

Letter to the Editor

Hydroxychloroquine preferentially induces apoptosis of CD45RO⁺ effector T cells by inhibiting autophagy: A possible mechanism for therapeutic modulation of T cells

To the Editor:

Autoimmune diseases, such as multiple sclerosis, systemic lupus erythematosus, inflammatory bowel disease, and rheumatoid arthritis, are the result of inappropriate immune responses against “self.” Hyperactivated, and likely autoreactive, effector T cells are common in autoimmunity, indicating that T-cell homeostasis is disturbed. Impaired apoptosis of self-reactive, effector T cells has been proposed as a driving mechanism of autoimmunity.¹ Consequently, a better understanding of the mechanisms regulating T-cell homeostasis and identifying possible ways to manipulate it pharmacologically for therapeutic purpose remain an unbridged knowledge gap.

T-cell homeostasis is, in part, regulated by the balance between apoptosis and autophagy.² During autophagy, double-membrane vesicles with cytoplasmic material (autophagosomes) are formed, which will fuse with lysosomes for degradation of their content. Autophagy has been described as a powerful mechanism of cell survival, postponing the onset of apoptosis upon cellular stress.³ Autophagy has furthermore been implicated in the development of immunity, and may therefore be an important therapeutic target for autoimmune diseases.⁴ Although the role of autophagy in the innate immune system is gaining clarity, its role in adaptive immunity remains poorly understood. Autophagy has been reported to occur in CD4⁺ T cells, although human data are scarce. Mice in which autophagy genes are specifically knocked-out in CD4⁺ T cells display reduced CD4⁺ T cell numbers, demonstrating that autophagy is important for T-cell survival.⁵

Here we investigated whether hydroxychloroquine (HCQ) can modulate autophagy in human CD4⁺ T cells and consequently cellular homeostasis, thereby exploring a novel therapeutic mechanism for this commonly used drug. HCQ is a potent autophagy inhibitor that affects lysosomal acidification, and thereby inhibits endogenous protein degradation, resulting in an increase in the number of autophagic compartments.⁶

HCQ is also used for the treatment of autoimmune diseases including rheumatoid arthritis, Sjögren syndrome, and systemic lupus erythematosus.⁷ Although HCQ has been demonstrated to inhibit proinflammatory cytokine production by macrophages and antigen presentation, potential effects on T cells remain elusive.⁷

To assess whether HCQ can modulate autophagy in T cells, human PBMCs were cultured in the presence of increasing concentrations of HCQ. The concentrations utilized in our experiments (0.7–20 $\mu\text{mol/L}$) are comparable to those in the blood of HCQ-treated patients (0–23 $\mu\text{mol/L}$).⁸ Autophagosomes were stained with a specific Cyto-ID autophagy detection kit⁹ (see the [Methods](#) section in this article’s Online Repository at www.jacionline.org), and the presence of autophagosomes in CD4⁺ T cells was determined by flow cytometry. HCQ treatment increased the number of autophagosomes in CD4⁺ T cells in a dose-dependent fashion, indicating that HCQ treatment indeed inhibits autophagy, resulting in the accumulation of

autophagosomes (Fig 1, A and B). To confirm and extend these data, sorted CD4⁺ T cells were treated with HCQ, stained for autophagosomes, and analyzed by confocal microscopy. The number of autophagosomes per cell was increased upon HCQ treatment (Fig 1, C and D). Furthermore, Western blot analysis showed that the autophagosome markers LC3-II and p62 increased upon HCQ treatment in sorted CD4⁺ T cells (Fig 1, E). Taken together, these data demonstrate that HCQ treatment inhibits the completion of the autophagy process in primary human CD4⁺ T cells, resulting in the accumulation of autophagosomes.

Because CD4⁺CD45RO⁺ memory and effector T cells are among the drivers in the pathogenesis of autoimmune diseases, we hypothesized that the rate of the autophagy process (also known as the autophagic flux) in these cells could differ from naive CD4⁺CD45RA⁺CD45RO[−] cells. Human PBMCs were treated with HCQ, and autophagosomes in CD45RA⁺ and CD45RO⁺ T cells were analyzed. While HCQ treatment led to a rapid accumulation of autophagosomes in CD4⁺CD45RO⁺ T cells, there was only a mild increase in CD4⁺CD45RA⁺ cells (Fig 1, F). These data demonstrate that the autophagic flux is lower in CD45RA⁺ naive cells compared with CD45RO⁺ memory and effector T cells.

Autophagy can function as a survival mechanism and prevent apoptosis.³ Furthermore, reduced apoptosis of T cells has been proposed as a key mechanism of autoimmunity.¹ Because we observed that HCQ can abrogate autophagy in CD4⁺ T cells, we determined whether HCQ treatment would result in more apoptosis of these cells as a consequence of impaired autophagy. Human PBMCs were treated with HCQ, and apoptosis of CD4⁺ T cells was determined by Annexin V staining. HCQ treatment dose-dependently increased apoptosis in CD4⁺ T cells (Fig 2, A and B). Because the autophagic flux was differentially regulated in CD45RA⁺ and CD45RO⁺ populations, the percentage of Annexin V positive cells was analyzed in these 2 subsets. Although HCQ only mildly induced apoptosis in CD45RA⁺ cells, apoptosis in CD45RO⁺ cells was significantly greater (Fig 2, C). These results were confirmed by using a propidium iodide staining (Fig 2, D). To verify that this difference in apoptosis induction between CD4⁺CD45RA⁺ and CD4⁺CD45RO⁺ T cells would also result in different survival patterns of T-cell subsets after HCQ treatment, CD4⁺CD45RA⁺ and CD4⁺CD45RO⁺ T cells were sorted, both stained with a different cell tracker dye for identification, and cultured at a 1:1 ratio in the presence of HCQ for 4 days. As expected, the CD45RA/CD45RO ratios were significantly altered after HCQ treatment, favoring CD45RA⁺ T-cell survival (Fig 2, E and F). Importantly, the cellular carboxyfluorescein succinimidyl ester or cell tracker content was not diluted, indicating that the observed differences were not the result of altered proliferation. Collectively, these data demonstrate that CD4⁺CD45RO⁺ cells normally undergo more autophagy than do CD4⁺CD45RA⁺ T cells and that inhibition of autophagy in these cells by HCQ results in apoptosis.

Here we demonstrate that in human T cells there is a basal level of autophagy and that the inhibition of autophagy by HCQ induces apoptosis. Autophagic flux and the rate of apoptosis induced by autophagy inhibition with HCQ are markedly higher in CD45RO⁺ T cells than in naive CD45RA⁺ T cells. These

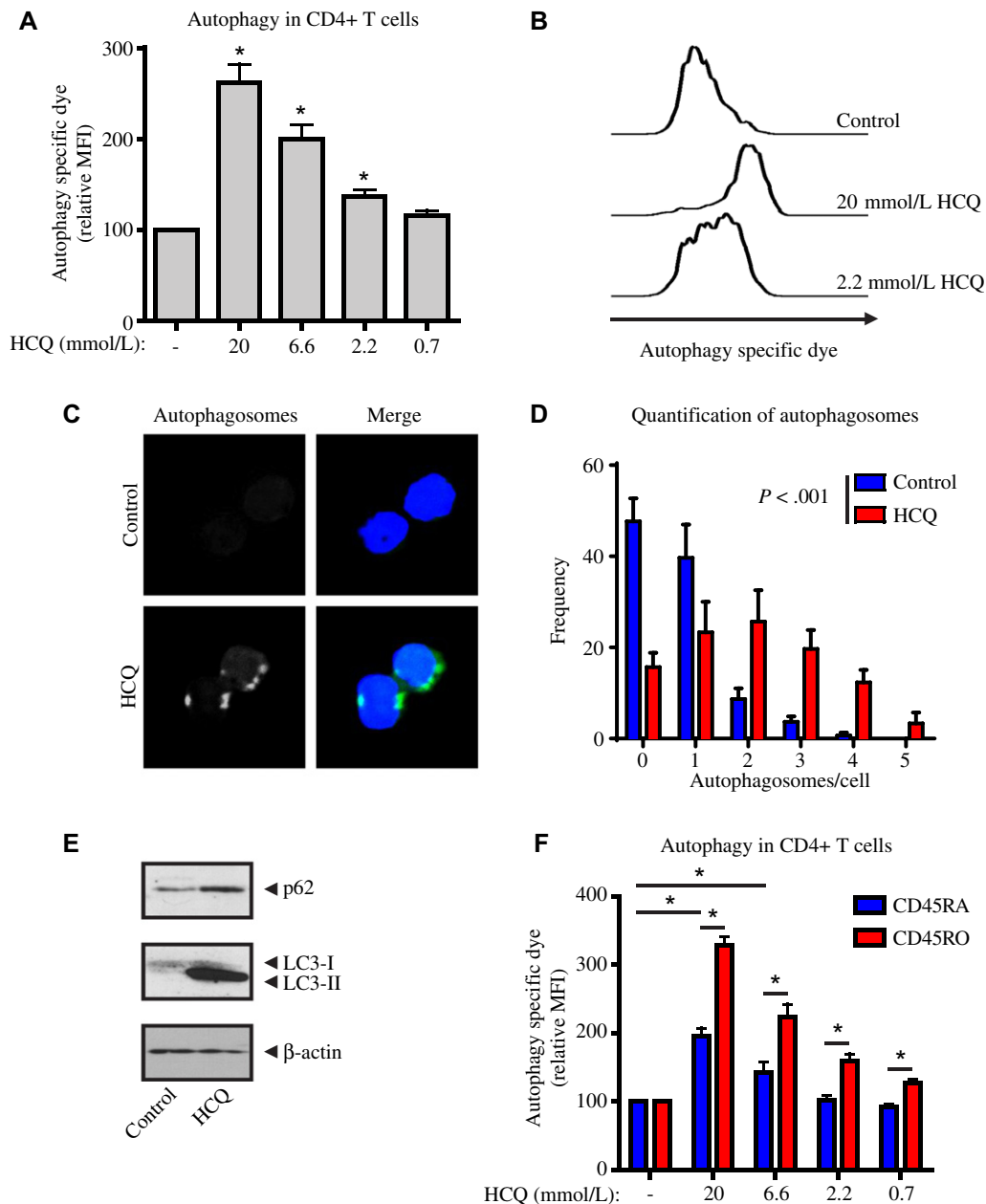


FIG 1. HCQ impairs autophagy in CD4⁺ T cells. **A**, Autophagy in human CD4⁺ cells, cultured in the presence of HCQ, $n = 8$. **B**, Histograms of Fig 1, **A**. **C**, Autophagosome visualization in CD4⁺ cells, cultured with 20 μ mol/L of HCQ. **D**, Quantification of Fig 1, **C**, $n = 3$. **E**, p62, LC3, and β -actin expression in CD4⁺ cells, $n = 4$. **F**, Autophagy in human CD4⁺CD45RA⁺CD45RO⁻ and CD4⁺CD45RA⁻CD45RO⁺ cells, cultured with HCQ, $n = 7$. *MFI*, Mean fluorescent intensity. * $P < .05$.

results improve our understanding of the mechanism of action of HCQ, a drug used in several autoimmune diseases. Impaired apoptosis of T cells has previously been reported to be involved in the development of autoimmunity.¹ Because virtually all activated self-reactive T cells in patients with autoimmune disease are CD45RA-negative and CD45RO-positive, HCQ treatment will preferentially induce apoptosis in these cells while only mildly affecting the naive T-cell repertoire. These data provide a novel mechanism by which autoimmunity can be modulated by using HCQ and validate autophagy as a promising therapeutic target.

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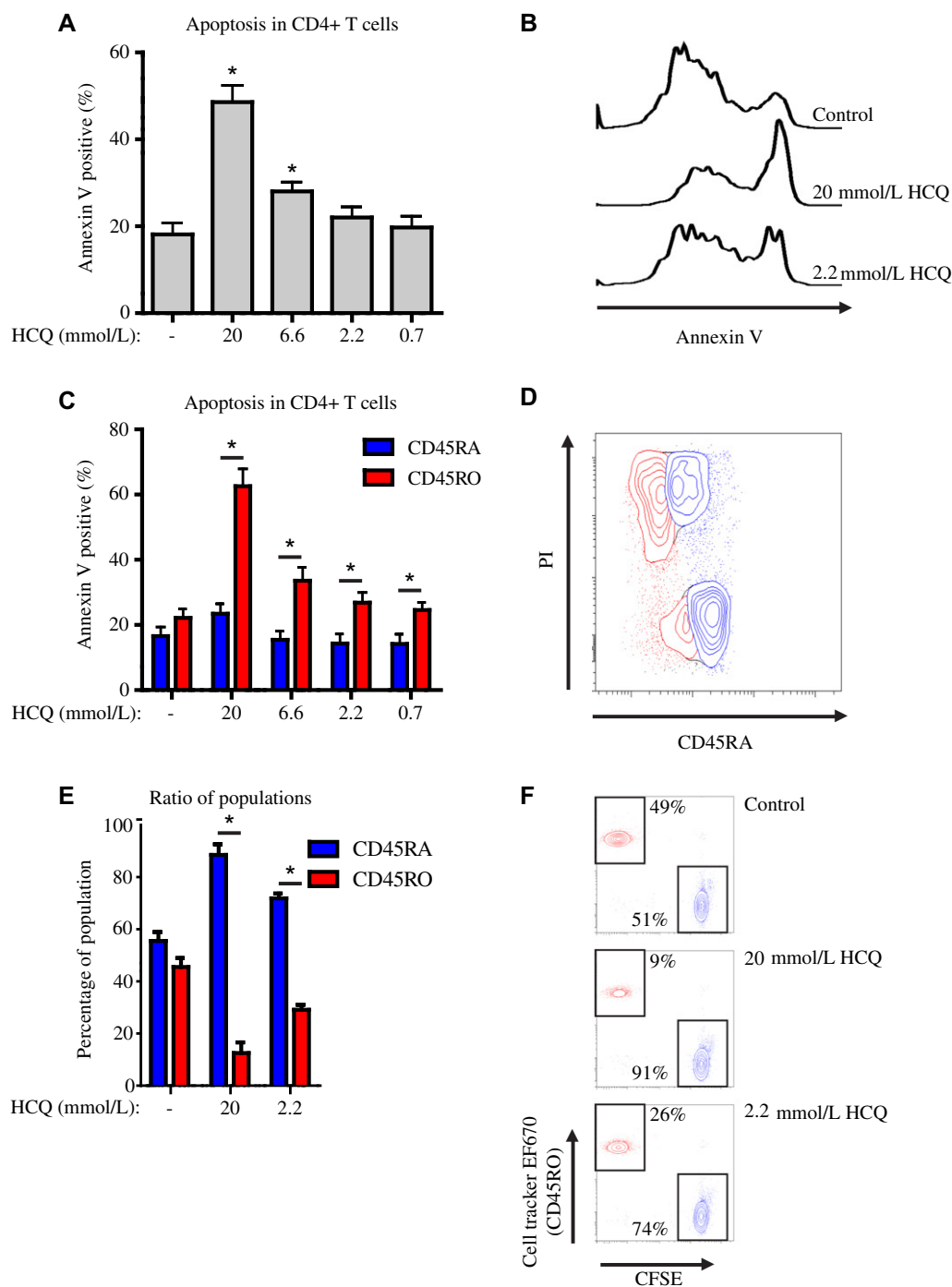


FIG 2. HClQ treatment preferentially induces apoptosis in CD4⁺CD45RA⁻CD45RO⁺ T cells. **A**, Apoptosis (by Annexin V staining) in human CD4⁺ cells, cultured in the presence of HClQ, n = 8. **B**, Histograms of Fig 2, **A**. **C**, Apoptosis in human CD4⁺CD45RA⁺CD45RO⁻ and CD4⁺CD45RA⁻CD45RO⁺ cells, n = 7. **D**, FACS plot of HClQ-treated CD4⁺CD45RA⁺CD45RO⁻ (blue) and CD4⁺CD45RA⁻CD45RO⁺ (red) cells stained with propidium iodide (PI). **E** and **F**, Sorted CD4⁺CD45RA⁺ (CFSE) or CD4⁺CD45RO⁺ (EF670) cells were mixed at a 1:1 ratio and cultured in the presence of HClQ (n = 4). CFSE, Carboxyfluorescein succinimidyl ester; FACS, fluorescence-activated cell sorting.

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METHODS**Autophagy detection for fluorescence-activated cell sorting**

Sorted CD4⁺ T cells or total PBMCs were cultured in the presence of HCQ for 18 hours. Subsequently, cells were stained with Cyto-ID autophagy detection kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's protocol. In short, cells were washed twice and stained with the autophagy specific dye diluted in supplemented culture medium (1:500) at 37°C for 30 minutes. Cells were washed 3 times and analyzed directly by flow cytometry.

Statistical analysis

Hypotheses were tested with 2-tailed *t* tests in single pairwise comparisons or multiple orthogonal comparisons. ANOVA *post hoc* tests were

used for correction of multiple nonorthogonal comparisons: Dunnett's when multiple conditions were tested against a single control condition; Sidak's when a subset of conditions was selected for pairwise comparisons; and Tukey's when all possible combinations were tested. One-sample 2-tailed *t* tests were used for comparisons against a fixed value (100%). Dependent samples were analyzed with paired *t* tests or repeated-measures ANOVA. *P* values, calculated with Prism (GraphPad, San Diego, Calif), are coded by asterisks: <.05 (*). Statistical difference in Fig 1, *D*, was calculated based on its Poisson distribution:

$$E_1 = \lambda t_1 = (x_1 + x_2) \times t_1 / (t_1 + t_2)$$

$$V_1 = (x_1 + x_2) \times t_1 \times t_2 / (t_1 + t_2)^2$$

$$TS = (X_1 - E_1)^2 / V_1$$