

Chloroquine enhances human CD8⁺ T cell responses against soluble antigens in vivo

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The presentation of exogenous protein antigens in a major histocompatibility complex class I–restricted fashion to CD8⁺ T cells is called cross-presentation. We demonstrate that cross-presentation of soluble viral antigens (derived from hepatitis C virus [HCV], hepatitis B virus [HBV], or human immunodeficiency virus) to specific CD8⁺ T cell clones is dramatically improved when antigen-presenting dendritic cells (DCs) are pulsed with the antigen in the presence of chloroquine or ammonium chloride, which reduce acidification of the endocytic system. The export of soluble antigen into the cytosol is considerably higher in chloroquine-treated than in untreated DCs, as detected by confocal microscopy of cultured cells and Western blot analysis comparing endocytic and cytosolic fractions. To pursue our findings in an in vivo setting, we boosted groups of HBV vaccine responder individuals with a further dose of hepatitis B envelope protein vaccine with or without a single dose of chloroquine. Although all individuals showed a boost in antibody titers to HBV, six of nine individuals who were administered chloroquine showed a substantial CD8⁺ T cell response to HBV antigen, whereas zero of eight without chloroquine lacked a CD8 response. Our results suggest that chloroquine treatment improves CD8 immunity during vaccination.

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Abbreviations used: CLSM, confocal laser scan microscope; FC, flow cytometry; HBenvAg, hepatitis B envelope antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; i, immature; LB, latex beads; NS3Ag, non-structural 3 antigen; TAP, transporters associated to antigen presentation; VV, vaccinia virus; WB, Western blot.

MHC class I molecules generally present peptides derived from endogenous antigens (i.e., viral, tumor, or self-proteins) that have been synthesized and processed within cells (1). However, some specialized APCs are capable of presenting exogenous antigens to CD8⁺ T cells (2–15). This process, termed cross-presentation (16), is primarily performed by DCs in vivo (2, 17–20). In the steady state, cross-presentation by immature (i) DCs leads to cross-tolerance (21–25). In contrast, upon DC maturation by a variety of innate immunity signals, cross-presentation primes CD8⁺ T cell responses against tumor, virus-infected, apoptotic cells, or those pathogens that do not infect or functionally impair APCs (11–15, 23–32). Two independent, but not necessarily exclusive, pathways have been proposed for explaining cross-presentation, but the revised model of the cytosolic pathway seems to reconcile the endosomal and the conventional cytosolic models (11–15, 28). Accord-

ing to this model, phagosomes would fuse with the ER membrane, thus acquiring both ER molecules (i.e., the protein translocation channel Sec61 and the transporters associated to antigen presentation [TAP]) and, ultimately, the capacity to function as organelles autonomously cross-presenting exogenous antigens via a phagosomal proteasome-/TAP-dependent route (33–36). Although particulate antigens (i.e., those associated with pathogens, immune complexes, apoptotic cells, virus-infected cells, or antigen/heat shock protein complexes [11, 12, 14, 15, 28, 37]) are efficiently cross-presented via this ER-like phagosomal pathway, cross-presentation of soluble antigens is considerably less efficient (11, 12, 14, 15, 28, 38–40), despite their ability to access macropinocytotic compartments with similar ER-like characteristics (33, 41).

We demonstrate that by inhibiting endosomal acidification with lysosomotropic agents (i.e., chloroquine or NH₄Cl), the efficiency of cross-presentation is substantially improved in vitro via an increased export of soluble antigens

The online version of this article contains supplemental material.

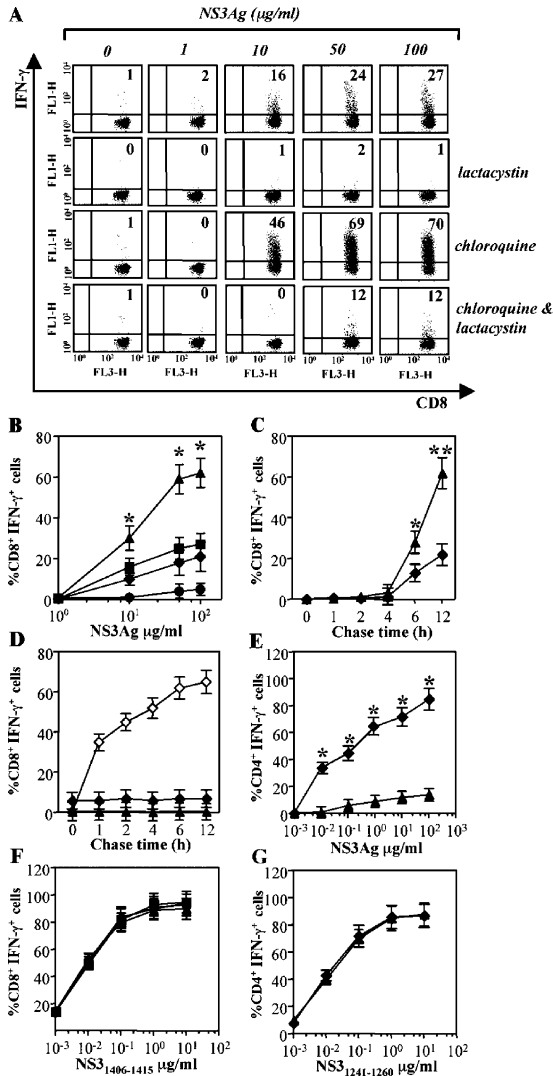


Figure 1. Cross-presentation efficiency is dependent on the inhibition of antigen degradation. (A) FC analysis of an HLA-A2-restricted NS3₁₄₀₆₋₁₄₁₅-specific CD8⁺ T cell clone producing IFN-γ in response to NS3Ag cross-presented by HLA-A2⁺ iDCs, in the presence or absence of the indicated compounds. Dot plots show double CD8 and IFN-γ staining. Results are expressed as percentages of cells and are indicated in each quadrant. One representative out of six different experiments is shown. (B) NS3Ag cross-presentation after 12 h of pulsing by six independent HLA-A2⁺ iDC populations to an NS3₁₄₀₆₋₁₄₁₅-specific CD8 clone (mean ± SD), in the absence (diamonds) or presence of chloroquine (triangles), leupeptin (squares), or lactacystin (circles). *, P < 0.0001 in the presence versus absence of chloroquine. (C) Chase time of 50 μg/ml NS3Ag cross-presentation after 30 min of pulsing by six independent iDC populations (mean ± SD), in the presence (triangles) or absence (diamonds) of chloroquine. *, P = 0.001 and **, P < 0.0001 in the presence versus absence of chloroquine. (D) NS3Ag + chloroquine (triangles), or peptide (open diamonds) presentation by three independent HLA-A2⁺ EBV-B cells to an NS3₁₄₀₆₋₁₄₁₅-specific CD8 clone (mean ± SD). (E) Presentation of NS3Ag (*, P < 0.0001 in the presence versus absence of chloroquine) by four independent DC populations to an NS3₁₂₄₁₋₁₂₆₀-specific CD4 T cell clone (mean ± SD), in the presence (triangles) or absence (diamonds) of chloroquine. (F and G) Presentation of the indicated peptides to the NS3₁₄₀₆₋₁₄₁₅-

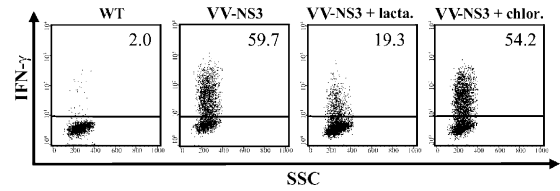


Figure 2. Efficient direct presentation of endogenous NS3Ag. FC analysis of 2×10^4 HLA-A2⁺ NS3₁₄₀₆₋₁₄₁₅-specific CD8⁺ T cell clones producing IFN-γ in response to endogenous NS3Ag directly presented by 2×10^4 autologous EBV-B cells that had been infected by either 5 PFU/cell of WT VV or NS3Ag-expressing VV (VV-NS3), in the presence or absence of lactacystin (lacta.) or chloroquine (chlor.). Dot plots show IFN-γ staining. Results are expressed as percentages of cells and are indicated in each quadrant. One representative out of three different experiments is shown. SSC, side scatter.

into the cytosol of DCs. The importance of these results encouraged us to carry out an in vivo study, which proved that a short course of chloroquine treatment followed by a booster dose of anti-hepatitis B virus (HBV) vaccine enhanced antigen-specific CD8⁺ T cell responses in HBV-vaccinated healthy individuals. Our data imply that this well-tolerated antimalarial drug can be used for the design of innovative vaccines aimed at eliciting protective CD8 immunity in humans.

RESULTS

Cross-presentation of soluble antigens is up-regulated by inhibitors of intravesicular acidification in vitro

We devised a system in which human HLA-A2⁺ iDCs cross-presented soluble r nonstructural 3 antigen (rNS3Ag) of hepatitis C virus (HCV) to an HLA-A2-restricted NS3₁₄₀₆₋₁₄₁₅-specific CD8⁺ T cell clone (Fig. 1). Cross-presentation of soluble NS3Ag (Fig. 1, A and B), but not NS3₁₄₀₆₋₁₄₁₅ peptide presentation (Fig. 1 F), was drastically blocked by lactacystin, a potent proteasome inhibitor (11–15, 28). This supports the idea that soluble NS3Ag cross-presentation requires proteasome-dependent processing. Surprisingly, cross-presentation efficiency improved when iDCs were pulsed in the presence of chloroquine (Fig. 1, A–C) or NH₄Cl (not depicted), whereas the antigen presentation to CD4⁺ T cells was strongly inhibited (Fig. 1 E). Notably, lactacystin drastically blocked cross-presentation of soluble antigens, even in the presence of chloroquine (Fig. 1, A and B). Chloroquine did not affect the peptide presentation of DCs to either CD8⁺ or CD4⁺ T cells (Fig. 1, F and G), ruling out the possibility that it improved cross-presentation by up-regulating the costimulatory capacities of DCs. Both chloroquine and NH₄Cl are lysosomotropic agents that diffuse across membranes and inhibit intravesicular acidification, which is critical to activate several acid proteases that induce proteolysis of antigens in the endocytic compart-

specific CD8 clone (F) or the NS3₁₂₄₁₋₁₂₆₀-specific CD4 T cell clone (G) in the absence (diamonds) or presence of chloroquine (triangles) or lactacystin (circles).

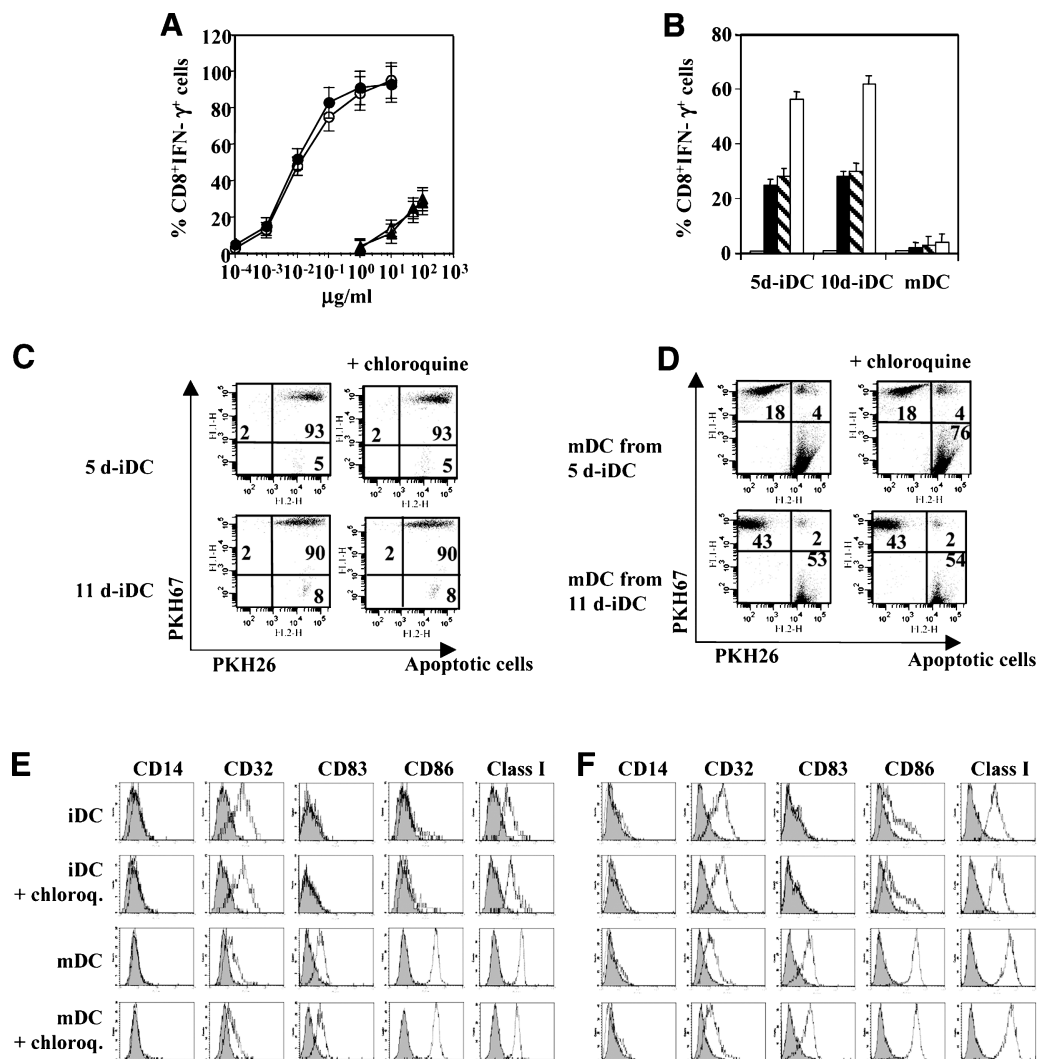


Figure 3. Cross-presentation and its improvement are restricted to iDCs. (A) IFN- γ production by an HLA-A2-restricted NS3₁₄₀₆₋₁₄₁₅^T-specific CD8 T cell clone in response to either four independent HLA-A2⁺ early (day 5; closed circles and triangles) or late (day 11; open circles and triangles) iDC populations cross-presenting either NS3Ag (triangles) or peptide (circles). Values represent mean \pm SD. (B) IFN- γ production by an NS3₁₄₀₆₋₁₄₁₅-specific CD8 T cell clone in response to four independent HLA-A2⁺ 5-d iDCs, 10-d iDCs, or mDCs, which had been unpulsed (dotted bars) or pulsed with 50 μ g/ml NS3Ag alone (closed bars), in the presence of leupeptin (hatched bars) or chloroquine (open bars). Values represent mean \pm SD. (C and D) Capacity of PKH67 (green)-dyed 5-d iDCs, PKH67

(green)-dyed 11-d iDCs (C), or PKH67 (green)-dyed mDCs (derived from either 5- or 11-d iDCs; panel D) to phagocytose PKH26 (red)-apoptotic cells as detected by FC. Phagocytosis was assessed as DCs double positive for apoptotic cells. The numbers of cells in C and D were similar. One representative out of three different experiments is shown. (E and F) Surface phenotype analysis of 5-d iDCs, mDCs derived from 5-d iDCs (E), 11-d iDCs, or mDCs derived from 11-d iDCs (F), in the presence or absence of chloroquine. Shaded histograms represent the isotype-matched control mAbs; open histograms represent stainings with mAbs specific to the surface molecules indicated in the figure. One representative out of three different experiments is shown.

ments (14, 42). Furthermore, it has been reported that chloroquine causes direct lysosomal membrane permeabilization, with the subsequent release of lysosomal products (i.e., cathepsins) from the lysosomal lumen into the cytosol (43). In contrast, only a slight, or no, improvement in cross-presentation was observed with compounds that inhibit various proteases without interfering with the pH level, such as leupeptin (Fig. 1 B), pepstatin A, EDTA, or peflabloc (unpublished data). Further studies are required to verify whether

this finding is caused by the protease inhibitors used not being readily membrane permeable (as opposed to chloroquine and NH₄Cl), which may thus cause them to accumulate at lower concentrations inside cells. Nonprofessional APCs, such as autologous EBV-transformed B cells (EBV-B cells; Fig. 1 D), were unable to cross-present even in the presence of chloroquine. In addition, EBV-B cells, but not iDCs, were unable to internalize 40-kD FITC-conjugated (FITC)-dextran, which is known to be efficiently captured via man-

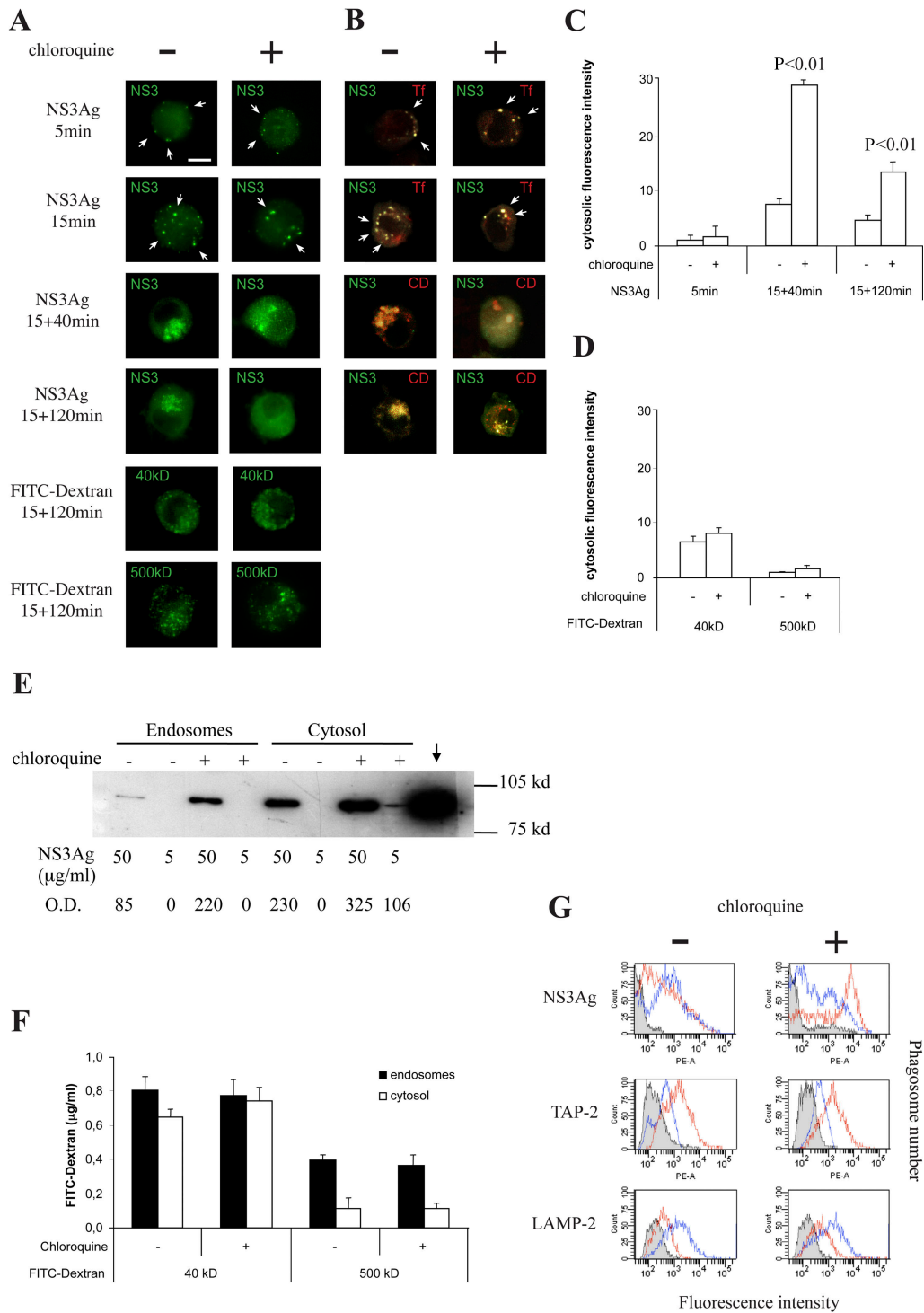


Figure 4. Improvement of soluble antigen export from endosomes into cytosol in chloroquine-treated iDCs. (A and B) iDCs were pulsed/chased with either NS3Ag alone or NS3Ag and TRITC-Tf, in the presence or absence of chloroquine, fixed, and stained at different times with human anti-NS3 alone or with anti-NS3 + rabbit anti-cathepsin D (C and D), followed by staining with the appropriate secondary antibodies. The signal of NS3 alone is green. The signals of double staining of Tf or CD and NS3 are red and green, respectively. The extent of colocalization is shown in yellow after merging. Arrows indicate NS3 enrichment or colocalization

with Tf or CD. In control experiments, DCs were incubated for 1 h at 37°C in the presence of 1 mg/ml FITC-dextran with the indicated molecular masses and then fixed. One representative out of three different experiments is shown. (C) Quantitative analysis of the fluorescence intensity of cytosolic NS3Ag in iDCs pulsed/chased with NS3Ag in the presence or absence of chloroquine. (D) Quantitative analysis of the fluorescence intensity of cytosolic FITC-dextran in iDCs pulsed/chased with FITC-dextran of the indicated molecular masses, in the presence or absence of chloroquine. (E) One representative out of three WB analyses on both endocytic- and

nose receptors by DCs (unpublished data) (44–47). Thus, the low cross-presentation efficiency by EBV–B cells could be caused by their lower antigen uptake capacity, which precludes the possibility of internalizing the high amounts of soluble antigens generally required for cross-presentation (37–40). Control experiments showed that chloroquine improved cross-presentation of soluble NS3Ag in a dose-dependent fashion (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20051106/DC1>), which further supports the data above. In contrast, bafilomycin A1, which increases the endosomal pH (via its capacity to inhibit the vacuolar proton ATPase) (48) but not membrane permeabilization (43), did not improve, but instead inhibited, cross-presentation by DCs (Fig. S2). Experiments simultaneously performed with those shown in Fig. 1 revealed that cross-presentation (Fig. 1, A and B) was less efficient than the direct presentation of NS3Ag, endogenously-synthesized by APCs, to the same NS3_{1406–1415}-specific CD8⁺ T cell clone (Fig. 2). This suggests that endogenous, more than exogenous, soluble antigens have preferential access to proteasome-dependent processing. Further control experiments confirmed that chloroquine substantially improved cross-presentation of hepatitis B envelope antigen (HBenvAg) to an HLA–A2–restricted HBenvAg_{213–221}-specific CD8⁺ T cell clone by HLA–A2⁺ DCs and that lactacystin drastically inhibited cross-presentation (Fig. S3).

Professional APCs are required for cross-presentation (and its improvement)

iDCs, taken up at day 5 or 10–11 of culture with GM-CSF and IL–4, maintained similar cross-presentation (Fig. 3, A and B), stimulation (as detected by their capacity to present the high affinity HLA–A2–binder NS3_{1406–1415} peptide; Fig. 3 A), or phagocytosis capacity (Fig. 3, C and D), as well as surface phenotype (Fig. 3, E and F), over time in vitro (46). Cross-presentation efficiency was confirmed as being substantially improved when both early and late iDCs were pulsed in the presence of chloroquine but not leupeptin (Fig. 3 B). The finding that iDCs were better at cross-presenting antigens than completely matured DCs (taken up 40 h after addition of the maturational CD40L stimulus in the iDC culture; Fig. 3 B) is most likely caused by the low capacity to internalize antigens via both phagocytosis (Fig. 3, C and D) and macropinocytosis (as detected by FITC–dextran uptake; not depicted) of the latter (44–46). Chloroquine did not affect phagocytosis (Fig. 3, C and D) or surface phenotype (Fig. 3, E and F) by/of DCs, and NS3Ag alone was totally incapable of inducing DC maturation in vitro (Fig. S4, avail-

able at <http://www.jem.org/cgi/content/full/jem.20051106/DC1>), ruling out the possibility that cross-presentation improvement was caused by either chloroquine- or NS3Ag-dependent artifacts. The specificity of cross-presentation was supported by the finding that the CD8 cell response was HLA–A2 restricted even when DCs were treated with chloroquine (Fig. S5).

Confocal microscopy evaluation of soluble antigen trafficking in DCs

To support the hypothesis that chloroquine increases the export of soluble antigens from endosomes to cytosol, NS3Ag trafficking was evaluated using a confocal laser scan microscope (CLSM) in whole DCs or EBV–B cells. After the initial 5–15 min of pulsing with NS3Ag, both untreated iDCs and the chloroquine-treated iDCs revealed colocalization of NS3Ag with transferrin (Tf) R (a marker of early endosomes; Fig. 4, A and B). After 40 min of chase, the majority of NS3Ag migrated from TfR⁺ into cathepsin D⁺ (a marker of late endosomes) compartments, and only a tiny part was exported into the cytosol in untreated DCs (Fig. 4 B). Conversely, chloroquine treatment considerably increased NS3Ag export into cytosol in the later phases, and only a small amount of antigen was colocalized with cathepsin D in late endosomes (Fig. 4 B). The magnitude of fluorescence intensity in cytosolic NS3Ag was much greater in chloroquine-treated than untreated DCs, after both 40 and 120 min of chase (Fig. 4 C). In control experiments, DCs were pulsed with different molecular masses of FITC–dextran. Dextran is nondegradable molecules that are internalized via mannose receptors and transported into cytosol in a size-dependent fashion (49). Only the 40-kD FITC–dextran, not the 500 kD, was visualized in the cytosol by confocal microscopy, irrespective of the presence or absence of chloroquine (Fig. 4, A and D). This finding rules out the possibility that the improvement in cytosolic NS3Ag export was caused by a trivial endosomal membrane rupture by chloroquine. Consistent with their incapacity to cross-present, both chloroquine-treated and untreated EBV–B cells showed only a low NS3Ag export from the endocytic compartments into the cytosol (Fig. S6, available at <http://www.jem.org/cgi/content/full/jem.20051106/DC1>).

Biochemical analyses of soluble antigen trafficking in DCs

To confirm the evidence that chloroquine favors the export of soluble antigens from endosomes into cytosol, we performed Western blot (WB) analyses of both cytosol- and endocytic-enriched preparations from DCs that had been pre-

cytosol-enriched preparations, purified from iDCs, which had been previously pulsed/chased (20'/90') with the indicated concentrations of NS3Ag, in the presence or absence of chloroquine. The two preparations were then separated by SDS-PAGE and immunoblotted for NS3Ag with the relevant human mAb and a secondary peroxidase-conjugated goat anti-human IgG antibody. NS3Ag alone (↓) was used as positive control. (F) Fluorometric analyses of different FITC–dextran in endocytic or

cytosolic fractions from iDCs pulsed with the indicated FITC–dextran in the presence or absence of chloroquine. (G) One representative kinetic out of four detected by FC of NS3Ag, TAP-2, or LAMP-2 staining in phagosomes containing a mixture of soluble NS3Ag and LB. Analyses were performed on gated phagosomes containing LB. Isotype control, green; staining after 20' of pulsing, red; staining after 20' of pulsing + 90' of chase, blue.

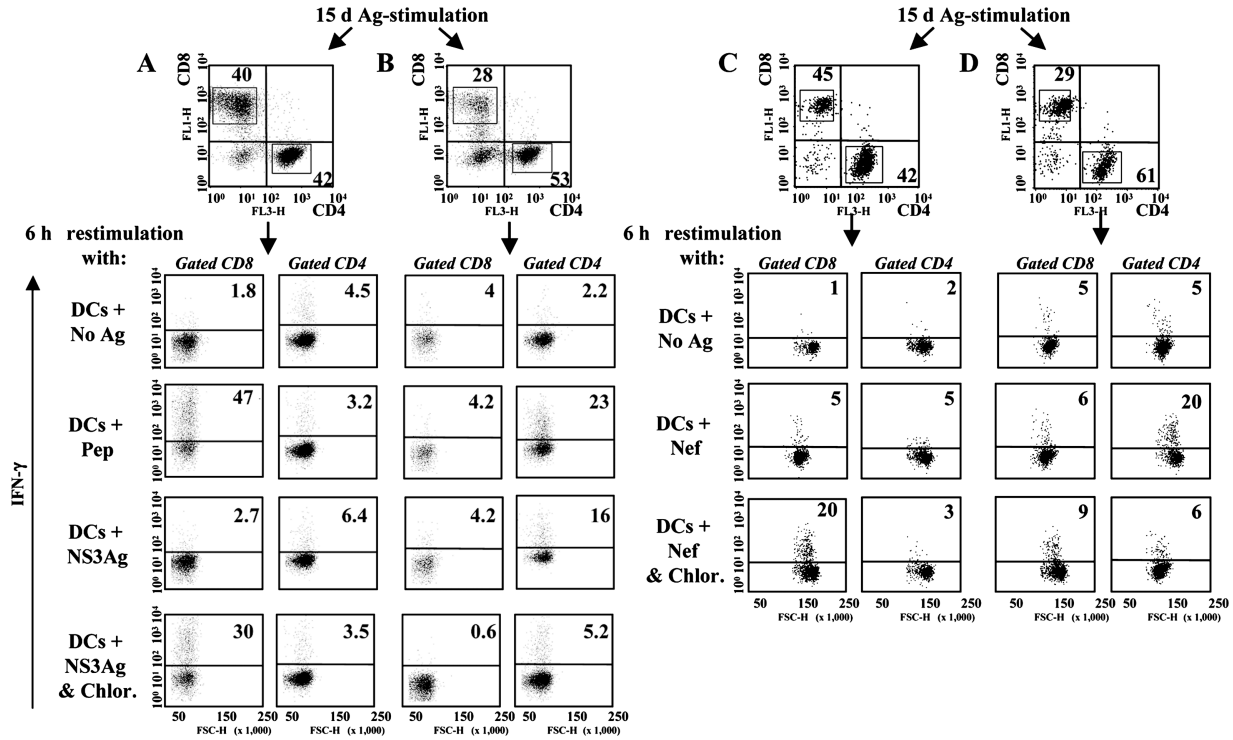


Figure 5. Chloroquine-boosted cross-presentation elicits antigen-specific CD8⁺ T cells ex vivo. (A and B) Double CD4 and CD8 FC staining in PBMCs from one representative HLA-A2⁺ HCV-infected individual out of three studied on 15-d stimulation with NS3Ag in the presence (A) or absence (B) of chloroquine. PBMCs were then restimulated (6 h) with autologous DCs, which had been pulsed or not as indicated, and tested for IFN- γ production. The NS3₁₄₀₆₋₁₄₁₅ peptide was used for detecting the CD8 cell-dependent IFN- γ production, whereas the NS3₁₂₄₁₋₁₂₆₀ peptide was

used for detecting the CD4 cell-dependent IFN- γ production. (C and D) Double CD4 and CD8 FC staining in PBMCs from one representative HIV-infected individual out of three studied on 15-d stimulation with 50 μ g/ml Nef in the presence (C) or absence (D) of chloroquine. Cells were then restimulated with autologous iDCs, which had been pulsed or not as indicated, and tested for IFN- γ production. Dot plots are gated on CD8 or CD4 cells and show IFN- γ staining. Results are expressed as percentages of IFN- γ ⁺ cells and are indicated in each quadrant.

viously pulsed/chased with NS3Ag, in the presence or absence of chloroquine. After 60–120 min of chase, chloroquine increased antigen accumulation in both the endocytic and cytosolic fractions (Fig. 4 E). The chloroquine effect at the level of the cytosolic fractions was particularly evident at the lowest antigen concentrations (5 μ g/ml) used, which were visible only in the chloroquine-treated DCs (Fig. 4 E). In control experiments, the cytosolic and endocytic fractions derived from DCs incubated with 40- or 500-kD FITC-dextran, in the presence or absence of chloroquine, were analyzed by a fluorometer. After 1 h of incubation, the 40-kD FITC-dextran was found in both endocytic and (more importantly) cytosolic fractions in considerably higher amounts than the 500-kD FITC-dextran, irrespective of the presence of chloroquine (Fig. 4 F). This finding further corroborates the interpretation that the greater antigen accumulation in cytosolic fractions of chloroquine-treated DCs (as detected by WB analysis; Fig. 4 E) was not determined by an endosomal membrane rupture by chloroquine. In addition, flow cytometry (FC) analysis was performed on phagosomes isolated from iDCs (50, 51), which had been previously pulsed and chased with a combination of soluble NS3Ag and latex

beads (LBs) in the presence or absence of chloroquine. In the early phases, the expression of NS3Ag was substantially higher in phagosomes from chloroquine-treated DCs, confirming that chloroquine also increases antigen accumulation in early phagosomes (Fig. 4 G). In contrast, chloroquine treatment did not affect the kinetics of expression of either phagosomal TAP-2 (as a marker of early phagosomes) (33–36) or LAMP-2 (as a marker of late phago/endosomes), suggesting that it does not interfere with the phago/endosome maturation process (Fig. 4 G).

Chloroquine elicits antigen-specific CD8⁺ T cells ex vivo

To verify whether chloroquine might expand memory CD8⁺ T cell responses ex vivo, PBMCs isolated from three HCV- (52) and three HIV-infected patients were cultured in the presence or absence of chloroquine with rNS3Ag or rNef, respectively, in IL-2-conditioned medium for 15 d. Under conditions in which PBMCs were first elicited with NS3Ag in the presence (Fig. 5 A), but not in the absence, of chloroquine (Fig. 5 B), a noteworthy expansion of CD8⁺ (but not of CD4⁺) T cells was observed. More important, CD8⁺ T cells, which were primarily elicited with NS3Ag in

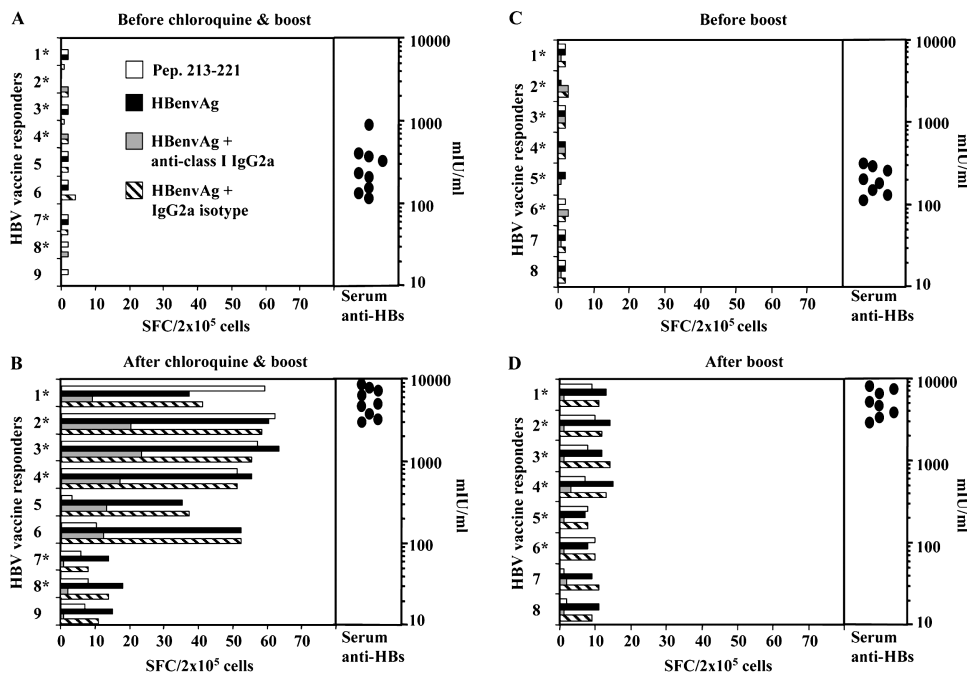


Figure 6. Chloroquine-boosted cross-presentation elicits antigen-specific CD8⁺ T cells in vivo. ELISPOT assays on fresh CD8⁺ T cells isolated from PBMCs of high responders to HBV vaccine, performed before (A) and after (B) chloroquine treatment and anti-HBV vaccine boost, or before (C) and after (D) only the anti-HBV vaccine boost. Fresh CD8⁺ T cells were tested for their capacity to form IFN- γ spots within 6 h of contact with autologous irradiated APCs, which had been previously pulsed or not with entire HBenvAg plus chloroquine or HLA-A2-binding HBenvAg₂₁₃₋₂₂₁ peptide, in the presence or absence of blocking anti-HLA-A,B,C mAb or the corresponding isotype antibody, as indicated in A. Results are expressed as spot-forming

cells (SFC) in 2×10^5 CD8⁺ T cells. The SFC values were subtracted by background, which was below 10 SFC in 2×10^5 cells in each test. SFC values obtained in response to the entire HBenvAg were significantly higher in chloroquine-treated than in untreated high responders ($P < 0.001$), as well as those obtained in response to the HLA-A2-binder HBenvAg peptide ($P < 0.05$) calculated among the HLA-A2⁺ high responders. The numbers with asterisks represent HLA-A2⁺ individuals. Histograms, attached to the right of each panel, report serum anti-HBenvAg (anti-HBs) antibody levels determined before and after the anti-HBV vaccination boost in all individuals studied (either chloroquine-treated or untreated).

the presence of chloroquine (Fig. 5 A), produced large amounts of IFN- γ in response to a secondary stimulation with the relevant NS3₁₄₀₆₋₁₄₁₅ peptide plus autologous APCs. Under these conditions, the generation of antigen-specific CD4⁺ T cells was completely abrogated. It is noteworthy that CD8⁺ T cells efficiently responded to a secondary stimulation with the entire NS3Ag only when the latter was cross-presented by chloroquine-treated DCs (Fig. 5 A). Meanwhile, stimulation of the NS3Ag-specific CD8⁺ T cell clone, used in the cross-presentation assays shown in Figs. 1 and 3 and Figs. S1, S2, and S5 above, was already obtained in steady state cross-presentation conditions and was considerably augmented by adding chloroquine. Furthermore, the short-term NS3Ag-specific CD8⁺ T cell line, shown in Fig. 5, responded to the same peptide concentration several folds less than the clone used in the cross-presentation assays (see Fig. 1 F and Fig. S2). This means that the efficiency of CD8⁺ T cell stimulation by cross-presentation is not only dependent on the capacity of APCs to cross-present but also on the affinity/avidity of CD8⁺ T cells. In contrast, PBMCs, which were first antigen stimulated in the absence of chloroquine (Fig. 5 B), only generated CD4⁺ T cells capable of

recognizing (on a secondary stimulation) both the entire NS3Ag and the MHC class II-restricted promiscuous NS3₁₂₄₁₋₁₂₆₀ epitope. The response of the same antigen-specific CD4⁺ T cells to a secondary stimulation with the entire NS3Ag in the presence of chloroquine was strongly inhibited. Comparable results were obtained by Nef stimulation of PBMCs from HIV-infected patients (Fig. 5, C and D).

Chloroquine elicits antigen-specific CD8⁺ T cells in vivo

Finally, we wondered whether chloroquine might elicit memory CD8⁺ T cell responses in vivo. PBMCs of healthy individuals vaccinated with the hepatitis B envelope protein have been previously shown to contain HBenvAg-specific CD8⁺ T cells (10, 53, 54). This finding is one of the first to evidence that exogenous viral proteins can enter the class I processing pathway (55-58) and that the “class I/II discrimination” paradigm is not necessarily a rule (59). These data served as a basis for the experiments reported here, which were designed to elucidate the role of chloroquine in improving the generation and expansion of HBenvAg-specific CD8⁺ T cell responses in vivo. Thus, we selected 17 healthy individuals who had efficiently responded to an anti-HBV

vaccination protocol 8–10 yr ago, in terms of both specific antibody and T cell responses (high responders). In particular, nine of them received a brief chloroquine treatment per os, followed by a booster dose of anti-HBV vaccine, whereas eight underwent the same vaccine boost without the chloroquine treatment (controls). The majority of individuals studied (13/17) were HLA-A2⁺ in order to minimize the possible diversity of antigen presentation caused by the HLA polymorphism (Fig. 6). A noteworthy expansion of HBenvAg-specific effector CD8⁺ T cells was detected by ELISPOT assay in six of the nine high responders who received both chloroquine treatment and the booster dose of anti-HBV vaccine (Fig. 6, A and B), but was not observed in the controls (Fig. 6, C and D). The generation of antigen-specific CD8⁺ T cell responses after the anti-HBV vaccination boost plus the chloroquine treatment was corroborated by an efficient CD8⁺ response to the immunodominant HLA-A2-related HBenvAg_{213–221} epitope in the HLA-A2⁺ individuals (Fig. 6, A and B). These data suggest that chloroquine can enhance the antigen-specific CD8⁺ T cell response to a soluble antigen *in vivo*. In addition, serum anti-HBenvAg antibody levels were not affected by chloroquine treatment (Fig. 6), suggesting that although chloroquine is not detrimental to the generation of strong antibody responses, it nonetheless exerts a specific effect on cross-presentation rather than a general adjuvant effect.

DISCUSSION

The most important finding of this paper is that we demonstrate for the first time that human CD8⁺ T cell responses can be efficiently enhanced *in vivo* with a short course of treatment with chloroquine followed by a booster dose of a soluble antigen immunization. Furthermore, we provide evidence that lysosomotropic agents (i.e., chloroquine or NH₄Cl) increase the cross-presentation efficiency in our model *in vitro*. The most probable explanation of this finding is that, in the presence of the lysosomotropic agents, internalized antigens are less efficiently degraded, accumulate to a higher level in the endosomes, and are ultimately more efficiently exported into cytosol, as indicated by CLSM and WB analyses. The finding that cross-presentation was not enhanced by bafilomycin A1 or various protease inhibitors supports the hypothesis that the exclusive capacity of the lysosomotropic agents to induce membrane permeabilization (43), in concert with the capacity to inhibit the endosomal acidification and, thus, antigen degradation, allows an increased and rapid export of nondegraded antigens from the endosomes into cytosol. Recent evidences demonstrated that cross-presentation of soluble antigens that are captured by DCs via macropinocytosis requires direct delivery into the perinuclear ER, where they can access the ER-dependent class I processing machinery, precluding their destruction by endo/lysosomal proteolysis (41). Whether the improved cross-presentation by chloroquine is determined by increased delivery into perinuclear ER (41) remains an interesting and relevant issue that requires further study. None-

theless, our data highlight that the efficiency of cross-presentation seems directly related to the level of antigen escape from endocytic processing (60–62). Recently, it has been suggested that DCs accumulate and process antigens for presentation to T cells more efficiently than macrophages because DCs contain considerably lower levels of lysosomal proteases, which rapidly destroy internalized antigens (63). However, even if limited, the lysosomal degradation capacity of DCs may be sufficient to restrain cross-presentation of soluble antigens. This hypothesis is supported by our finding that chloroquine improves cross-presentation of soluble antigens by DCs and by the well-known evidence that internalization of soluble antigens only results in cross-presentation at very high concentrations of antigen (11, 12, 14, 15, 28, 38). In contrast, the efficient cross-presentation of particulate antigens (11, 12, 14, 15, 28, 37) may be explained by the phagocytosis uptake of particulate antigens being more efficient than fluid-phase uptake of soluble antigens (12, 38, 64). Alternatively, the evidence that phagosomes export proteins into the cytosol more efficiently than endosomes may explain the efficient cross-presentation of particulate antigens (33–36, 65, 66), which possibly require minimal degradation (by the partial protease repertoire of DCs [63]) to be cross-presented (49). This may account for previous observations reporting that chloroquine did not affect or even inhibit cross-presentation of particulate antigens (49, 67). The contradictory results between our study demonstrating that chloroquine enhances CD8⁺ T cell responses against soluble antigens both *in vitro* and *in vivo* in humans and findings obtained in various mouse models (66, 68) may be partially explained by differences between human and mouse models (69) and/or by the different DC populations used. It is difficult to establish whether chloroquine enhanced antigen-specific CD8⁺ T cell responses in HBV-vaccinated individuals *in vivo* via its capacity to improve cross-presentation, as our *in vitro* studies indicated. However, putting together the latter with the indication that chloroquine reduces protein degradation in human beings *in vivo* (70), we are tempted to hypothesize that the mechanism of chloroquine in our vaccination trials might be that of improving cross-presentation via the inhibition of antigen degradation.

On the basis of data obtained with three different soluble proteins (NS3Ag, Nef, and HBenvAg), we can, on the other hand, deduce that the endosomal processing machinery plays a critical role in limiting cross-presentation of soluble antigens. The endosomal processing machinery may thus represent a valid checkpoint that contains excessive autoimmune CD8⁺ T cell responses against soluble self-antigens. In contrast, the high cross-presentation efficiency of DCs reported for particulate antigens will provide either control of infections or an efficient tolerance toward inert apoptotic cells in physiological conditions.

Finally, our observations may represent a major breakthrough in “vaccinology.” In general, conventional vaccines (constituted by soluble proteins) used in humans are unable to prime efficient CD8⁺ T cell responses. The evidence that

chloroquine favors strong CD8⁺ T cell responses in vivo suggests that it could be exploited for the design of innovative strategies eliciting protective CD8 immunity.

MATERIALS AND METHODS

Antigens. The rNS3Ag_{187–1465} expressed in *Escherichia coli* and affinity purified was purchased from Biodesign International. The rHBenvAg (ad subtype), containing the preS1_{15–52}, the preS2_{133–145}, and the entire S sequences, was obtained from GlaxoSmithKline Biologicals. The full-length rNef_{1–206} protein of HIV-1 was expressed in *E. coli* and purified as previously described (71).

Cell preparation and surface phenotype. PBMCs were isolated, and antigen-specific T cell clones were generated as previously described (10). CD8⁺ cells were purified from PBMCs by positive selection with anti-CD8 mAb coupled to magnetic beads (Miltenyi Biotec). FACS analysis demonstrated >99% CD8⁺ cells in the positively purified population and <5% in the CD8-depleted population. iDCs were derived from peripheral monocytes, which had been purified by positive selection with anti-CD14 mAb coupled to magnetic beads. CD14⁺ cells were then cultured for 5 or 10–11 d in RPMI 1640 with 5% FCS medium containing 2 mM glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 50 µg/ml kanamycin (GIBCO BRL), 50 ng/ml GM-CFS (Novartis Pharma), and 1,000 U/ml rIL-4 (provided by A. Lanzavecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland). Mature DCs were obtained by a 40-h stimulation of iDCs with CD40L-transfected J558L cells (DC/J558L ratio = 1:1) provided by P. Lane (University of Birmingham Medical School, Birmingham, UK), as previously described (13, 46). Both iDCs and mDCs were stained with anti-CD14, anti-CD86 (Caltag Laboratories), anti-CD32, anti-CD83 (Becton Dickinson), or anti-MHC class I molecule (Serotec, Ltd.) mAbs, followed by staining with the appropriate secondary labeled antibodies (BD Biosciences), and were analyzed by a flow cytometer (FACSCalibur; Becton Dickinson) using CellQuest software (Becton Dickinson).

DC phagocytosis. The phagocytosis capacity of DCs was evaluated according to methods described previously (72). In brief, activated T cells (derived from a CD8 clone) were dyed red with PKH26 (Sigma-Aldrich), induced to undergo Fas-mediated apoptosis as previously described (13), and co-cultured with iDCs or mDCs, which were dyed green with PKH27 (Sigma-Aldrich) at a 1:1 ratio. After 12 h at 4°C or 37°C, phagocytosis was detected as double-positive cells by FC.

Cross-presentation and antigen stimulation assays. DCs or EBV-B cells, as APCs, were pulsed with increasing concentrations of soluble antigen or peptide in the presence or absence of 10 µg/ml chloroquine, NH₄Cl, leupeptin, 2 ng/ml pepstatin A, 10 mM EDTA, 1 mM penicillin, 80 µM lactacystin (Sigma-Aldrich), or bafilomycin A1 (Qbiogene), fixed with 0.05% glutaraldehyde (Sigma-Aldrich), and co-cultured with 2–3 × 10⁴ cells/well of antigen-specific CD8⁺ or CD4⁺ T cell clones for 6 h at 37°C. At hour 2, 10 µg/ml Brefeldin A (Sigma-Aldrich) was added. Cells were stained with tricolor-labeled anti-CD8 (Caltag) or PE-cyanin 7 (PC7)-labeled anti-CD4 (Beckman Coulter), processed, and analyzed for detecting the intracellular IFN-γ with FITC-labeled anti-IFN-γ (BD Biosciences) by FC, as previously described (52). Negative controls were obtained by staining cells with an irrelevant isotype-matched mAb. In some experiments, the direct presentation of endogenously synthesized NS3Ag to an NS3_{1406–1415}-specific CD8⁺ T cell clone was determined by using 2 × 10⁴ autologous EBV-B cells as APCs, which had been infected by either 5 PFU/cell of WT vaccinia virus (VV) or NS3Ag-expressing VV (VV-NS3) in the presence or absence of lactacystin or chloroquine.

Ex vivo experiments. In the ex vivo experiments, whole PBMCs from HCV- or HIV-infected individuals were stimulated with 50 µg/ml rNS3Ag or rNef in the presence or absence of 10 µg/ml chloroquine. 50 U/ml rIL-2 was added after both 5 and 10 d. After a further 5 d, live cells were stained with FITC-labeled anti-CD8 (Caltag) and PC7-labeled anti-

CD4 (Beckman Coulter). A tiny fraction of them was promptly analyzed for both CD4 and CD8 expression by FC. The residual fraction was restimulated with 10 µg/ml antigen or peptide and autologous DCs for 6 h and analyzed for detecting intracytoplasmic IFN-γ in both CD4 and CD8 populations by FC (52).

In vivo experiments. Of the 17 high HBV vaccine responders selected for the in vivo experiments, 9 (7 HLA-A2⁺) assumed 500 mg per os of Chloroquine Bayer (Bayer AG), corresponding to a 300-mg base chloroquine, followed by a booster dose of anti-HBV vaccine (Engerix-B; Glaxo-SmithKline) on day 2. As a control, the remaining eight high responders (six HLA-A2⁺) only underwent the booster dose of their regular procedure of anti-HBV vaccination. The study was performed according to the ethical guidelines of the 1975 Declaration of Helsinki and a priori approval by our Institutional Review Board. PBMCs were collected in both groups before and 10 d after the vaccination booster and tested for the HLA-A2 expression by FC analysis. CD8⁺ T cells were positively selected from PBMCs and tested in an ELISPOT assay, as previously described (52), for the IFN-γ spot formation in response to a 6-h stimulation with irradiated autologous CD8-depleted PBMCs as APCs, previously pulsed or not with rHBenvAg or peptide, in the presence of chloroquine, blocking anti-HLA-A,B,C mAb (W6/32, IgG2a; Serotec, Ltd.), or the corresponding isotype. Spots were quantified using an ELISPOT reader (cod. 99022004; AID GmbH).

Confocal microscopy. APCs were pulsed and chased with 50 µg/ml rNS3Ag alone or together with 50 µg/ml tetraethylrhodamine isothiocyanate-labeled transferrin (TRITC-Tf; Molecular Probes) in the presence or absence of 10 µg/ml chloroquine for 15 min, washed, and chased for 40 or 120 min at 37°C. They were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and stained with different combinations of the following primary antibodies: human anti-HCV-NS3_{1363–1454} mAb (1:100 in PBS; CM3.B6, IgG1) (73) and rabbit anti-cathepsin D polyclonal antibodies recognizing the active form (46 kD) of human cathepsin D (1:50 in PBS; UBI). The primary antibodies were visualized using (a) FITC-conjugated goat anti-human IgG (1:80 in PBS; Sigma-Aldrich); (b) Texas red-conjugated goat anti-rabbit IgG (1:50 in PBS; Jackson ImmunoResearch Laboratories); and (c) Texas red-conjugated rabbit anti-goat IgG (1:20 in PBS; Sigma-Aldrich). Nonspecific fluorescence was assessed in control samples by omitting the secondary antibodies from the staining procedure. In some experiments, DCs were incubated with 1 mg/ml 40- or 500-kD FITC-dextran (Sigma-Aldrich) in the presence or absence of chloroquine at 37°C and fixed. Fluorescence signals were analyzed by recording staining images using a cooled CCD color digital camera (SPOT-2; Diagnostic Instruments Inc.) and IAS 2000/H1 software (Delta Sistemi). Colocalization of fluorescence signals was evaluated using a CLSM (LSM 5 PAS-CAL; Carl Zeiss MicroImaging, Inc.). The multitrack function was used to prevent cross talk between the two signals. Quantitative analysis of the fluorescence intensity was performed by evaluating three different cytoplasmic areas (1 mm² each) per cell in 200 cells for each condition and randomly taken from three different experiments. The results represent the mean values ± SD. The fluorescence intensity of the control samples was subtracted from that of the positive-stained samples for each time point.

Purification and FC of phagosomes. DCs were previously pulsed/chased with a combination of 0.8 µm LBs (Sigma-Aldrich) and 50 µg/ml rNS3Ag in the presence or absence of chloroquine. They were then homogenized, and phagosomes were purified as described previously (28, 34, 50). LB-containing phagosomes were isolated at the 25–10% interface of a discontinuous sucrose gradient and analyzed by FC as previously described (51). In brief, LB⁺ phagosomes were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences) at 4°C for 20 min and rewashed with Perm Wash Buffer (BD Biosciences). They were then stained with human anti-NS3 mAb (73), followed by secondary PE-labeled anti-human IgG (BD Biosciences) and goat anti-TAP-2 polyclonal antibody (Santa Cruz Biotechnology, Inc.), followed by PE-labeled anti-goat IgG antibody-

ies (Caltag) or mouse FITC-labeled anti-LAMP-2 mAb (Santa Cruz Biotechnology, Inc.), and analyzed by FC.

Analyses of cytosolic- and endocytic-enriched fractions. To detect NS3Ag in both cytosolic and endocytic enriched fractions, $10\text{--}15 \times 10^6$ DCs were pulsed with different concentrations of rNS3Ag in the presence or absence of $10 \mu\text{g/ml}$ chloroquine for 20 min, washed, and chased for 90 min at 37°C . In some experiments, they were pulsed with 1 mg/ml of 40- or 500-kD FITC-dextran for 1 h at 37°C . DCs were then homogenized (49, 50), and the cytosolic and vesicular fractions were isolated as described previously (49, 50) and analyzed by WB or fluorometer as described below. The β -hexosaminidase activity (specific to endo/lysosomes), as detected with the fluorescent substrate 4-methyl-umbelliferyl-N-acetyl-D-glucosamide and lactate dehydrogenase by measuring the decrease in the absorbance of nicotinamide adenine dinucleotide (49, 50, 74, 75), was 10% below that of the whole cell lysate in the cytosolic fraction (unpublished data). The cytosolic- and endocytic-enriched fractions, obtained as described above, were separated by SDS-PAGE and immunoblotted for NS3Ag with the relevant human mAb and the secondary peroxidase-conjugated goat anti-human IgG antibody (Nordic Immunology). Optical density was calculated by the ImageQuant software (GE Healthcare). FITC-dextran expression in both endosomal and cytosolic fractions was analyzed using a fluorometer (Wallac 1420 Victor; PerkinElmer Life and Analytical Sciences, Inc.), and values were computed using the software provided (Victor Manager; PerkinElmer Life and Analytical Sciences, Inc.). The contents of FITC-dextran were determined from a standard curve of the values of different FITC-dextran concentrations. All values were converted into microgram per milliliter ($\mu\text{g/ml}$).

Statistical analysis. Differences in the cross-presentation assays in the presence or absence of chloroquine or other drugs (both in vitro and in vivo) were analyzed by the Mann-Whitney U test.

Online supplemental material. Fig. S1 shows that chloroquine enhances cross-presentation of NS3Ag by DCs in a dose-dependent fashion. Fig. S2 shows the inability of bafilomycin A1 to improve cross-presentation. Fig. S3 shows the improvement of soluble HBenvAg cross-presentation by chloroquine. Fig. S4 shows that rNS3Ag does not affect the surface phenotype of iDCs. Fig. S5 shows the HLA restriction of NS3Ag cross-presentation. Fig. S6 shows the inability of EBV-B cells to export soluble NS3Ag from endosomes into the cytosol, as detected by CLSM. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20051106/DC1>.

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