Hydroxychloroquine-Mediated Autophagy Inhibition Shifts Monocyte-Derived Dendritic Cells Toward Immunogenic Phenotype In Vitro

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KEYWORDS

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ABSTRACT

Dendritic cells (DCs) are recognized as the most potent antigen-presenting cells (APCs) and act as intermediaries between the innate and adaptive immune systems. The maturation and function of DCs are significantly influenced by autophagy. Hence, the current research evaluated the effect of hydroxychloroquine (HCQ), an autophagy inhibitor, on the maturation and activation of monocyte-derived DCs. Monocytes were cultured and induced to differentiate into monocyte-derived DCs by the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) cytokines. After five days, the immature DCs were exposed to HCQ and Lipopolysaccharides (LPS), followed by a 24-hour incubation period. On the sixth day, the flow cytometry technique was employed to assess the impact of HCQ on the phenotypic characteristics of the DCs. Finally, the expression of both pro- and anti-inflammatory genes in the HCQ-treated and untreated DCs groups was examined using the real-time PCR method. DCs that were subjected to treatment with HCQ exhibited a notable augmentation in the expression of CD11c, CD86, and HLA-DR, which are markers associated with maturation and antigen presentation. Furthermore, the DCs treated with HCQ demonstrated a significant reduction in the expression of IL-10, TGF-β, and IDO genes, while displaying an increase in the expression of TNF-α and IL-12 when compared to the control group. These findings indicate that targeting autophagy can induce a shift in DCs towards an immunogenic state. However, further investigations are necessary to assess the impact of HCQ-treated DCs on T cell responses both in vitro and in vivo in tumor-bearing animal models.

1. Introduction

Dendritic cells (DCs) play crucial roles in the initiation and control of both innate and adaptive immune responses, serving as a diverse group of specialized antigen-presenting cells (APCs) (1). Currently, there is significant focus on regulating the function of DCs to enhance the efficacy of immunotherapy for a range of ailments such as cancer and autoimmune disorders. As professional APCs, DCs play a crucial role in the immune system by facilitating either immunity or tolerance. They achieve this by capturing and presenting antigens to T cells, as well as by transmitting immunomodulatory signals through direct cell-to-cell interactions and the release of cytokines (2). Based on the developmental stage of the DCs, they can be categorized into two primary groups: immature and mature. The majority of immature DCs are found on mucosal surfaces, where they serve as guardians to detect antigens by means of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) using pattern recognition receptors (PRRs) (3). These DCs exhibit reduced levels of major histocompatibility complex (MHC)-I and MHC-II, adhesion molecules, and T cell co-stimulation factors including CD40, CD80, CD83, and CD86. Immature DCs do not release proinflammatory cytokines such as IL-12, IL-6, IL-1β, and TNF-α, yet they retain the ability to migrate (4). In contrast, mature DCs exhibit a diminished capability to internalize and process antigens, yet they possess an augmented ability to migrate. Furthermore, mature DCs have been documented to display elevated levels of several co-stimulatory molecules, such as CD40, CD80, and CD86, along with an enhanced production of proinflammatory cytokines and chemokines (5). Based on activation status, DCs are divided into immunogenic and tolerogenic subsets. Immunogenic DCs indicate increased expression of costimulatory molecules as well as inflammatory cytokines including IL-12 and TNF-α, which stimulate anti-tumor immune responses (6). However, tolerogenic DCs express high amounts of immunosuppressive factors including IL-10, TGF-β, IDO, and immune checkpoint molecules such as PD-L1 and CTLA-4, enhancing regulatory T cell responses, and have been considered a treatment strategy for autoimmune diseases (6).

Autophagy is an essential cellular mechanism that facilitates the removal of various molecules and subcellular components, such as nucleic acids, proteins, lipids, and organelles, through degradation mediated by lysosomes. This process plays a crucial role in maintaining cellular balance, supporting cellular differentiation, facilitating development, and ensuring cell survival (7). In DCs, the autophagy machinery is recognized for its significance in regulating the processing of both endogenous and exogenous antigens for MHC-II presentation. This is accomplished either through the fusion of autophagosomes with MHC-II loading compartments (MIIC) or via LC3-associated phagocytosis (LAP) (8, 9). Numerous recent studies have examined the role of autophagy in the functions of DCs across a range of physiological and pathological contexts, resulting in paradoxical results. Morris et al. have shown that the maturation of bone marrow-derived DCs (BMDCs) infected with respiratory syncytial virus (RSV) was hindered by the blockade of autophagy, as determined by the reduced expression of MHC-II and co-stimulatory molecules CD40, CD80, and CD86 (10). Autophagy induction through starvation has been observed to enhance the expression of CD86 and HLA-DR, along with the secretion of IL-10 and IL-6, in human dendritic cells infected with Bacillus Calmette-Guerin (BCG) (11). In a mouse model of allogeneic hematopoietic stem cell transplantation, Hubbard-Lucey and colleagues have indicated that mice lacking an autophagy component, ATG16L1, exhibit elevated expression of CD80 and CD86 costimulatory molecules in the DCs (12). Alissafi and colleagues have demonstrated that Foxp3+ Tregs effectively suppress the autophagic process in DCs through a mechanism that relies on cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in order to reduce their immunogenic potential (13).

Hydroxychloroquine (HCQ) is classified as a 4-aminoquinoline drug, characterized by its flat aromatic core structure. The presence of a basic side chain in this structure is believed to play a role in the drug's accumulation within intracellular compartments, particularly lysosomal compartments. This accumulation is considered vital for the drug's efficacy and its potential interaction with nucleic acids (14). HCQ can inhibit autophagy by lysosomal acidification, subsequently blocking the fusion of autophagosomes with lysosomes (15). Since autophagy plays a paradoxical role in DC maturation and activation, the aim of this study was to evaluate the effects of HCQ-mediated autophagy suppression on the maturation status as well as gene expression profile of various pro-inflammatory and anti-inflammatory markers in human monocyte-derived DCs.

2. Methodology

Materials

The media used in this study consisted of RPMI 1640 (Gibco, New York, USA) supplemented with 15% heat-inactivated Fetal Bovine Serum (FBS) (Gibco, New York, USA), L-glutamine (2 mmol/L), Streptomycin (100 µg/mL), and Penicillin (100 IU/mL), all of which were provided by Gibco. 2-mercaptoethanol (2ME) was obtained from Sigma Chemical Co (Munich, Germany), while human recombinant granulocyte-macrophage colony-stimulating factor (rh GM-CSF) and recombinant human interleukin-4 (rh IL-4) cytokines were purchased from BioLegend (San Diego, United States). Lipopolysaccharide (LPS), HCQ, and Ficoll were provided by Sigma Chemical Co (Munich, Germany), and the human monocyte isolation kit was purchased from BioLegend (San Diego, United States). Antibodies used for phenotypical characterization of DCs included Anti HLA-DR-APC and anti-CD86-PE, which were obtained from BioLegend (San Diego, United States), and Anti CD11c-FITC and Anti CD14-FITC, which were ordered from Immunostep (Salamanca, Spain). The Apoptosis Detection Kit used in this study was obtained from Immunostep (Salamanca, Spain). The TRIzol reagent, which is utilized for RNA extraction, was supplied by Roche Diagnostics, located in Mannheim, Germany. In order to synthesize complementary DNA (cDNA), the Addscript cDNA synthesis kit from AddBio, based in Korea, was employed. To evaluate gene expression through Real-Time PCR, the SYBER Green master Mix from Amplicon, situated in England, was utilized.

Monocyte isolation from peripheral blood mononuclear cells (PBMCs)

Fresh peripheral blood was obtained from healthy donors using sterile falcons containing heparin. To separate peripheral blood mononuclear cells (PBMCs), a Ficoll density gradient centrifugation technique was employed. The isolation of monocytes from PBMCs was achieved using the MACS technique, which involved the utilization of biotinylated Anti-CD14 antibody and streptavidin-nanobeads for positive selection. Initially, cells other than monocytes were eliminated from the MACS column and collected as unlabeled cells. However, due to the presence of the CD14 marker on their surface and their binding to the antibody, monocytes remained attached to the beads within the column. Subsequently, monocytes were collected from the column using MACS buffer and piston. The MACS technique's efficacy in isolating monocytes with high purity was confirmed by evaluating the expression of the CD14 marker on the separated cells using the MACSQuant cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany).

Generation of human monocyte-derived DCs

Monocytes were cultured in a 6-well plate with complete media, which consisted of RPMI-1640 enriched with 15% FBS, 100 µg/mL streptomycin, 100 IU/mL penicillin, and 2 mM L-glutamine. The concentration of monocytes was 1.5×10^6 per mL, and the plate was supplemented with 50 µM of 2-mercaptoethanol (2ME) solution and cytokines rh GM-CSF and rh IL-4 at concentrations of 40 and 25 ng/mL, respectively. On the third day, fresh complete media containing rh GM-CSF and rh IL-4 was added to the plate, replacing half of the medium. This resulted in the generation of immature DCs on day 5.

The determination of the optimal dose of HCQ through an apoptosis assay

The Annexin V-FITC/PI staining method was employed to determine the optimal dosage of HCQ for the experiments. On the fifth day of DC culture, the control group of iDCs did not receive any drug, while the other two groups of iDCs were treated with HCQ doses of 10 and 100 µM acquired from similar studies (16, 17). These cells were then incubated for 24 hours at a temperature of 37°C and 5% CO2. Following incubation, the cells were collected in 1.5 mL microtubes and centrifuged at 300g for 10 minutes. They were then washed using PBS and 200 µL of 1X Apoptosis kit buffer was added to each microtube after removing the supernatants. The microtubes were incubated on ice and in darkness for 10 minutes with the addition of Annexin V-FITC antibody. Subsequently, PI dye was added to the microtubes and the incubation was continued for an additional 5 minutes. The microtubes were then centrifuged at 300g at 4°C for 10 minutes after adding PBS (2X). After removing the supernatants, 200 µL of PBS was added to each microtube, and the apoptosis rate of the cells was evaluated using a flow cytometry instrument.

Treatment of DCs with HCQ and their phenotypic study

On the fifth day of the experiment, RPMI-1640 enriched with 15% FBS was used to replace half of the culture media in each well. The treatment group of DCs was administered with the optimal dose of HCQ. After a minimum of 3 hours of incubation time, both the treatment and control groups were exposed to 100 ng/mL LPS and incubated for 24 hours to produce mDCs. On day 6, mDCs (control group) and HCQ-treated mDCs (HCQ-mDCs) were collected for further evaluation. The cells were resuspended in FACS buffer and incubated with antibodies including anti-CD11c-FITC, anti-HLA-DR-APC, anti-CD14-FITC, and anti-CD86-PE in the dark at 4°C for 25 minutes. After incubation, PBS was added and microtubes were centrifuged at 300g for 10 minutes (2X). The cells were resuspended in 200 µL PBS and analyzed using a MACSQuant cytometer (Miltenyi Biotec, Auburn, CA, USA). The obtained data was analyzed using FlowJo v10.5.3 software to determine the expression of CD11c, HLA-DR, and CD86 surface markers based on Mean Fluorescent Intensity (MFI).

RNA isolation and gene expression evaluation by qRT-PCR

After extracting total cellular RNA using the TRIzol reagent from Roche Diagnostics, Mannheim, Germany, the concentration of RNA was determined through the use of a spectrophotometer. The RNA was then stored at a temperature of -80°C, and the production of complementary DNA (cDNA) was

carried out with the assistance of BioFACT 2step 2X RT-PCR Pre-Mix (Taq). Gene expression was evaluated using the Applied Biosystems StepOnePlusTM Real-Time PCR System (Life Technologies, Carlsbad, CA, USA), and the expression of target mRNAs was normalized using the 18s gene as an internal control. The primer sequences can be found in Table 1. All experiments were conducted in triplicate, and the relative mRNA expression was measured using the $2^{-\Delta\Delta Ct}$ method.

Table 1. The sequences of primers used for gene expression analysis

Gene	Forward/Reverse	Sequence
TNF-α	F	TTCTCCTTCCTGATCGTGGCA
	R	TAGAGAGAGGTCCCTGGGGAA
IL-10	F	AGGAAGAGAAACCAGGGAGC
	R	GAATCCCTCCGAGACACTGG
IL-12	F	TCAGAATTCGGGCAGTGACTATTG
	R	ATCCTTCTTTCCCCCTCCCTA
TGF-β	F	CTTGCCCTCTACAACCAACACA
	R	AGCGCACGATCATGTTGGAC
IDO	F	AGCTTATGACGCCTGTGTGAA
	R	TCCTTTGGCTGCTGGCTTG
18S	F	CTACGTCCCTGCCCTTTGTACA
	R	ACACTTCACCGGACCATTCAA
NF-Kb	F	AACAGAGAGGATTTCGTTTCCG
	R	TTTGACCTGAGGGTAAGACTTCT

Statistical analysis

GraphPad Prism v8.0.2, developed by GraphPad Software in San Diego, California, USA, was employed for the purpose of data analysis. To compare the data between the control and treatment groups, the student's t-test was utilized. Each parameter was evaluated in triplicate, and the mean ± SD values were reported for each group. The significance cut-off was set at a p-value of ≤ 0.05, where "ns" denoted not significant, "*" represented a p-value ≤ 0.05, "**" indicated a p-value ≤ 0.01, "***" signified a p-value ≤ 0.001, and "****" denoted a p-value ≤ 0.0001.

3. Result and Discussion

Determination of HCQ optimum dose for DCs' treatment by apoptosis assay

In order to determine the most effective dose of HCQ, the Annexin V-FITC/PI staining and flow cytometry techniques were employed. The results showed that the percentage of viable cells in the control group and groups treated with 10 and 100 μM doses were 97.9%, 89.5%, and 9.09%, respectively. Based on these findings, it was concluded that the optimal dose for the treatment of DCs in subsequent experiments was 10 μM (Figure 1).

HCQ treatment increased the expression of maturation-related markers on DCs

Flow cytometry analysis demonstrated that the purity of monocytes obtained from PBMCs using the

MACS method exceeded 90% (Figure 2). Following a 6-day culture in the presence of IL-4 and GM-CSF cytokines, along with the addition of LPS on day 5, the monocytes adhered to the plate differentiated into mDCs (Figure 3a). To evaluate the phenotypic characteristics of the mDC group and the HCQ-mDC group, markers associated with maturation and antigen presentation in DCs (CD11c, HLA-DR, and CD86) as well as the monocyte marker (CD14) were assessed. A minimal percentage of differentiated cells exhibited expression of the monocyte marker CD14, however, a great percentage of cells revealed the expression of CD11c, HLA-DR, and CD86 markers, indicating successful differentiation from monocytes to DCs (Figure 3b). Subsequently, the disparity in the surface expression of these markers between the mDC and HCQ-mDC groups was investigated using MFI. Flow cytometry findings indicated a significant increase in the expression of CD11c ($P \leq 0.01$**) and CD86 ($P \leq 0.0001$****) in the HCQ-mDC group compared to the mDC group (Figure 3c). Although the expression of the HLA-DR marker was higher in the HCQ-mDC group than in the mDC group, the difference was not statistically significant ($P > 0.05$*) (Figure 3c).

HCQ treatment decreased the expression of genes related to tolerogenic phenotype in DCs

The impact of HCQ treatment on the activation status of DCs was assessed by examining the expression profile of specific genes associated with both tolerogenic and immunogenic phenotypes. Real-Time PCR was utilized to determine the levels of TGF-β, IL-10, IDO (tolerogenic phenotype), as well as TNF-α, IL-12, and NF-KB (immunogenic phenotype). The results obtained from this analysis revealed that HCQ treatment led to a significant reduction in the expression of IL-10, TGF-β ($P \leq 0.01$), and IDO ($P \leq 0.05$). Conversely, it was found that the expression levels of TNF-α ($P \leq 0.05$) and IL-12 ($P \leq 0.0001$) were increased in comparison to the control group. Notably, no remarkable differences were observed in the expression of NF-KB between the control and treatment groups ($P > 0.05$) (Figure 4).

Discussion

DCs have garnered significant attention due to their distinctive characteristics, positioning them as highly promising therapeutic agents for combatting both cancer and autoimmune disorders. DCs possess the ability to migrate to lymph nodes and activate naive T cells, making them a highly efficient type of APCs for the induction of $CD8^+$ T cell immunity in cancer immunotherapies. Additionally, the secretion of cytokines and chemokines by DCs can elicit an appropriate immune response (18). DCs are categorized into two subsets, immunogenic and tolerogenic, based on their activation status. Immunogenic DCs exhibit elevated expression of costimulatory molecules and inflammatory cytokines like IL-12 and TNF-α, which stimulate anti-tumor immune responses (19). Conversely, tolerogenic DCs express high levels of immunosuppressive factors such as IL-10, TGF-β, IDO, and immune checkpoint molecules like PD-L1 and CTLA-4, which enhance Treg responses. Tolerogenic DCs have been considered as a treatment strategy for autoimmune diseases (20).

Autophagy, a cellular physiological process, has been demonstrated to play a role in various aspects of DC functions. These include DC maturation, antigen presentation, cytokine production, DC migration, as well as T-cell activation (21). Several recent studies have investigated the involvement of autophagy in the operations of DCs within various physiological and pathological conditions, leading to contradictory findings. Therefore, the objective of this research was to assess the impact of HCQ, an autophagy inhibitor, on the maturation status and gene expression patterns of different pro-inflammatory and anti-inflammatory markers in DCs derived from human monocytes.

Our results indicated that HCQ treatment of DCs along with LPS stimulation synergistically enhances the expression CD11c, HLA-DR, and CD86, markers related to maturation and activation, compared with the control group (Figure 3c). Moreover, HCQ treatment could significantly increase the expression of pro-inflammatory genes related to the immunogenic phenotype of DCs including IL-12 and TNF-α, whereas it reduced the expression of genes associated with the tolerogenic state of DCs including IL-10, TGF-β, and IDO (Figure 4).

Similar to our outcomes, various studies have shown the immunogenic impacts of numerous autophagy

inhibitors on DCs. Autophagy suppression in mice via targeting Atg16L1 resulted in the upregulation of the co-stimulatory molecules CD40, CD80, and CD86 on the $CD11c^+$ DCs compared to the wild-type mice (12). In a study by Liu et al., it has been shown that siRNA-mediated suppression of lung carcinoma cells' exosomal MALAT1 downregulated the autophagy process in DCs, and subsequently increased the expression levels of co-stimulatory molecule CD80 as well as pro-inflammatory factors interferon (IFN)-γ and IL-12 (22). It has been shown that ATF4-dependent autophagy induction in DC-like THP-1 cells using the skin sensitizer 1-fluoro-2,4-dinitrobenzene resulted in the inhibition of CD86 co-stimulatory marker and pro-inflammatory cytokines IL-12β, IL-1β, and CXL10 expression (23). A study by Baghdadi et al. has indicated that the release of tumor cell-associated DAMPs as a consequence of chemotherapy enhances mucin domain-containing molecule-4 (TIM-4) expression on Tumor-Associated Macrophages and DCs, which in turn promotes autophagy-mediated degradation of ingested tumors, resulting in the diminished antigen presentation and devastated cytotoxic T lymphocyte responses (24). The inhibition of autophagy, either through pharmacological means or by ATG16L1 knockdown in DCs derived from human PBMCs, was found to hinder the production of the immunosuppressive cytokine IL-10. Consequently, this inhibition resulted in an enhanced proliferation of T-cells (25).

Inconsistent with our results, several investigations have reported that autophagy suppression in DCs shifts them into tolerogenic phenotype. Paludan et al. has reported that the effective presentation of MHC class II of an endogenously synthesized viral protein (Epstein–Barr virus nuclear antigen 1 (EBNA1) to $CD4^+$ lymphocytes is reduced by the pharmacological and genetic inhibition of autophagy in DCs (26). A study by Zang et al. has indicated that autophagy inhibition via Beclin-1 targeting in mice infected with the influenza A (H1N1) virus diminishes cytokine levels of IL-6, TNF-α, IFN-β, IL-12p70, and IFN-γ secreted from bone-marrow-derived DCs compared to the bone-marrow-derived DCs from H1N1-infected wild-type mice (27). The autophagy machinery in DCs is suppressed by the inhibitory receptor CTLA-4 on $Foxp3^+$ Tregs. This suppression leads to the downregulation of MHC-II, and subsequently a decrease in the turnover of peptide-MHC-II complexes and a reduction in the stimulation of peptide-specific T cell responses. As a result, autoimmune responses are ameliorated (13). Fan and colleagues have shown that 3-MA and bafilomycin A1 mediated autophagy blockade diminishes ConA-induced expression of MHC-II, CD80, and CD86 as well as secretion of IL-12 and IFN-γ cytokines in mouse bone-marrow-derived DCs (28).

4. Conclusion and future scope

Over the last few decades, it has become increasingly evident that DCs are a valuable tool in the treatment of various medical conditions such as cancers and autoimmune diseases. This is due to their ability to either stimulate or suppress the immune system, depending on their immunogenic or tolerogenic status. Various studies have shown a paradoxical role for autophagy in terms of DCs' stimulation or suppression. Our results revealed that autophagy targeting via HCQ in monocyte-derived DCs could significantly elevate and reduce the gene expression of pro-inflammatory and immunosuppressive factors, respectively. Furthermore, HCQ treatment increased the expression of surface markers related to DCs' maturation. Overall, our findings indicate that autophagy inhibition along with LPS stimulation results in immunogenic phenotype of DCs which are beneficial for the treatment of cancers. There is a need for further preclinical investigations particularly in animal models in order to unravel the effects of autophagy targeting in DCs.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

All the data generated or analyzed during this study are included in the published article. Further inquire

can be directed to the corresponding author.

Ethics approval

All experiments and procedures were conducted in accordance with the ethical principles of Tabriz University, Tabriz, Iran, and approved by the regional ethical committee for medical research.

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