

Hydroxychloroquine decreases Th17-related cytokines in systemic lupus erythematosus and rheumatoid arthritis patients

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OBJECTIVES: Hydroxychloroquine is an antimalarial agent that has been used in systemic lupus erythematosus and rheumatoid arthritis treatment for many years. Recently, novel mechanisms of action have been proposed, thereby broadening the therapeutic perspective of this medication. The purpose of this study was to evaluate the immunomodulatory activity of hydroxychloroquine in T helper 17 (Th17) cytokines in healthy individuals and patients.

METHODS: Eighteen female patients with systemic lupus erythematosus (mean age 39.0 ± 12.9 years) and 13 female patients with rheumatoid arthritis (mean age 51.5 ± 7.7 years) were recruited from Universidade Federal de Pernambuco-Brazil. The patients were included after fulfilling four classification criteria for systemic lupus erythematosus or rheumatoid arthritis from the American College of Rheumatology. After being stimulated with phorbol 12-myristate 13-acetate and ionomycin in the absence or presence of different concentrations of hydroxychloroquine, the interleukin 6, 17 and 22 levels were quantified with an enzyme-linked immunosorbent assay in culture supernatants of peripheral blood mononuclear cells from healthy individuals and patients.

RESULTS: We demonstrated that in peripheral blood mononuclear cells from healthy volunteers and in systemic lupus erythematosus and rheumatoid arthritis patients, there was a significant reduction in the IL-6, IL-17 and IL-22 supernatant levels after adding hydroxychloroquine.

CONCLUSIONS Our *in vitro* results demonstrated that hydroxychloroquine inhibits IL-6, IL-17 and IL-22 production and contributes to a better understanding of the mechanism of action of this medication.

KEYWORDS: Systemic Lupus Erythematosus; Rheumatoid Arthritis; Th17 cells; Hydroxychloroquine.

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INTRODUCTION

Hydroxychloroquine (HCQ) is an antimalarial agent that has been used in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) treatments for many years. Recently, advances in our understanding of its mechanisms of action have expanded the therapeutic prospects of HCQ (1–3). Perhaps the most relevant discovery was of an

antagonistic effect of HCQ (inhibiting the immune response) on Toll-like receptors (TLRs) (4). Other mechanisms, such as interfering with antigen presentation and lysosomal acidification, blocking UV light in cutaneous reactions and inhibiting phospholipase A2, have been well described (5,6). All of these molecular effects could partially explain the immunomodulatory effect of HCQ upon proinflammatory cytokines, such as IL-6, IL-1 β and TNF- α (7).

Helper T cells (Th) are phenotypically heterogeneous and classified according to the cytokines produced by the innate immune system during the Th cell differentiation process. Four main lineages, Th1, Th2, regulatory T cells (TReg) and a novel cell named Th17, have been well described (8). The activation and maturation of Th17 lymphocytes relies on adequate T cell receptor (TCR) expression, co-stimulatory molecules and cytokines. Combined, TGF- β , IL-1 β , IL-6, IL-21 and IL-23 play a pivotal role in properly differentiating

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naïve CD4 positive cells. TGF- β and IL-6 are essential for inducing transcription factors retinoic acid receptor-related orphan receptor-t (ROR γ t) and STAT-3 in naïve CD4 positive cells, which are responsible for promoting proper maturation. IL-23 is another important cytokine that is required to expand and stabilize the Th17 cell population (8).

Recently, Th17 cells have been implicated in SLE pathogenesis, which identifies them as potential therapeutic targets (9). Previous studies with SLE patients have demonstrated an increase in serum Th17-related cytokines (IL-17 and IL-23). Th17 appears to infiltrate targeted organs, such as the kidneys and skin, which suggests an important role of these cells in SLE pathogenesis (10–12). Moreover, anti-DNAbs and total IgG production in 15 lupus nephritises were increased when the peripheral blood mononuclear cells (PBMCs) were cultured with IL-17 (13). In RA, activated T cells are a characteristic feature of synovial inflammation, which suggests a Th1- and Th17-mediated disease. Elevated levels of IL-17 have been observed in the synovial fluid of these patients (14). Additionally, IL-17A-producing CCR6⁺ memory T cells have been identified in the synovial fluid of RA patients (15). These studies have clearly shown that the Th17 pathway is implicated in the pathogenesis of SLE and RA, but whether HCQ is able to specifically down-regulate Th17 cytokines is unknown. To our knowledge, our study is the first to address this issue.

MATERIALS AND METHODS

Study Population

Eighteen female SLE patients (mean age 39.0 \pm 12.9 years) and 13 female RA patients (mean age 51.5 \pm 7.7 years) were recruited from the Rheumatology Division at the Hospital das Clínicas-Universidade Federal de Pernambuco. All patients answered a clinical questionnaire from which demographic, clinical, current medication and laboratorial data were collected (Table 1). Additional information was obtained from hospital records and reviewed by experienced physicians. The patients were included in the study after fulfilling at least four or more of the classification criteria for SLE or RA (16,17) from the American College of Rheumatology (ACR). After excluding any rheumatic disease, five healthy volunteers (mean age 25 \pm 1.64 years) were recruited as the control group. Peripheral blood samples were obtained from the patients and healthy volunteers. Tests for serum anti-DNAbs and serum comple-

Table 1 - Demographic, clinical and current drug of the patients with RA and SLE.

Diagnostic	SLE	RA
Number of patients	18	13
Age in years: mean \pm SD (range)	39 \pm 12.9 (22–61)	51.5 \pm 7.7 (40–69)
Disease duration in years: mean (range)	10.9 (0.1–40)	8.1 (0.8–29.6)
Treatment N(%)		
NSAIDS*	–	1 (8%)
Steroids	12 (67%)	10 (77%)
Antimalarial agents	10 (55%)	3 (23%)
Methotrexate	–	7 (53.8%)
Leflunomide	–	4 (30.8%)
Azathioprine	4 (22%)	–
Mycophenolate Mofetil	1 (5%)	–
Thalidomide	1 (5%)	–

*Non-steroidal anti-inflammatory drugs.

ment (C3, C4 and CH50) were conducted using standard methods.

PBMC purification

The PBMCs were obtained from the heparinized blood of five healthy, non-smoking donors who had not taken any drugs for at least 15 days prior to the sampling. The PBMCs were isolated using the standard Ficoll-Hypaque density-gradient centrifugation (GE Healthcare Biosciences, Pittsburgh, PA, USA) method. The cells were counted in a Neubauer chamber, and cell viability was determined using the Trypan blue (Sigma-Aldrich, St. Louis, USA) exclusion method. Cells were only used when their viability was >98%.

PBMC cultures

The PBMCs (1 \times 10⁶ cells/ml) were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), HEPES 10 mM (Gibco, Carlsbad, CA, USA) and penicillin (10.000 U/ml)/streptomycin (10.000 μ g/ml) (Gibco, Carlsbad, CA, USA). The cells were stimulated with PMA (Sigma-Aldrich, St. Louis, USA) + Ionomycin (Sigma-Aldrich, St. Louis, USA) in the presence or absence of HCQ (hydroxychloroquine sulfate, Sigma). Methylprednisolone (Pfizer, New York, NY, USA) was used as a positive control. The cells were incubated at 37°C in a humidified 5% CO₂ incubator.

Cytokine titration

The cytokine levels in the culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. In culture, supernatants from PBMCs, IL-6 (BD Biosciences, Franklinlakes, NJ, USA), IFN- γ (BD Bioscience) and TNF- α (BD Bioscience), levels were measured at 24 hours, and for IL-17A (R&D Systems, Minneapolis, MN, USA), IL-22 (eBiosciences, San Diego, CA, USA), and IL-23p19, (eBiosciences, San Diego, CA, USA) levels were measured at 48 hours. The lower detection limits for the ELISA analyses were as follows: 15.625 pg/ml for human IL-17 and human TNF- α , 9.375 pg/ml for human IL-6 and IFN- γ and 31.25 pg/ml for human IL-23p19 and IL-22.

Statistical analysis

All results in this article were analyzed with univariate comparisons using nonparametric tests (Wilcoxon matched-pairs test) with $p < 0.01$ considered a significant association and $p < 0.05$ a suggestive association. All quantitative data were plotted with GraphPad Prism 3.02 software.

Ethics

All subjects signed informed consent forms that were approved by the ethics committee of Universidade Federal de Pernambuco in accordance with the Helsinki Declaration.

RESULTS

Effect of HCQ on the synthesis of cytokines of the Th17 pathway in PBMCs of healthy volunteers

We investigated the effect HCQ on Th17 cytokines (IL-6, IL-17 and IL-22) in the PBMCs of healthy volunteers. After 48 hours of stimulation with PMA and ionomycin, the HCQ inhibited the production of IL-6, IL-17 and IL-22 (Figures 1

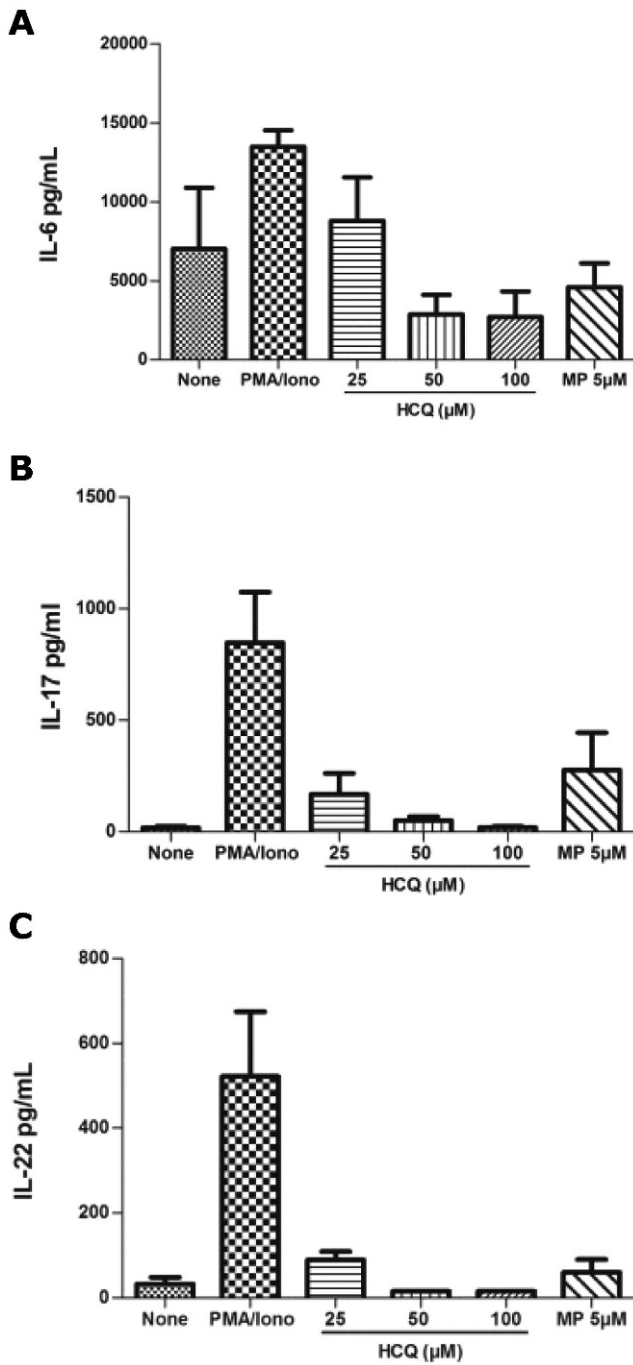


Figure 1 - Inhibition of IL-17A (A), IL-22 (B) and IL-6 (C) supernatants levels in PBMCs from five healthy individuals using HCQ at 25 μ M, 50 μ M and 100 μ M doses.

A, B and C) in a dose-dependent manner. We did not observe any significant inhibition in the production of these cytokines using the Wilcoxon test ($p > 0.05$). These results are most likely caused by the small number of healthy volunteers.

Effect of HCQ on the synthesis of Th17-related cytokines in the PBMCs of SLE and RA patients

The main objective of this study was to evaluate the effect of HCQ on the cytokine profile of SLE and RA patients.

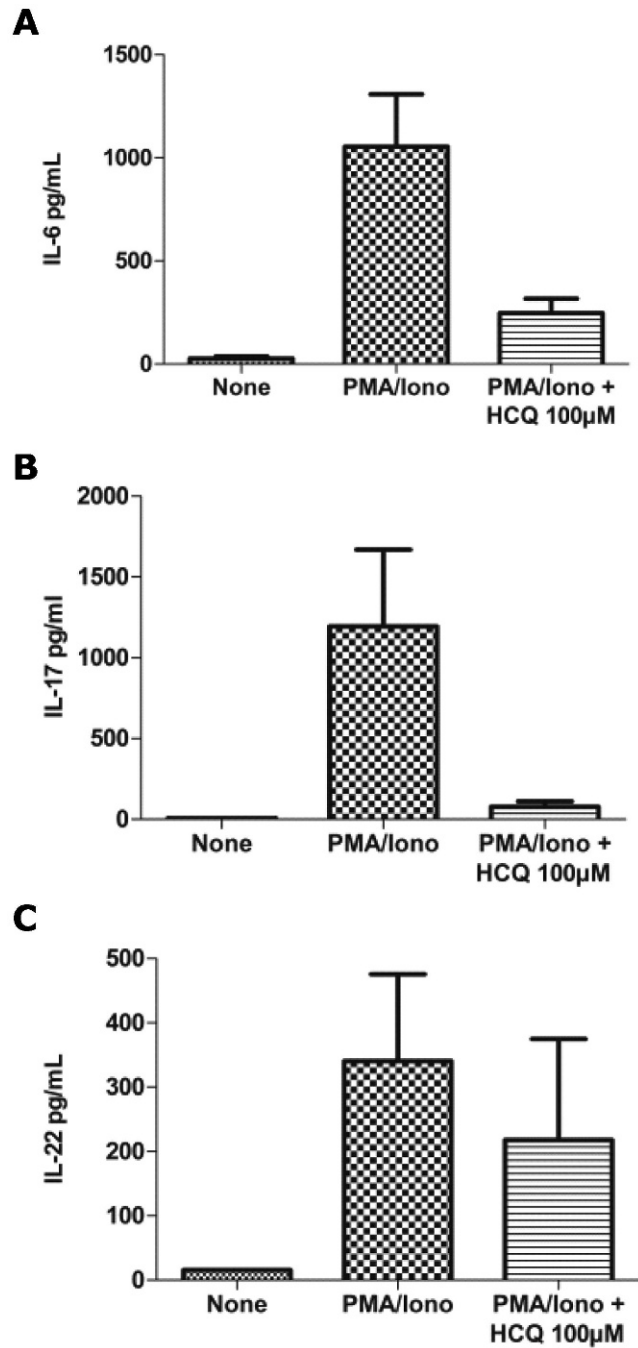


Figure 2 - Inhibition of IL-6 (A), IL-17 (B) and IL-22 (C) production using HCQ at a dose of 100 μ M in PBMCs from SLE patients.

Following our previous experiments in mice and healthy individuals, we analyzed the effect of HCQ (at a concentration of 100 μ M) on Th17 cytokine levels from the PBMCs of SLE and RA patients after PMA and ionomycin stimulation. The levels of IL-6, IL-17 and IL-22 were negligible in the non-stimulated cells. There were remarkable increases in the levels of these cytokines in the PBMCs from RA and SLE patients, most likely because of the addition of PMA and ionomycin.

Following HCQ administration in both the SLE and RA patients, we found significant inhibition in the levels of IL-6 ($p = 0.0001$ for SLE and $p = 0.0025$ for RA, Figures 2A and

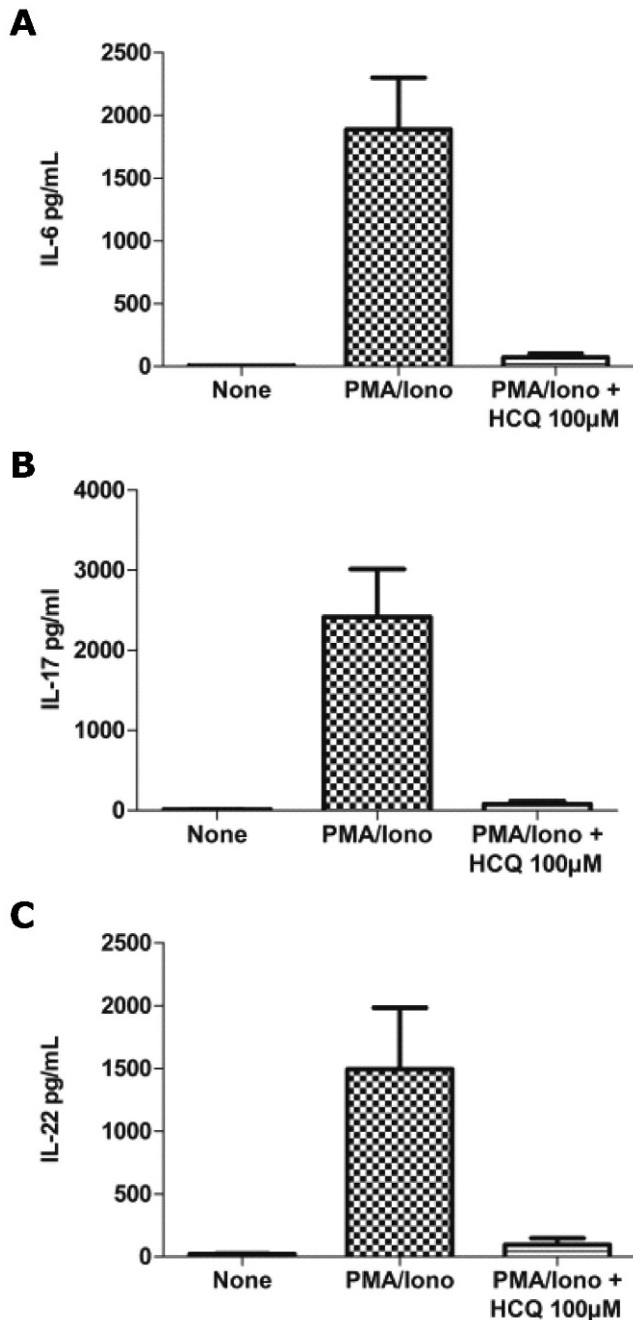


Figure 3 - Inhibition of IL-6 (A), IL-17 (B) and IL-22 production (C) using HCQ at a dose of 100 µM in PBMCs from RA patients.

3A), IL-17 ($p=0.0001$ for SLE and $p=0.0002$ for RA, Figures 2B and 3B) and IL-22 ($p=0.0067$ for SLE and $p=0.0002$ for RA, Figures 2C and 3C).

DISCUSSION

In the present study, we found that the antimalarial medication HCQ decreased pro-inflammatory Th17 cytokines levels in the PBMCs from healthy individuals and SLE/RA patients, suggesting a novel mechanism of action for these antirheumatic drugs. To our knowledge, this study is the first report of an inhibitory effect of antimalarial medication on IL-17 and IL-22 supernatant levels.

Increasingly, experimental and clinical evidence has indicated that IL-17 and Th17 cells play a central role in SLE and RA pathogenesis (10,18). IL-17 amplifies the immune response by increasing target organ inflammation and damage and by augmenting antibody production by B cells, an important player in SLE (19). In RA patients, the IL-17 serum and synovial levels are positively correlated with disease activity and upregulate osteoclast activation, which promotes bone erosion (20). Recently, our group described the upregulation of serum IL-22 levels in RA patients and identified an important correlation with disease activity and the presence of erosion (21).

Antimalarials have been widely used to treat SLE patients for many years, particularly for cutaneous and musculoskeletal manifestations (22,23). HCQ should also be used as a maintenance treatment in patients with stable disease, as previously reported in a randomized controlled trial in which the placebo group presented more lupus flares than the HCQ group (24,25). These results suggest that an immunomodulatory 'status' is induced by HCQ, which could contribute to adequate disease control over many years. Another important beneficial effect of HCQ is a well-demonstrated reduction in cardiovascular risk in SLE patients. Previous cohort studies have shown a significant reduction in cholesterol levels, carotid plaque formation and thrombosis frequency (26,27). Recently, antimalarials have also improved survival in different populations of SLE patients (28,29).

For RA treatment, controlled trials have demonstrated the efficacy of HCQ in controlling arthritis symptoms as antimalarial agents are classified as disease-modifying antirheumatic drugs (DMARD) (30). Compared with other antirheumatic drugs, DMARDs are relatively well tolerated and safe to use during pregnancy and lactation. Additionally, these medications could improve glycemic control and lipid profiles, thereby decreasing cardiovascular risks in RA patients. An observational study described a 38% risk reduction of developing diabetes in RA patients after a long follow-up period (31).

In the present study, HCQ exhibited an anti-inflammatory effect on Th17-related cytokines in healthy individuals and SLE and RA patients. Previous studies using different cell populations have demonstrated that HCQ inhibits pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), IL-1- α and IL-6 (32,33). These results are in accordance with our findings, but few studies have evaluated the *in vitro* effect of HCQ upon supernatant levels of T-cell related cytokines.

An interesting issue regarding our study is whether '*in vivo*' treatment using antimalarial agents could influence the *in vitro* HCQ effect. In our sample, although only a minority of patients was currently using HCQ, we found no differences when comparing patients with or without HCQ treatment (data not shown). In fact, in our study design, we compared the cytokine supernatant levels before and after administering HCQ to the same patient. Thus, each patient was his or her own control, and '*in vivo*' HCQ use was not a confounding variable.

The exact mechanism by which HCQ reduces IL-6, IL-17 and IL 22 levels is unknown, but one possible explanation is that it occurs by reducing Th17 cells through a decrease in the antigen presentation. Antimalarials classically enter lysosomes, causing an increase in cell pH, which interferes with protein processing and secretion (6). Moreover, the



inhibitory effect of HCQ on TLR expression decreases the aberrant immune response typically found in rheumatic diseases. In fact, there is growing evidence that TLRs, particularly TLR 9, play pivotal roles in SLE and RA (34,35).

One limitation of our study was the inability to identify the Th17 cells through a specific technique, such as flow cytometry. However, we identified a consistent inhibitory effect in mice, healthy individuals and patients, suggesting a significant inhibitory effect upon cytokines involved in Th17 activation. Regarding IL-6, our results are in accordance with a previous study of 14 SLE patients, which showed a decrease in IL-6 mRNA expression in skin samples after three months of chloroquine treatment (7).

Although different mechanisms of action have been previously described, it has not been established whether HCQ is able to suppress the cytokines produced by Th17 cells. Our *in vitro* results demonstrated that this medication can inhibit IL-6, IL-17 and IL-22 production, which may be a novel pathway to understanding the immunomodulatory properties of HCQ.

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AUTHOR CONTRIBUTIONS

Silva JC performed the human cell culture, ELISA-related experiments, data acquisition and statistical analysis and drafted the manuscript. Mariz HA drafted the manuscript, performed the statistical analysis, recruited the patients and acquired the clinical data. Rocha Jr LF recruited the patients and acquired the clinical data. Oliveira PS provided support to Silva JC during the experiments and performed the statistical analysis. Dantas AT recruited the patients and acquired the clinical data. Duarte AL was involved in the study elaboration, design, and coordination, as well as the patient recruitment and acquisition of clinical data. Pitta IR participated in the study elaboration, design and coordination. Galdino SL participated in the study elaboration, design and coordination, collaborated on the draft version of the manuscript and provided final approval of the submitted version of the manuscript. Pitta MG participated in the study elaboration, design and coordination, collaborated on the draft version of the manuscript and provided final approval of the submitted version of the manuscript.

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