CORRESPONDENCE

Chloroquine reduces Th17 cell differentiation by stimulating T-bet expression in T cells

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Chloroquine (CQ) is a weak base that was originally used to treat malaria infection.[1] However, recent findings have pinpointed the modulatory effect of CQ in models of chronic inflammation and viral infections. The antiviral effect of CQ and its derivative hydroxychloroquine has attracted great attention due to the recent Sars-CoV-2 virus outbreak.[2,3] As an immunomodulatory agent, CQ reduces the severity of experimental autoimmune encephalomyelitis (EAE), likely by inducing regulatory T cells and tolerogenic dendritic cells (DCs).[4,5] EAE and multiple sclerosis (MS) are demyelinating disorders of the CNS in which interleukin (IL)-17-producing helper T (Th17) cells play a major role in disease severity. To date, there has been no report of the effect of CQ on T cell differentiation; thus, we aimed to investigate whether CQ interferes with Th17 cell differentiation.

We isolated $CD4^+$ T cells from the spleens of wild-type (WT) C57BL/6 mice and activated the cells with agonistic anti-CD3/CD28 antibodies under Th17 polarizing conditions in the presence or absence of CQ. We found that cultures with CQ had a significant decrease in IL-17 production (Fig. 1a). Moreover, CQ at low concentrations was toxic to Th17 cells but not to Th1 cells or DCs (Fig. 1b). Indeed, CQ augmented Th1 cell differentiation (Fig. 1b). These results show that Th17 cells are more susceptible to CQ toxicity than Th1 cells and DCs. We then investigated whether CQ also impacts Tc17 cell differentiation and found that IL-17 production by $CD8^+$ T cells was also reduced by CQ treatment (Fig. 1c). Because T-bet and STAT1 are major suppressors of Th17 cell differentiation,[6,7] we aimed to investigate T-bet and STAT1 expression in CQ-treated cells. Cultures of CQ-treated Th17 cells with the least toxic dose of CQ had a significant increase in T-bet but not STAT1 expression (Fig. 1d). This result is interesting because we recently showed that CQ-induced tolerogenic DCs rely on STAT1 expression to maintain their suppressive function.[8] Collectively, our current results indicate that, in T cells, CQ suppresses Th17 cell differentiation by stimulating T-bet rather than STAT1 expression.

Finally, we investigated whether EAE suppression in CQ-treated mice is associated with an increase in T-bet expression in Th17 cells. We immunized WT mice with MOG_{35-55} peptide in complete Freund's adjuvant to induce EAE and monitored the mice daily for disease development. CQ treatment started once the mice showed the first signs of EAE (approximately day 10 p.i.) and continued for 7 consecutive days. Similar to what we previously published,[4] CQ-treated mice displayed significantly less severe EAE than PBS-treated mice (Fig. 1e). Analysis of CNS cells at day 21 p.i. revealed that CQ-treated mice had a significant decrease in IL-17-producing Th17 cells and no effect on IFN-γ-producing Th1 cells compared with cells from PBS-treated mice (Fig. 1f). Further analyses showed that STAT1 expression was unchanged, while T-bet expression was increased in Th17 cells in the CNS of CQ-treated mice compared with those treated with PBS (Fig. 1g).

Collectively, our results demonstrate a novel mechanism by which CQ reduces inflammation in EAE through inducing T-bet expression in T cells and reducing Th17 differentiation. Interestingly, the shift in the balance between Th17/Th1 cells towards IFN-γ production may reflect another pathway by which CQ augments antiviral responses. Nevertheless, further studies are necessary to confirm this hypothesis. Our results provide further evidence that CQ may be a powerful therapeutic agent in MS and the other chronic inflammatory diseases of the CNS.

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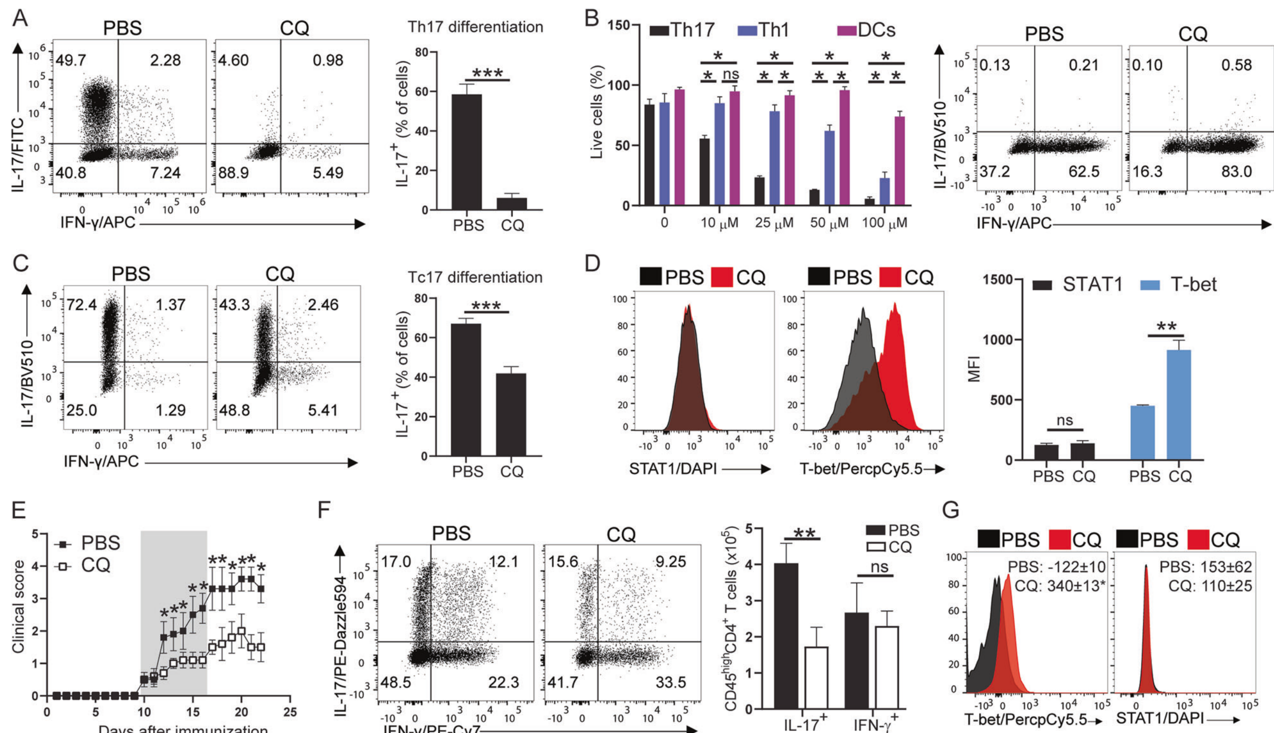

Fig. 1 CQ inhibits Th17 cell differentiation by stimulating T-bet expression. **a** $CD4^+$ T cells from WT mice were isolated using magnetic beads according to the manufacturer's instructions (Miltenyi Biotec.). $CD4^+$ T cells (200,000) were plated on 96-well U-bottom plates with anti-CD3/CD28 (1 µg/mL each) under Th17 polarizing conditions (recombinant mouse TGF-β1: 2.5 ng/mL; recombinant mouse IL-6: 20 ng/mL; anti-IFN-γ: 10 µg/mL and anti-IL-4: 5 µg/mL) in the presence or absence of increasing concentrations of CQ (10, 25, 50, and 100 µM) for 72 h. At the end of the culture period, the cells were collected and stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL, Sigma-Aldrich), ionomycin (500 ng/mL, Sigma-Aldrich) and GolgiPlug (1 µg/mL, BD Biosciences) for 3 h at 37 °C. The cells were then surface stained with fluorescent antibodies against CD45 and CD4 for 20 min at 4 °C, fixed and permeabilized with commercial kits (Perm buffer, Thermo Fisher) and stained overnight with antibodies against IL-17, IFN-γ, GM-CSF, RORγt, T-bet, and STAT1. **b** Th17 cells were prepared as described in (**a**). Th1 cells were prepared by stimulating $CD4^+$ T cells with anti-CD3/CD28 (1 µg/mL each) and recombinant mouse IL-12 (25 ng/mL) for 72 h, and DCs were generated from bone marrow precursors in the presence of recombinant mouse GM-CSF (10 ng/mL) for 7 days. The cells were treated with increasing doses of CQ as described in (**a**), after which cell viability was measured by propidium iodide incorporation analysis by flow cytometry and trypan blue staining analysis by hemocytometer. **c** Tc17 cells were generated as described in (**a**) with $CD8^+$ T cells that were magnetically isolated from WT mice. **d** Flow cytometry analysis of T-bet and STAT1 from samples described in (**a**). **e** WT mice were s.c. immunized with 200 µg of $MOG_{35–55}$ peptide (GenScript) emulsified in complete Freund's adjuvant containing 5 mg/mL *M. tuberculosis* H37RA and 240 ng of pertussis toxin at days 0 and 2 p.i. Half of the mice ($n = 5$/group) were treated with CQ (3.5 mg/kg), while the remaining mice received PBS daily via i.p. administration for seven consecutive days (shaded area in the plot). Disease development was monitored daily. **f** EAE mice were euthanized at day 21 p.i. and their CNS cells were collected and processed for flow cytometry analysis. Cells that infiltrated the CNS were activated with PMA, ionomycin and GolgiPlug and stained for flow cytometry as indicated in (**a**). **g** Flow cytometry analysis of T-bet and STAT1 expression in Th17 cells from (**f**). The results are expressed as a representative flow cytometry graph and/or cumulative graphs depicting the mean ± SEM of each group. Analyses between two groups were performed with Student's *t* tests, while analyses between three or more groups were performed with one-way ANOVA followed by Bonferroni post tests. For analysis of clinical scores, we employed two-way ANOVA with Sidak post tests. In all analyses, *p* values < 0.05, < 0.01, and < 0.001 were considered statistically significant (denoted with *, **, and ***, respectively; Ns not significant). All experiments were repeated at least three times with three replicates in each culture experiment. All experiments with animals were approved by the institutional animal welfare committee

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