cytometry analysis, 1×10^6 cells were labeled with fluorochrome-conjugated monoclonal antibodies. Cells were incubated with antibody cocktail for 30 min at 4 °C and fixed with 1% formaldehyde. The antibodies used were as follows: anti-CD11c/APC (clone N418), anti-major histocompatibility complex-II/PECy7 (clone M5/114.15.2), anti-CD80/FITC (clone 16-10A1), anti-CD86/PE (clone GL-1), all from eBioscience (San Diego, CA, USA). 50 000 events were acquired from each sample in flow cytometer (FACSCanto, BD Biosciences, Franklin Lakes, NJ, USA) and analyzed in FlowJo 7.5 software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

For clinical score and weight comparisons, collected data were analyzed by two-way analysis of variance with Bonferroni *post hoc* test. Analyses between two groups were performed by Student's *t*-test. All analyses were performed using GraphPad prism version 5.0 (GraphPad software, San Diego, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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