Hydroxychloroquine sensitizes chronic myeloid leukemia cells to Vγ9Vδ2 T cell-mediated lysis independent of autophagy

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Abstract. Hydroxychloroquine (HCQ) is the only autophagy inhibitor in clinical use and it has shown great potential in treating chronic myeloid leukemia (CML). By inhibiting autophagy, HCQ enhances the anti-CML efficiency of chemotherapy. In the present study, we demonstrated that HCQ sensitized CML cells to Vγ9Vδ2 T cell-mediated lysis. HCQ inhibited autophagy in CML cells, but the sensitizing effects of HCQ were autophagy-independent. Since the sensitization was not mimicked by ATG7 knockdown and even occurred in the absence of ATG7. We revealed that in a time-dependent manner HCQ induced the expression of NKG2D ligand ULBP4 on the surface of CML cells. This marks the leukemia cell for recognition by Vγ9Vδ2 T cells. Blocking the interaction of NKG2D with its ligands deleted the sensitizing effects of HCQ. In addition, we showed that HCQ did not affect the synthesis or degradation of ULBP4, but induced the translocation of ULBP4 from the cytoplasm to the cell membrane. Our results uncovered a previously unknown mechanism for HCQ in CML treatment that underlines the ability of HCQ to modulate the immune visibility of CML cells, and pave the way to the development of new combination treatments with HCQ and Vγ9Vδ2 T cells.

Introduction

Chronic myeloid leukemia (CML) is a hematological malignancy arising from hematopoietic stem cell transformation (1,2). Targeting BCR-ABL protein with tyrosine kinase inhibitors (TKIs) has profoundly improved the survival of CML patients. However, there are still challenges: firstly, approximately 40% CML patients relapse with BCR-ABL mutation and these patients are resistant to most TKIs; secondly, CML patients in blast crisis (BC) are insensitive to TKIs (3,4); thirdly, CML stem cells are intrinsically insensitive to TKIs. It is urgent to explore novel strategies for CML treatment. Combination therapy and immunotherapy are potential way to circumvent these problems. Combination treatments with hydroxychloroquine (HCQ) and traditional agents have been investigated recently (5-12). HCQ enhances the sensitivity of CML cells to TKIs, even in primary CML stem cells (13,14). The combination of HCQ with imatinib almost completely eliminates CML stem cells *in vitro*. Based on this finding, a randomized phase II clinical trial (NCT01227135) is now underway (2,15). HCQ can also potentiate the anti-CML efficiency of other agents including suberoylanilide hydroxamic acid, perifosine, asparaginase and diosgenin (6-12). In addition, HCQ can strengthen the efficacy of immunotherapy (16-19). By inhibiting hypoxia-induced autophagy in breast cancer cells, HCQ facilitates natural killer cell (NK)-mediated elimination of tumor cells (18). HCQ also enhances cytotoxic T lymphocyte (CTL)-mediated lysis of melanoma cells (19).

Vγ9Vδ2 T cell is important immune cell in peripheral blood and are attractive candidate for the elimination of leukemia cells (20-23). Consistent with an earlier study, we have previously shown that Vγ9Vδ2 T cells can effectively recognize and kill CML cells (23-25). Both HCQ and Vγ9Vδ2 T cells are promising in CML treatment, but the effects of HCQ on the elimination of CML cells by Vγ9Vδ2 T cells are unknown.

The anti-CML effects of HCQ mainly rely on autophagy inhibition (26). However, some studies have revealed that HCQ also can exert anticancer effects independent of autophagy (27,28). In the present study, we uncovered a previously unknown autophagy-independent mechanism by which HCQ enhanced the sensitivity of CML cells to Vγ9Vδ2 T cell-mediated lysis. HCQ enhanced the sensitivity by promoting the translocation of ULBP4 from the cytoplasm of CML cells to the membrane, which is important for the recognition of cancer cells by Vγ9Vδ2 T cells (24,29,30). Our results revealed an unknown mechanism of HCQ in treating CML, and provide the first evidence that combining HCQ with Vγ9Vδ2T immunotherapy represents a promising treatment for CML.

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Materials and methods

Reagents. Hydroxychloroquine sulfate (HCQ) was purchased from Selleck Chemicals (Houston, TX, USA). Zoledronate was from Novartis (Basel, Switzerland). IL2 was purchased from PeproTech (Rocky Hill, NJ, USA).

Vγ9Vδ2 T cell preparation. Vγ9Vδ2 T cells were prepared from peripheral blood samples. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples of healthy volunteers and cultured in RPMI-1640 medium (Corning Costar, Corning, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) for 10-14 days. PBMCs were stimulated with 400 nM zoledronate and 300 IU/ml recombinant human IL2 for 72 h. IL2 was renewed every 3 days at the same concentration and lymphocytes were kept at $1.5x10^6$ cells/ml. Ten to fourteen days later, the purity of Vγ9Vδ2 T cells was determined by flow cytometry using mAbs for TCRVδ2 and indicated a purity ≥90%.

Leukemia cell culture. Human CML cell lines K562 and K562/GO1 were purchased from the Institute of Hematology at the Chinese Academy of Medical Sciences (Tianjin, China). K562/GO1 cell is an imatibin resistant CML cell line, which showed increased level of BCR-ABL (31). The cell lines were cultured (0.5 cells $x10^6$/ml) in complete RPMI-1640 medium (Corning Costar) containing 10% FBS (Gibco).

Bone marrow samples were taken from two CML patients and bone marrow mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation (Haoyang Biological Manufacture, Co., Ltd., Tianjin, China). CML stem cells were labelled with anti-CD34 FITC antibody and selected using anti-FITC magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The percentage of CD34-positive cells was >85%. Informed consent was obtained in accordance with the Declaration of Helsinki from all patients and volunteers. Approval for the study was obtained from the Ethics Committee of the First Affiliated Hospital of Zhejiang University.

Vγ9Vδ2 T cell cytotoxicity assay. To test the cytotoxicity of Vγ9Vδ2 T cells, a flow cytometric cytotoxicity assay was performed using CFSE (Life Technologies, Grand Island, NY, USA) and propidium iodide (PI; Multi Science, Hangzhou, China) (32). K562 and K562/GO1 cells were pretreated with 30 μM HCQ for 8 h and control cells were pretreated with an equal volume of phosphate-buffered saline (PBS). Then cells were harvested and washed twice with PBS. After CFSE staining, the cells were resuspended in serum-free medium and plated into 96-well plates, and Vγ9Vδ2 T cells were added at effector target ratios (E:T) of 20:1, 10:1, or 5:1. In addition, 150 IU/ml IL-2 was added to the wells. Wells containing only labelled target cells were also prepared to evaluate background levels of cell death. Cells were incubated for 4 h at 37˚C and 5% CO_2, then stained with PI and analyzed by flow cytometry. Cytolytic activity was calculated based on the percentage of dead target cells ($CFSE^+$ PI^+). In some experiments, effector cells were pretreated with 10 μg/ml anti-NKG2D neutralizing mAb (eBioscience, Inc., San Diego, CA, USA) or isotype control mAb (eBioscience) for 1 h at room temperature. Fluorescence was analyzed using a Flow Cytometry FC500 system (Beckman Coulter, Inc., Miami, FL, USA) and data were analyzed using the CXP flow cytometry software.

Vγ9Vδ2 T cell degranulation assay. Degranulation of Vγ9Vδ2 T cells was evaluated using the lysosomal marker CD107a as previously described (33). CML cell lines and primary CML cells were pretreated with HCQ or PBS. Cells were incubated with Vγ9Vδ2 T cells at an E:T ratio of 1:1 in a 96-well plate, and 5 μl Alexa647-CD107 (BioLegend, San Diego, CA, USA) was added to all wells. As positive control wells, 0.05 μg/ml phorbol ester (PMA) and 1 μg/ml ionomycin (Multi Science) were added. Wells containing Vγ9Vδ2 T cells only were prepared as negative controls to establish background levels of degranulation. Cells were incubated for 1 h at 37˚C, then 3 μl GolgiStop (BD Biosciences, Franklin Lakes, NJ, USA) was added. Cells were further incubated for 3 h, and then washed and incubated with an anti-Vδ2 TCR antibody (eBioscience) to label Vγ9Vδ2 T cells for analyzing by flow cytometry. The level of Vγ9Vδ2 T cell degranulation was determined by the percentage of CD107-positive Vγ9Vδ2 T cells.

Toxicity assay of HCQ to CML cell lines. CML cell lines were cultured at $2x10^5$ cells/ml in the presence of 30 μM HCQ for 8 h at 37˚C and 5% CO_2. Control CML cells were treated with equal volumes of PBS. Cells were washed twice with PBS, and then stained with 7.5 μM PI for 0.5 h. Cell viability was tested by flow cytometry and PI-positive cells were considered dead.

Flow cytometric analysis. Cells were washed and resuspended in 100 μl PBS, then incubated with 5 μl fluorophore-conjugated mAb at 4˚C in the dark for 30 min. Mouse anti-human PE-ULBP1, PE-ULBP2/5/6, PE-ULBP3, APC-ULBP4 and PE-MICA/B (R&D Systems, Minneapolis, MN, USA), FITC-CD34 and PE-Vδ2TCR (eBioscience) antibodies were used. Isotype antibodies were used to assess non-specific staining. For intracellular staining, cells were fixed and permeabilized using a fixation/permeabilization kit (BD Biosciences) according to the manufacturer's instructions before mAb labelling.

Protein extraction and western blot analyses. Equal numbers of cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing phenylmethane sulfonyl fluoride. Cell lysates were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred onto nitrocellulose membranes. The membranes were blocked in 5% BSA (BBI Life Science, Shanghai, China), then incubated with primary antibodies and secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA). The following primary antibodies were used: Anti-P62/SQSTM1 (Abcam, Cambridge, MA, USA), anti-ULBP4 (R&D Systems), anti-LC3, anti-ATG7 and anti-β-actin (Cell Signaling Technology, Danvers, MA, USA). Immunoreactive bands were visualized using an Odyssey infrared imaging system (LI-COR Biosciences).

Short hairpin RNA (shRNA) preparation and transfection. Lentiviral vectors containing shRNA against ATG7 or the corresponding control shRNA were synthesized by Shanghai

GenePharma, Co., Ltd. (Shanghai, China). Lentiviruses were produced in 293T cells by transfecting the lentiviral expression vector and packaging vectors (psPAX2 and pMD2.G; Addgene, Cambridge, MA, USA) using Attractenen transfection reagent (Qiagen, Valencia, CA, USA). After enriching, the lentiviruses were transfected into K562 and K562/GO1 cells. Transfection efficiency was estimated by evaluating GFP expression. The effects of autophagy inhibition were tested by western blot analysis.

Confocal immunofluorescence microscopy. HCQ- or PBS-treated K562 cells were placed onto adhesion microscope slides (Citoglas, Haimen, China) and fixed in 4% methanol (Biotech Well, Shanghai, China). After fixation, cells were permeabilized with 0.1% Triton X-100 and incubated in a primary anti-ULBP4 (Abcam) antibody followed by a CY3-tagged secondary antibody (Wuhan Boster Biological Technology Ltd., Wuhan, China) to stain ULBP4 protein. The green fluorescent dye Dio (Invitrogen, Carlsbad, CA, USA) was used to stain the cell membrane. The nucleus was counterstained with DAPI. Images were captured using a two-photon confocal microscope (Olympus Corp., Tokyo, Japan).

Transmission electron microscopy. K562 and K562/GO1 cells were treated with 30 μM HCQ or PBS for 8 h. Harvested cells were fixed with 2.5% glutaraldehyde overnight at 4˚C and then post-fixed in a solution containing 1% osmium tetroxide and dehydrated through an alcohol series. Fixed samples were sectioned and stained with 3% uranyl acetate and Reynolds lead citrate. Samples were imaged using a transmission electron microscope (Philips TECAN 10; Philips Electronic N.V, Amsterdam, The Netherlands).

Statistical analyses. Data were from three independent experiments and expressed as mean ± SD. All data were analyzed using SPSS 7.0 software with ANOVA or two-tailed Student's t-test. $P<0.05$ was considered statistically significant.

Results

HCQ sensitizes CML cells to Vγ9Vδ2T cell-mediated lysis. To investigate whether HCQ can affect the susceptibility of CML cells to Vγ9Vδ2 T cell-mediated lysis, the human CML cell lines K562 and K562/GO1 pretreated with HCQ or PBS were used as target cells and Vγ9Vδ2 T cells as effector cells. CML cells were co-cultured with Vγ9Vδ2 T cells for 4 h at different effector target ratios (E:T) and cell death was quantified by PI staining. Background cell death was similar in HCQ-pretreated cells and control cells, but HCQ-pretreated cells showed higher sensitivity to Vγ9Vδ2 T cell-mediated lysis than control cells. The specific lysis of K562 by Vγ9Vδ2 T cell increased from 35.9±1.64 to 47.1±5.97% ($P<0.05$) and from 18.72±3.73 to 24.95±2.345% ($P<0.05$) at E:T ratios of 20:1 and 10:1 (Fig. 1A). Similarly, the lysis of K562/GO1 increased from 31.7±3.9 to 46.6±1.85% ($P<0.05$) and from 17.4±2.2 to 26.2±3.06% ($P<0.05$) at E:T ratios of 20:1 and 10:1 (Fig. 1B). To confirm that enhanced CML cell death was not caused by toxicity of HCQ to CML cells, we performed a toxicity assay. HCQ treatments (30 μM) for 8 h did not reduce cell viability (Fig. 1C).

To confirm the afore-mentioned results, we examined degranulation of Vγ9Vδ2 T cells using CD107a as a marker. CD107a expression was evaluated by flow cytometry after Vγ9Vδ2 T cell interaction with HCQ or PBS pretreated K562 and K562/GO1 cells. The expression of CD107a increased in Vγ9Vδ2 T cells co-cultured with HCQ-pretreated CML cell lines compared with Vγ9Vδ2 T cells co-cultured with control CML cells. The percentage of CD107a positive Vγ9Vδ2 T cells co-cultured with K562 cells increased from 12.45±4.66 to 23.05±4.98% ($P<0.05$), and from 18.3±3 to 28.2±3.5% ($P<0.05$) in Vγ9Vδ2 T cells co-cultured with K562/GO1 (Fig. 1D). We validated this result in primary CML stem cells isolated from two CML patients. HCQ pretreated primary CML cells accelerated Vγ9Vδ2 T cell degranulation (Fig. 1E). Collectively, our results demonstrate that HCQ can enhance the susceptibility of CML cell lines and CML primary stem cells to Vγ9Vδ2 T cell-mediated lysis.

HCQ inhibits autophagy in CML cells. HCQ is an important autophagy inhibitor and some studies have proven that inhibiting autophagy promotes the elimination of cancer cells by NK and CTL cells (18,19). To explore the mechanisms involved in the sensitizing effects of HCQ, we first tested whether HCQ can inhibit autophagy in CML cells. During autophagy, microtubule-associated protein light chain-3II (LC3II) is selectively expressed on the autophagosomal membrane and can be easily detected as autophagosome biomarkers (34,35). We evaluated LC3II expression in K562 and K562/GO1 cells after HCQ treatment. As shown in Fig. 2A, LC3II accumulation was dependent upon the exposure time to HCQ. After 2 h of exposure LC3II begins to accumulate, and with increasing exposure time autophagy inhibition increased. HCQ treatment also increased P62 expression (Fig. 2A), which correlated with autophagy inhibition (36). Furthermore, transmission electron microscopy showed that autophagic vacuoles were not present in control CML cells. HCQ treatment induced the accumulation of autophagic vacuoles in CML cells containing some organelles and electron-dense inclusions (Fig. 2B). In summary, immunoblotting and transmission electron microscopy showed that HCQ significantly inhibits autophagy in CML cells.

HCQ sensitizes CML cells to Vγ9Vδ2T cell-mediated lysis independent of autophagy. HCQ can inhibit autophagy in CML cells, but whether HCQ sensitizes CML cells to Vγ9Vδ2 T cell-mediated lysis by inhibiting autophagy is unknown. We targeted the autophagy protein ATG7 with shRNA to inhibit autophagy. Contrast to control shRNA, shATG7 significantly silenced ATG7 protein expression, and inhibited autophagy as shown by the disappearance of LC3II even in the presence of HCQ (Fig. 3A). Autophagy inhibition with shATG7 did not increase the sensitivity of K562 and K562/GO1 cells to Vγ9Vδ2 T cytotoxicity (Fig. 3B). This suggests that HCQ sensitizing CML cells to Vγ9Vδ2 T cytotoxicity is not through autophagy inhibition. ATG7 knockdown prevents autophagy in early stages while HCQ blocks later stages of autophagy. To further confirm that the sensitizing effects of HCQ are autophagy-independent, we tested whether HCQ enhances the sensitivity of autophagy-defective CML cells to Vγ9Vδ2 T cells. After knocking down

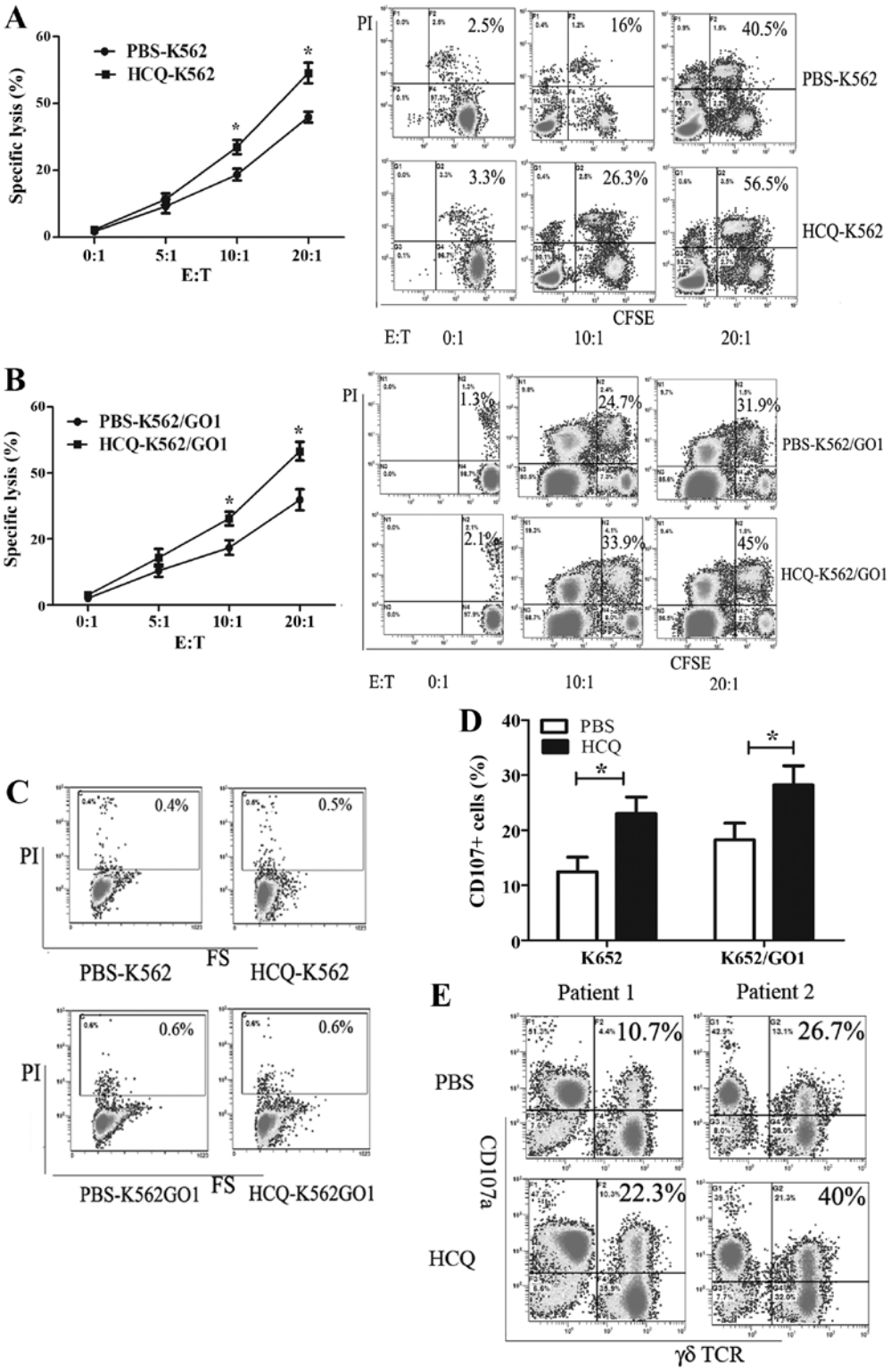

Figure 1. HCQ enhances the sensitivity of CML cells to Vγ9Vδ2T-mediated lysis. HCQ pretreated (A) K562 and (B) K562/GO1 cells are more sensitive to Vγ9Vδ2 T mediated lysis at E:T ratio of 10:1 20:1. Data are shown as mean ± SD; (*P<0.05) (left panel). Respective results are shown (right panel), number in the top right corner represents the specific lysis of CML cells by Vγ9Vδ2 T cells. (C) The toxicity of HCQ to K562 and K562/GO1 were tested, and PI positive cells were considered dead. HCQ treatments (30 μM) for 8 h do not reduce CML cell viability. Results are representative of 3 independent experiments. (D) HCQ pretreated-K562 and K562/GO1 can accelerate the degranulation of Vγ9Vδ2 T cells. Data are presented as mean ± SD; (*P<0.05). All experiments were performed in triplicate. (E) Degranulation of Vγ9Vδ2 T cells cocultured with HCQ- or PBS pretreated CML primary stem cells are shown, number in the top right corner is the percentage of CD107a positive Vγ9Vδ2 T cells.

ATG7 in CML cells, we pretreated these cells with PBS or HCQ and co-cultured with Vγ9Vδ2 T cells. HCQ sensitized autophagy-defective K562 and K562/GO1 cells to Vγ9Vδ2 T cell cytotoxicity (Fig. 3C). We conclude that HCQ sensitizes K562 and K562/GO1 cells to Vγ9Vδ2 T cell-mediated lysis independent of autophagy.

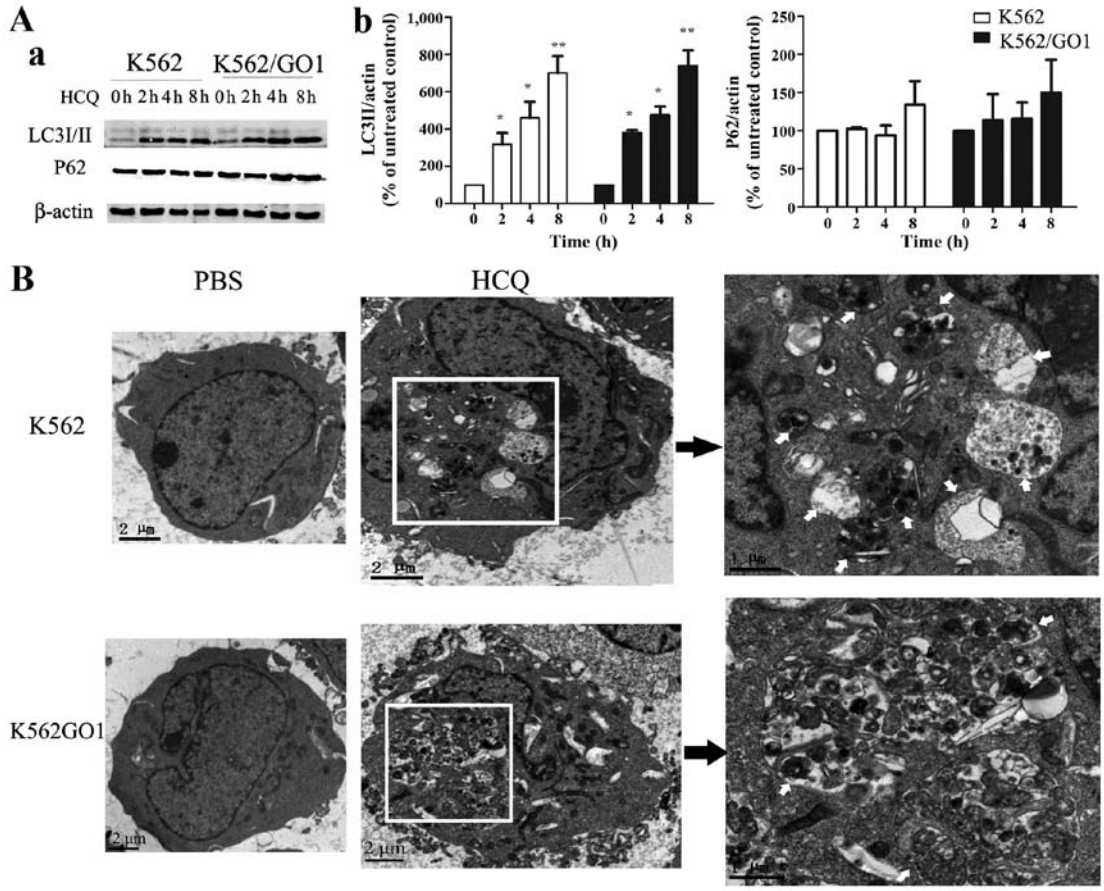

Figure 2. HCQ inhibits autophagy in CML cell lines. (A) LC3 and P62 were detected by western blot analysis after K562 and K562/GO1 exposure to HCQ for indicated time. β-actin was measured as loading control. (a) Representative results of 3 experiments are shown, (b) densitometric analysis was performed using odyssey software and results are expressed as mean ± SD; *P<0.05 **P<0.01. (B) To detect autophagosome, representative electron microscopy images are shown for HCQ- or PBS-treated K562 and K562/GO1. Arrowheads point to autophagic vesicles.

HCQ sensitizes CML cells to Vγ9Vδ2 T cell-mediated lysis by inducing ULBP4 expression on CML membrane. Although the recognition of target cell is mainly TCR mediated, Vγ9Vδ2T cells also can utilize activating receptor NKG2D. NKG2D is a NK cell-activating receptor, which recognizes stress-inducing ligands including MICA, MICB and UL16-binding proteins (ULBP1-6) in cancer cells (37-39). We and others have previously shown that the interaction of NKG2D with its ligands is important for recognition of leukemia cells by Vγ9Vδ2 T cell and activation of Vγ9Vδ2 T cells. We speculated that HCQ may affect the expression of NKG2D ligands in CML cells. Consistent with our hypothesis, flow cytometric analysis showed that HCQ induced ULBP4 expression on the cell membrane, and the expression of other NKG2D ligands was not affected (Fig. 4A and B). ULBP4 was not expressed on the membrane of K562 and K562/GO1 cells, but after HCQ treatment for 8 h the mean fluorescence intensity (MFI) of ULBP4 increased from 0.359±0.072 to 2.157±0.78 in K562 cells and from 0.41±0.04 to 1.9±0.19 in K562/GO1 cells (Fig. 4A and B). Next, we investigated whether HCQ-induced expression of ULBP4 is involved in sensitizing CML cells to Vγ9Vδ2 T cell-mediated lysis. We incubated effector cells with anti-NKG2D antibody to block the interaction of NKG2D with its ligands and then performed cytotoxicity assays. HCQ increased the sensitivity of CML cells to Vγ9Vδ2 T cell-mediated lysis, and blocking the interaction of NKG2D with its ligands almost completely abrogated this sensitizing effect (Fig. 4C). Notably, control CML cell did not express ULBP4 on the membrane, and blocking interaction of NKG2D with its ligands did not affect the sensitivity of control CML cells to Vγ9Vδ2 T cell-mediated lysis (Fig. 4C). This implies that ULBP4 is much more important than other NKG2D ligands in Vγ9Vδ2 T cell activation and CML cell elimination, consistent with previous findings (39). Taken together, these results indicate that HCQ sensitizes CML cells to Vγ9Vδ2 T cell-mediated cytotoxicity by inducing ULBP4 expression on the CML cell membrane.

HCQ induces ULBP4 translocation from the cytoplasm to the cell membrane. To explore the mechanisms behind HCQ-induced expression of ULBP4 on the CML cell membrane, we monitored ULBP4 expression over time after HCQ treatment. HCQ induced the expression of ULBP4 in a time-dependent manner (Fig. 5A and B). ULBP4 was not expressed on K562 and K562/GO1 cells before HCQ treatment but the percentage of ULBP4-positive cells began to increase after HCQ exposure for 2 h. After 8 h of HCQ exposure, the percentage of ULBP4-positive cells rose to 52.6±9.7% in K562 cells and 61.4±8.3% in K562/GO1 cells and remained relatively stable for 24 h (Fig. 5A and B). Next, we tested the regulation of ULBP4 synthesis or degradation by HCQ. Western blotting showed that HCQ does not affect the total amount of ULBP4 protein in CML cells (Fig. 5C). To verify this result,

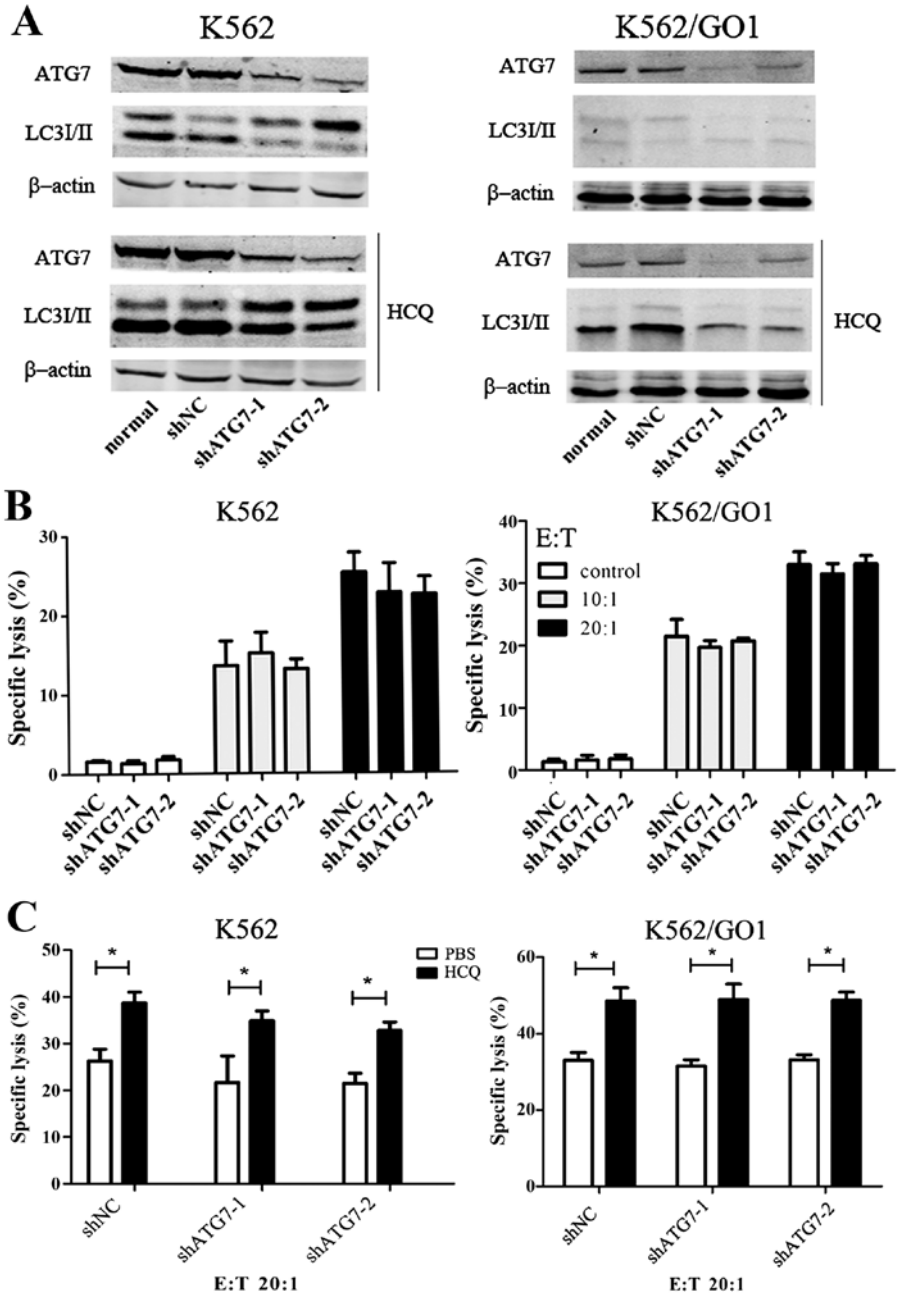

Figure 3. HCQ sensitizes CML cells to Vγ9Vδ2T cell-mediated lysis independent of autophagy. (A) Knockdown of ATG7 in K562 and K562/GO1 were evaluated, and LC3 proteins were tested in these cells to verify the inhibition of autophagy. (B) Vγ9Vδ2 T mediated lysis to K562 and K562/GO1 following ATG7 knockdown at effector target ratio of 20:1. (C) HCQ increased the sensitivity of autophagy-defective K562 and K562/GO1 to Vγ9Vδ2T mediated lysis at effector target ratio of 20:1. Data are presented as mean ± SD (*P<0.05). All experiments were performed in triplicate independently.

we examined ULBP4 expression by flow cytometry after cells were fixed and permeabilized. HCQ exposure increased the expression of ULBP4 on the cell surface but did not affect the total expression of ULBP4 on the cell surface plus that within the cell (Fig. 5D). Furthermore, we found that ULBP4 was not expressed on the cell membrane of HCQ-untreated K562 and K562/GO1 cells, but it accumulated in the cytoplasm (Fig. 5E). These results implied that HCQ may induce ULBP4 translocation from the cytoplasm to the membrane. To confirm this hypothesis, we evaluated the expression of ULBP4 in K562 cells by confocal microscopy. HCQ treatment redistributed ULBP4 from the cytoplasm to the cell membrane. Without HCQ treatment, ULBP4 was distributed throughout the cytoplasm and HCQ treatment relocalized ULBP4 to the membrane (Fig. 5F). Taken together, these findings show that HCQ does not affect ULBP4 synthesis and degradation, but relocates ULBP4 from the cytoplasm to the membrane.

Discussion

HCQ is known to be an effective autophagy inhibitor. However, HCQ has multiple functions (28,40). HCQ suppresses antigen processing and presentation, inhibits prostaglandin and cytokine synthesis, modulates toll-like receptors, and affects matrix metalloproteinase levels in serum (40-42). In the present study, we revealed that HCQ enhances the susceptibility of CML

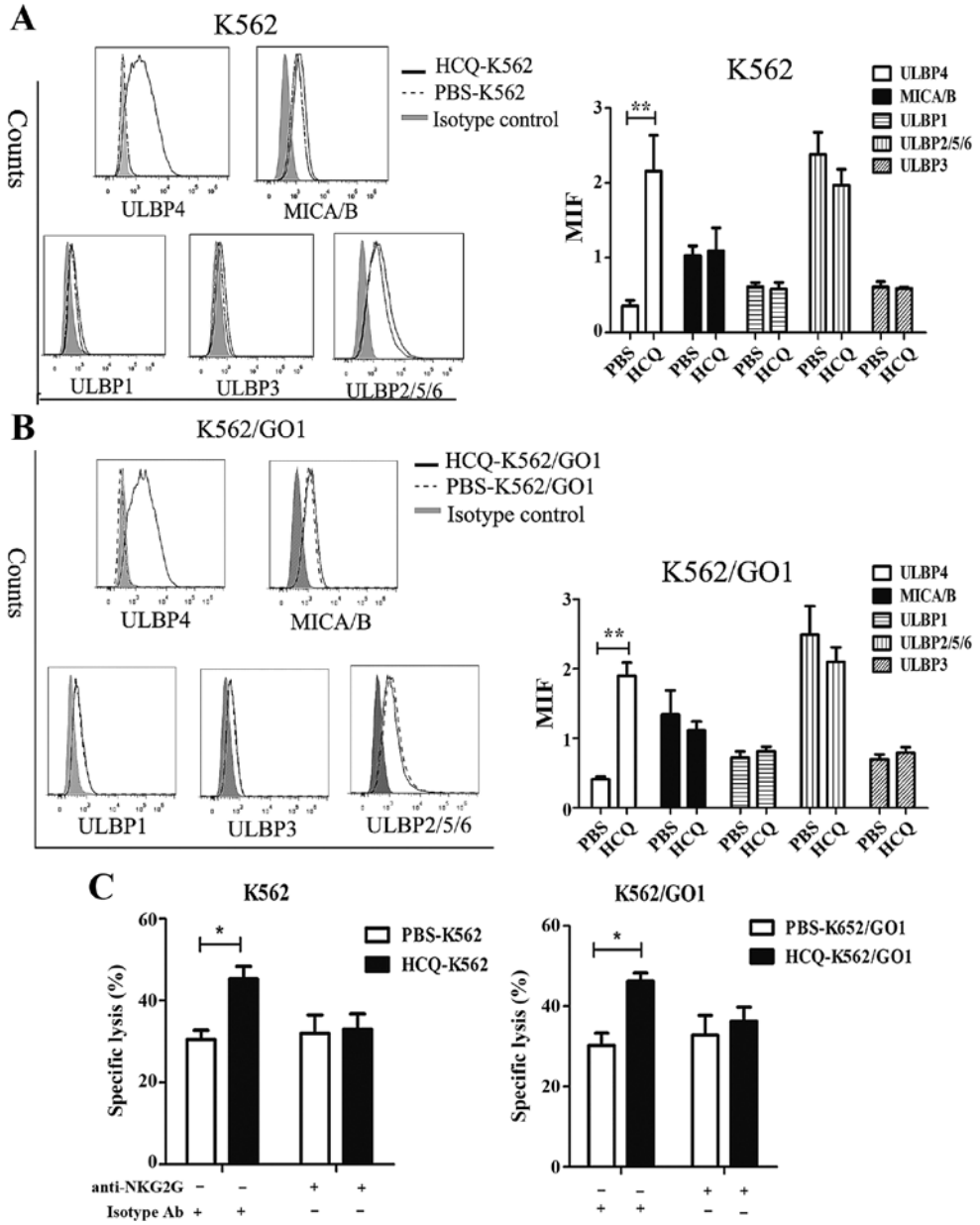

Figure 4. HCQ sensitizes K562 and K562/GO1 by inducing ULBP4 expression on cell membrane. Expression of NKG2D ligands (MICA/B, ULPB1, ULBP2/5/6, ULBP3 and ULBP4) were tested in (A) K562 and (B) K562/GO1 following HCQ treatment; $^{**}P<0.01$. Representative results of 3 independent experiments are shown (left panel), mean fluorescence intensity was expressed as mean ± SD (right panel). (C) Vγ9Vδ2T cells were incubated with 10 μg/ml anti-NKG2D mAb or isotype mAb for 1 h. Then the specific lysis of HCQ- or PBS-pretreated K562 and K562/GO1 by Vγ9Vδ2T cells was tested at E:T ratio of 20:1. Data are presented as mean ± SD ($^{*}P<0.05$).

cells to Vγ9Vδ2 T cell-mediated cytotoxicity. HCQ sensitized imatinib-resistant and imatinib-sensitive CML cells to Vγ9Vδ2 T cell-mediated lysis, and HCQ pretreated CML cells accelerated the degranulation of Vγ9Vδ2 T cells. These results indicate that HCQ combination with immunotherapy may be an effective and safe choice for CML patients, regardless of responsiveness to imatinib.

Autophagy in carcinoma is involved in immunotherapy resistance. Hypoxia-induced autophagy compromised the susceptibility of cancer cells to CTL cytotoxicity (19,43). In breast cancer cells and renal carcinoma cells, hypoxia-induced autophagy selectively degraded NK cell-derived granzyme B and impaired the susceptibility of carcinomas to NK-mediated lysis (18,44). Inhibiting autophagy can enhance the sensitivity of carcinoma cells to NK- and CTL-mediated lysis under hypoxic conditions. We have shown that HCQ can inhibit autophagy in CML cells. However, HCQ sensitizes CML cells to Vγ9Vδ2 T cell-mediated lysis in an autophagy-independent manner. Knockdown of ATG7 effectively blocked autophagy, but did not enhance the sensitivity of CML cells to Vγ9Vδ2 T cells. Furthermore, HCQ increased the sensitivity of autophagy-incompetent CML cell to Vγ9Vδ2 T cell-mediated cytotoxicity. These findings indicated that autophagy is not involved in resistance to Vγ9Vδ2 T cell-mediated lysis in CML cells. We speculate that the level of basal autophagy in CML cells is too low to have an effect. Consistent with this hypothesis, previous studies have demonstrated that BCR-ABL protein, which is a hallmark of CML, can downregulate

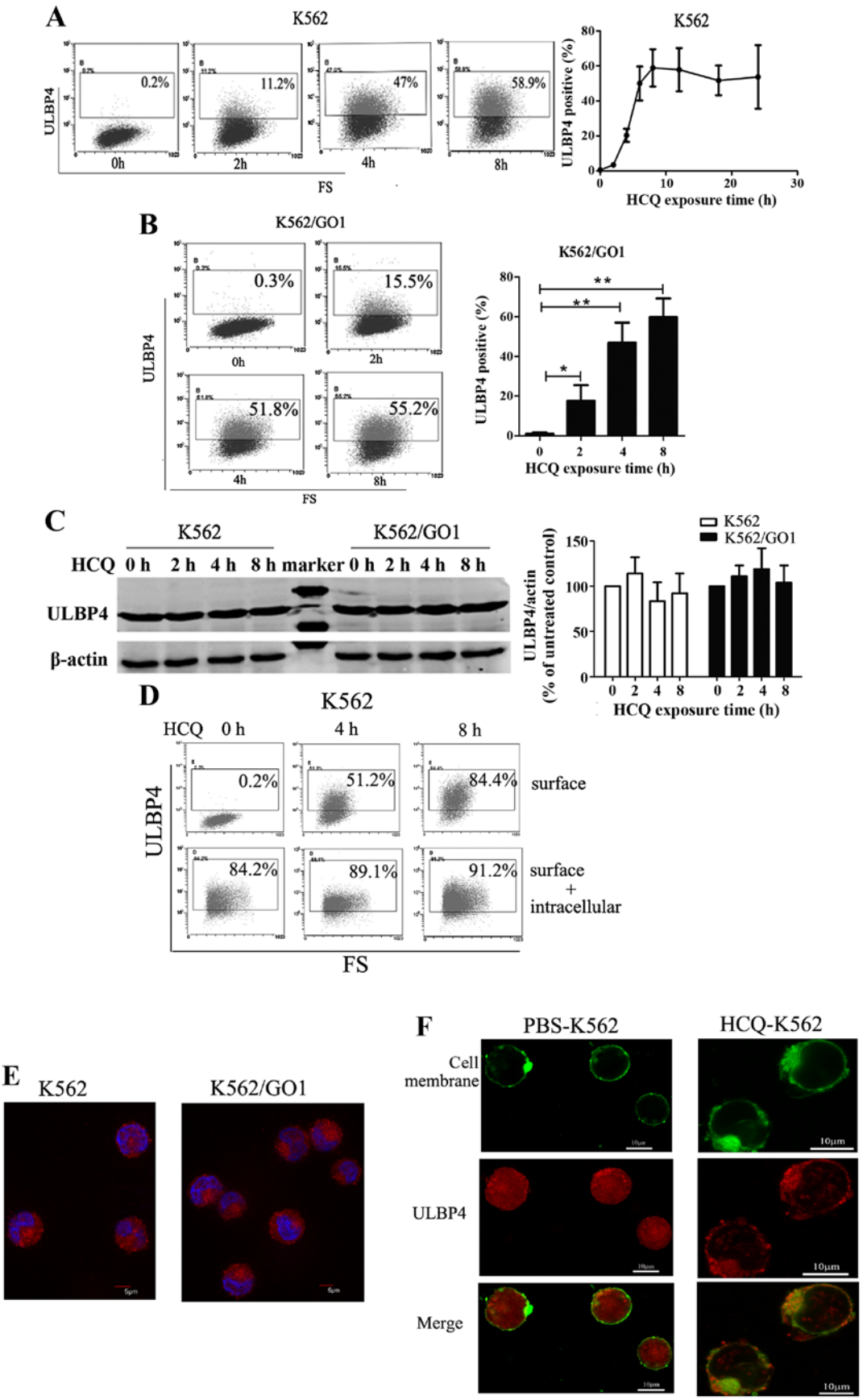

Figure 5. HCQ induces ULBP4 translocation from cytoplasm to CML cell membrane. Expression of ULBP4 was tested in (A) K562 and (B) K562/GO1 following HCQ treatment for indicated time. Representative images of 3 independent experiments are shown (left panel), and ANOVA was performed (right panel; $^{*}P<0.05$ $^{**}P<0.01$. (C) ULBP4 protein in K562 and K562/GO1 was tested with western blot analysis, (D) and the total expression of ULBP4 on membrane and in plasma were tested with flow cytometry. (E) Immunofluorescence was performed to test the expression of ULBP4 in K562 and K562/GO1. DAPI (blue) stain for cell nucleus, Cy3 (red) conjugated secondary antibody for ULBP4. (F) Immunofluorescence was performed to test the location of ULBP4 in K562 treating with PBS (left panel) or HCQ (right panel). Cell membrane was stained with Dio (green) and ULBP4 was stained with Cy3 (red) conjugated secondary antibody.

autophagy in PI3K/AKT/mTORC1-dependent or -independent manner (45,46), and BCR-ABL-expressing cells exhibit low basal autophagy (47).

In addition to TCR-dependent activation, Vγ9Vδ2 T cell activity is tightly regulated by the NK-like active receptor NKG2D (20,48,49). The NKG2D ligands MICA/B and ULBP1-6 are specifically expressed on microorganism-infected cells and various tumor cells. They mark these cells as targets for Vγ9Vδ2 T and other immune cells (50-52). Previous studies have shown that ULBP4, one of NKG2D ligands, plays a key role in γδT cell activation (39). Consistent with this, we have demonstrated that specific lysis of leukemia cells by Vγ9Vδ2 T cells correlates positively with ULBP4 expression on the leukemia cell surface (unpublished data). We tested the expression of NKG2D ligands in CML cells after HCQ treatment. Expression of NKG2D ligands was absent (ULBP4, ULBP1 and ULBP3) or only weakly detected (MICA/B and ULBP2/5/6) in CML cells. HCQ treatment specifically induced the expression of ULBU4 on the membrane of CML cells. Blocking the interaction of NKG2D with its ligands completely deleted the sensitizing effects of HCQ. This result suggests that HCQ sensitizes CML cells to Vγ9Vδ2 T cytotoxicity by inducing ULBP4 expression on the cell membrane. Vγ9Vδ2 T cells recognize tumors via three mechanisms: preferential involvement of the TCR, preferential involvement of NKG2D, or a combination of both (24,50). We showed that blocking interaction of NKG2D with its ligands decreased Vγ9Vδ2 T cell-mediated lysis to HCQ-pretreated CML cells expressing ULBP4 on the membrane, but not in control cells that did not show ULBP4 expression at the membrane (Fig. 4B). This indicates that ULBP4 is major NKG2D ligand in CML cells recognized by NKG2D during Vγ9Vδ2 T cell activation, and Vγ9Vδ2 T cells recognize CML cells pretreated with or without HCQ in different ways. Vγ9Vδ2 T cells may recognize PBS-treated CML cells through preferential involvement of the TCR and recognize HCQ-treated CML cells through a combination of NKG2D and TCR.

NKG2D ligands mark cancer cells for recognition by immune cells, but tumor cells can detain NKG2D ligands intracellularly to evade immune surveillance (53-55). Human melanomas prevent NK cell-mediated cytotoxity through sequestration of MICA in the endoplasmic reticulum (53). ULBP4 was not detected at the membrane of HCQ-untreated CML cells, but it accumulated in the cytoplasm. This may indicate an immune escape mechanism of CML cells to avoid elimination by Vγ9Vδ2 T and NK cells. Fortunately, HCQ can overcome this obstacle and induces ULBP4 expression at the cell surface. Chemotherapy strategies using HCQ promote NKG2D-dependent elimination of CML cells by Vγ9Vδ2 T cells. Recently published studies have emphasized the importance of post-translational regulation in NKG2D ligand synthesis (54-56). The present study reveals that HCQ does not affect the synthesis or degradation of ULBP4 but promotes ULBP4 translocation from the cytoplasm to the cell membrane. However, the mechanisms behind ULBP4 translocation remain elusive. Some studies have demonstrated that N-linked glycosylation regulated the translocation of MICA/B from the cytoplasm to the membrane (57,58). However, we have shown that tunicamycin, a selective inhibitor of N-linked glycosylation, did not abolish HCQ-induced ULBP4 expression (data not shown). This suggests that N-linked glycosylation may not be involved in regulating ULBP4 translocation. Transportation of MICB and ULBP2 from the cytoplasm to the membrane is regulated through endosomal and lysosomal pathways (59,60). Whether these pathways are involved in ULBP4 transportation remains to be elucidated.

Our results uncovered a novel autophagy-independent mechanism of HCQ in CML treatment, and imply that combination treatments with HCQ and Vγ9Vδ2 T cells represent a potential strategy for CML, especially for the relapse and TKI-resistant patients.

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