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Cross-Presentation of the Long-Lived Lymphocytic Choriomeningitis Virus Nucleoprotein Does Not Require Neosynthesis and Is Enhanced via Heat Shock Proteins

Sameh Basta,2,3* Ricarda Stoessel,2* Michael Basler,* Maries van den Broek,† and Marcus Groettrup*

Many viral proteins that contain MHC class I-restricted peptides are long-lived, and it is elusive how they can give rise to class I epitopes. Recently, we showed that direct presentation of an epitope of the long-lived lymphocytic choriomeningitis virus nucleoprotein (LCMV-NP) required neosynthesis in accordance with the defective ribosomal products hypothesis. In this study, we report that LCMV-NP can be cross-primed in mice using either LCMV-NP-transfected human HEK293 or BALB/c-derived B8 cells as Ag donor cells. In addition, we established that contrary to direct presentation, cross-presentation required accumulation of the mature LCMV-NP and could not be sustained by the newly synthesized LCMV-NP protein, intermediate proteasomal degradation products, or the minimal NP396 epitope. Nevertheless, NP cross-presentation was enhanced by heat shock and was blunted by inhibitors of heat shock protein 90 and gp96. We propose that cross-presentation has evolved to sustain the presentation of stable viral proteins when their neosynthesis has ceased in infected donor cells. The Journal of Immunology, 2005, 175: 796–805.

Peptides presented on MHC class I molecules are normally generated through fragmentation of proteins that have been synthesized within the cell. It is therefore remarkable that several structural proteins of viruses are quite stable in cells and nevertheless give rise to sufficient quantities of peptide to induce a CTL response (1–3). A hypothesis which reconciles this apparent contradiction is the defective ribosomal products (DRiPs)3 hypothesis that states that CTL epitopes are not derived from properly folded proteins but from newly synthesized polypeptides that are degraded within a few minutes after their translation (4). The lymphocytic choriomeningitis virus nucleoprotein (LCMV-NP) is an example of such proteins since it is stable over 3–4 days but nevertheless contains immunodominant epitopes like NP396. Epitopes generated from LCMV-NP shortly after neosynthesis were the sole factor for its direct presentation on MHC class I (5). However, the mechanisms involved in the presentation of DRiPs via the MHC class I pathway are only just surfacing (6–8).

Professional APCs (pAPCs) can generate class I peptides by two different mechanisms. Either from internally synthesized proteins (9) via “direct-presentation” or by acquiring exogenous Ags through “cross-presentation.” Cross-presentation represents a vital pathway for the initiation of immune responses against tumors and viruses that do not have access to, or interfere with, the classical pathway for MHC class I presentation (10–15). Dendritic cells and macrophages are pAPCs that have the unique capability of generating and presenting peptides on MHC class I molecules from exogenous Ags (16–18). Activation of naive TCD8 cells by such pAPCs that have cross-processed Ags into the MHC class I pathway is known as cross-priming (13).

Although the DRiP hypothesis explicates how direct presentation of stable proteins in general can occur, it remains unknown whether stable viral proteins such as LCMV-NP can be cross-presented by pAPCs. This is particularly important since in vivo evidence questioned the ability of LCMV-NP to access the cross-priming pathway (19), yet, it was recently shown that proteins are the mediators of in vivo cross-priming (20–22). Moreover, immediate proteasomal products were reported to be the main source of Ags transferred to the pAPC in another virus system (23), while others substantiate that Ags could be transferred as peptides bound to heat shock proteins (HSPs) (24). It is possible that virus-specific differences, depending on the nature of virus proteins and their cellular localization, could exist during Ag processing. We therefore started our study by exploring the following avenues; are stable proteins, such as LCMV-NP, predisposed to access the cross-presentation pathway and, if so, what are the antigenic elements responsible for that (20–25)?

In this study, we report on the cross-presentation of LCMV-NP in vitro and in vivo. We show that accumulated mature proteins probably aided by molecular chaperones are the source of Ags fueling the cross-presentation pathway. Interestingly, neither proteasomal products nor nascent proteins nor DRiPs per se were capable of providing Ags to the cross-presentation pathway. Our data highlight the significant role of the cross-presentation machinery in degrading stable proteins that are otherwise resistant to processing via the proteasome in virus-infected cells.
Materials and Methods

**Mice, viruses, and media**

Female C57BL/6 (H-2b) mice were originally obtained from Charles River Breeding Laboratories. The animals were kept at the University of Constance in a pathogen-free facility in accordance with the rules of the veterinarian authority of Regierungsspräsidium Freiburg and used at 6–10 wk of age.

LCMV-WE was originally obtained from F. Lehmann-Grube (Hamburg, Germany) and propagated on the fibroblast line L929. Recombinant vaccinia virus encoding the LCMV nucleoprotein (rVV-NP) or the NP396 minigen was propagated on BSC40 cells (26). All media were purchased from Invitrogen Life Technologies and contained GlutaMAX, 10% FCS, and 100 μM penicillin/streptomycin.

HEK293 is a human embryonic kidney cell line and B8 is a BALB/c-derived, fibroblastic cell line (H-2b) obtained by SV40 infection in vitro (5). BMC-2 and DC2.4 are macrophage and dendritic cell lines, respectively (a kind gift from Dr. K. Rock, University of Massachusetts Medical School, Worcester, MA).

**Plasmids**

The plasmid pAT153.H-2-D^d was a kind gift from F. Momburg (German Cancer Research Center (DKFZ), Heidelberg, Germany). pCMV-NP and pCMV-UB-NP were a kind gift from Dr. L. Whitton (The Scripps Research Institute, La Jolla, CA).

**Transfection and establishment of stable cell lines**

HEK293 cells were transfected with the following plasmids pCMV-NP or pCMV-UB-NP (3 μg) and the plXSP plasmid for selection marker purmurycin (1 μg). The transfection was conducted with FuGENE 6 according to the manufacturer’s instructions (Roche Diagnostics). Cells were selected for growth with 2.5 μg/ml purumycin (Sigma-Aldrich), and several clones were tested positive for NP expression. Immunostaining revealed that the protein localized to the cytosol in the transfected cells. In the case of pCMV-UB-NP, the protein was only detected after inhibition of the proteasome with the inhibitor N-acetyl-s-leucyl-s-leucyl-norleucinal (LlNL: Sigma-Aldrich), 50 μM, overnight. For MHC expression, HEK-UB-NP (2 × 10^5) cells were transfected with the plasmid pAT153.H-2-D^d (2 μg) using FuGENE 6 reagent. The transfection efficiency was measured with the FITC-labeled anti-H-2-D^d Ab (clone KH95; BD Biosciences).

**Western blotting**

HEK293 cells (2 × 10^6, wild type or transfected) were lysed in 500 μl of lysis buffer (50 nM HEPES (pH 7.5), 2 mM MgCl2, 1 mM EDTA, 1% Triton X-100, and 10% glycerol) on ice for 10 min. After sonification and centrifugation, 10-μl aliquots of the crude lysates were boiled for 5 min at 95°C in 10 μl of 2× reducing Laemmli sample buffer and separated by SDS-PAGE (10% gel). Proteins were blotted onto nitrocellulose (Schleicher & Schüll BioSciences). After blocking overnight at 4°C, the membrane was incubated with the mouse Ab (anti-LCMV/NP, clone 2539 (27), kindly contributed by Dr. M. Buchmeier, The Scripps Research Institute, La Jolla, CA) in PBS in 0.2% Tween 20 and 5% milk for at least 2 h at room temperature, washed three times in PBS/0.2% Tween 20, and then exposed to the HRP-conjugated goat anti-mouse Ab (diluted 1/1000 in PBS/5% milk; DakoCytomation).

**Intracellular detection of LCMV-NP**

For the detection of LCMV-NP, cells (1 × 10^5) were harvested and resuspended in 200 μl of PBS, followed by fixation with 4% Formalin for 20 min at room temperature. After washing with 1× PBS, the cells were permeabilized with 1% Triton X-100 for 20 min at room temperature, followed by incubation overnight at 4°C with rat anti-LCMV-NP Ab (clone VL4/28) in PBS/2% FCS. After two further washing steps, FITC-conjugated polyclonal mouse anti-rat Ab (diluted 1/150 in PBS; BD Biosciences) was left with the cells for at least 1 h at room temperature. Data were acquired with a FACSscan flow cytometer (BD Biosciences) using CellQuest (BD Biosciences) and analyzed with FlowJo software (TreeStar).

**Intracellular cytokine staining (ICS)**

**In vitro**

To detect T^CD8+^, cell activation with ICS, effector cells were coincubated in round-bottom 96-well plates with 0.1 μM peptide-loaded APCs (3 × 10^5/well). Either DMSO or irrelevant peptide (gp33) were used as controls for background activation in all assays. The coinoculation ratio of 1:1 APCs (BMC-2) to responders was chosen when T cells were expanded for 5–6 days in vitro. When TCD8+ cell lines were used, the DC2.4 cell line as stimulator cells, brefeldin A (BFA) was added directly and the cells were left together for 3 h before staining.

**In vivo**

Ex vivo responders were incubated with the specific peptides for 2 h at 37°C and then left in the presence of BFA (Sigma-Aldrich) at 10 μg/ml for 3 h. The coinoculation ratio of 1:10 APCs (BMC-2) to responders was chosen in the case of direct analysis ex vivo. In general, cells were then stained with PE-Cy5 anti-CD8 rat IgG Ab clone 53-6.7 (BD Biosciences) on ice for 20 min, washed, and fixed with 1% paraformaldehyde in PBS at room temperature for 20 min. After washing, the cells were labeled with FITC-conjugated rat anti-IFN-γ Ab clone XGM1 (BD Pharmingen) in PBS/0.1% saponin at 4°C overnight. Stained cells were analyzed with a live gate on the CD8+ cells after 80,000 gated cells were acquired.

**Tetrameric MHC class I peptide analysis**

Tetrameric complexes containing biotinylated H-2Db β2-microglobulin, the NP396 peptide, and extravidin-FITC were generated as described previously (26, 29). Splenocytes (5 × 10^7 cells) were stained with 0.5–1 μg of tetramer in 50 μl of FACS buffer (PBS, 2% FCS, 0.01% NaN3, and 20 μM EDTA) at room temperature for 30 min, followed by addition of PE-Cy5 anti-CD8 rat IgG Ab clone 53-6.7 (BD Biosciences) on ice for 30 min. Cells were washed twice and data were acquired using CellQuest (BD Biosciences) as before by gating on CD8+ lymphocytes.

**TCD8+ cell priming and induction of T cell lines**

For in vivo priming, 8- to 10-wk-old female C57BL/6J mice were injected i.p. with 1 × 10^5 cells in PBS. In the case of primary responses, ICS measurements were performed directly 8 days after injection. In some experiments, spleens were harvested and restimulated with peptide-loaded APCs. On day 5 or 6 of the in vitro expansion, T cells were tested for IFN-γ production by ICS as before.

For generating NP396-specific T cell lines, C57BL/6 mice were injected i.v. with 2 × 10^6 cells or 10^5 cells of LCMV-WE (100 PFU/ml). The spleens were harvested 4 wk after injection and the splenocytes were purified by density centrifugation (Ficoll-Paque). The splenocytes were cultured in RPMI 1640 containing mouse IL-2 (40 U/ml) along with gamma-irradiated peptide-loaded APCs. An additional density centrifugation step was conducted 5–6 days later when they were used for in vitro Ag presentation experiments. In all assays, we used the T cell lines at this time point and their specificity corresponded to at least 85% when tested with peptide (50 nM).

After the detection of the epitope NP396, we resuspended the APCs in RPMI 1640 (1 × 10^7/ml), added them to NP396-specific TCD8+ cells, and stained for CD8 and IFN-γ as described above.

**Preparation of Ag donor or presenting cells (ADCs and APCs)**

ADCs were detached with trypsin-EDTA, washed with PBS, and resuspended in RPMI 1640 (1 × 10^5 cells/ml) and treated with different inhibitors where indicated. For interfering with HSPs, the HSP90-specific inhibitors geldanamycin (5 μM) and herbimycin A (5 μM) were used (Sigma-Aldrich). Cycloheximide (250 μg/ml) was obtained from Calbiochem. For testing different conditions, the cells were either further heated (14 min at ~45°C) or UV irradiated (8 min), or snap frozen in liquid nitrogen (~180°C) and UV irradiated for 8 min (lysed and UV treated (LyUV)). UV-triggered apoptosis was induced using the Stratalinker 2400 UV cross-linker that provides a radiation intensity of 4 mW/cm^2 per s. Interestingly, one round of LCMV resulted in intact cells incorporating trypan blue, whereas LyUV+ cells only 40% incorporated trypan blue. If the cells were subjected to repeated cycles of freezing and thawing, the outcome was mainly cell fragments.

As APCs, we used the macrophage cell line BMC-2 that was recently shown to possess competent phagosomes as organelles for Ag cross-presentation (30). Where indicated, the following inhibitors were used: BFA (10 μg/ml), chloroquine (100 μM), leupeptin (100 μM), pepstatin A (50 μg/ml), fucoidin (250 μg/ml; Sigma-Aldrich), lactacytin (LC, 50 μM; Biomol). During the cross-presentation assays, coinoculation of ADCs with BMCs was conducted in round-bottom 96-well plates (Greiner bio-one; Cellstar) at 37°C at a ratio of 1:1 for 16 h.

**Results**

**Expression of LCMV-NP in transfected cells**

To study LCMV-NP cross-presentation, we used human HEK293 cells as ADCs since they cannot directly present the H-2Db-restricted NP396 epitope to specific T cell lines that we used to
monitor cross-presentation. We generated stable transfectants expressing either the full-length LCMV-NP (designated HEK-NP) or a ubiquitin-LCMV-NP fusion protein (HEK-Ub-NP). The N-terminal ubiquitin moiety is a G76A mutant that cannot be cleaved off by ubiquitin-specific proteases and targets the fusion protein for rapid degradation by the proteasome (31). Western blot analyses of LCMV-NP in either LCMV-infected HEK293 cells (HEKi) or HEK-NP cells demonstrated that LCMV-NP was abundantly expressed (Fig. 1A). LCMV-NP expression was very low in untreated HEK-Ub-NP cells but proteasome inhibitors markedly enhanced the expression level, thus confirming rapid proteasomal degradation of the fusion protein (Fig. 1A, HEK-Ub-NP + LLnL) as previously demonstrated in transiently transfected cells (31). The relative NP expression levels as assessed by Western blot analysis were further confirmed by pulse-chase analysis as well as intracellular NP staining and flow cytometry (data not shown and Fig. 1B).

The long-lived form of LCMV-NP is needed for cross-presentation in vivo and in vitro

Initially, we compared the ability of HEK-NP and HEK-Ub-NP cells to induce T cells in vivo. T cell activation was higher in the spleen and peritoneum (Fig. 2A) when mice were injected with HEK-NP than with the HEK-Ub-NP cells. We detected the priming of T cells by cocultivating the responder cells with peptide-loaded APCs for a total of 5 h in a standard ICS assay. For controls, we used either the same concentration of DMSO or an irrelevant peptide, gp-33 in all assays. In some circumstances, ex vivo-activated T cells are underestimated with a functional assay such as ICS. We therefore conducted tetramer analysis ex vivo using the specific H-2 D^b-NP396-PE tetramer to measure the induced T cell frequencies. Interestingly we could detect significantly higher numbers in the HEK-NP condition compared with the HEK-Ub-NP cells (Fig. 2B).

However, because of the low signal intensity obtained ex vivo, we assessed the induction of T_{CD8^+} after restimulation and expansion for 5 days in vitro using ICS (Fig. 2C). Before animal inoculation, the ADCs were either left untreated (whole), or lysed to become necrotic (lysed), or LyUV; but for all three protocols, we found after in vitro expansion that HEK-NP cells expressing stable NP induced higher levels of activated T_{CD8^+} cells up to five times more than HEK-Ub-NP cells expressing the short-lived NP fusion protein. As stated in the legend to Fig. 2, we have resorted to two different pulsed APC lines (irradiated DC2.4 for expanding the T cells in vivo and BMCs as APCs for the ICS assay) to avoid any possible cross-reactive T cells and therefore reduce background noise.

To further explore the in vivo phenomena, we performed experiments in vitro where ADCs were left with APCs for 16 h before the addition of NP396-specific T cell lines. The data confirmed the observations made before: HEK-NP cells were a better source of Ag than HEK-Ub-NP cells (Fig. 3A). In addition, when protein degradation was blocked in HEK-Ub-NP cells with the proteasome inhibitor LC, we recovered the ability of the ADCs to cross-present (HEK-Ub-NP + LC), demonstrating that proteasomal products were less efficient than the stabilized protein as a source of Ag for cross-presentation. As expected, the addition of LC to HEK-NP cells did not significantly alter the cross-presentation signals, probably due to the fact that LCMV-NP is an inherently long-lived protein. To make sure that the inferior response in HEK-Ub-NP cells was not due to a defect in epitope generation, we transiently transfected these cells with an H-2D^b expression plasmid and confirmed NP396 direct presentation which correlated with the H-2D^b expression level (data not shown).

To confirm these findings independently and to assess the role of peptide epitopes for cross-presentation, we infected either HEK293 cells or J74 cells (H-2^d) with rVV encoding either the full-length LCMV-NP protein or the NP396 minigene. In this situation, donor cells were subjected to UV irradiation to prevent secondary refection of the APCs. As shown in Fig. 3B, only ADCs that were expressing the full-length protein served as an efficient source of Ag for the activation of NP396-specific T_{CD8^+} cells. In marked contrast, the rVV encoding the NP396 minigene failed to induce activation via cross-presentation, suggesting that the minimal peptide Ags cannot be efficiently cross-presented.

Heat shock or lysis and UV irradiation increases cross-presentation of LCMV-NP but not of LCMV-Ub-NP

To test different factors that may affect cross-presentation, we subjected HEK-NP and HEK-Ub-NP cells to either heat shock or to lysis by freezing/thawing followed by UV irradiation (LyUV). The heat shock treatment (14 min at 45°C) did not significantly affect the viability of HEK cells as determined by immuno blotting with anti-NP Ab. HEK-Ub-NP cells were also left overnight in the presence of the proteasome inhibitor LLnL (50 μM, HEK-Ub-NP + LLnL). As a positive control, LCMV-infected L292 cells were used (L292). B. The intensity of NP expression as determined by flow cytometry. HEK-Ub-NP cells are represented by the black-filled histogram that overlapped the negative control. L292 are depicted by the filled histogram with the black line, HEK-NP by the unfilled histogram with the black line, and HEK-Ub-NP + LLnL, depicted by unfilled histogram with the gray line.

FIGURE 1. Characterization of stable transfectants expressing the NP (LCMV-NP) and a Ub-LCMV-NP fusion protein. A. Western blot analysis of the LCMV-NP steady-state levels. HEK293 cells transfected (wild type (WT)), LCMV-infected (HEKi), and stably transfected with either NP (HEK-NP) or Ub-NP (HEK-Ub-NP) were lysed, and their NP content was determined by immunoblotting with anti-NP Ab. HEK-Ub-NP cells were also left overnight in the presence of the proteasome inhibitor LLnL (50 μM, HEK-Ub-NP + LLnL). As a positive control, LCMV-infected L292 cells were used (L292). B. The intensity of NP expression as determined by flow cytometry. HEK-Ub-NP cells are represented by the black-filled histogram that overlapped the negative control. L292 are depicted by the filled histogram with the black line, HEK-NP by the unfilled histogram with the black line, and HEK-Ub-NP + LLnL, depicted by unfilled histogram with the gray line.

The HSP90 family enhances cross-presentation of LCMV-NP

Because initial experiments indicated that treatment of the ADCs leading to increased expression of HSPs enhanced cross-presentation, we examined the outcome of interfering with a prominent class of the HSP families. HEK-NP cells were treated with the very well-characterized HSP90 inhibitors geldanamycin and herbinycin before heat shock or LyUV treatment. As shown before, when
ADCs were either heat shocked or LyUV treated, there was an increase in the signals (Fig. 3C). When we used the inhibitors that are specific for both HSP90 and gp96, the signals were almost at background levels (Fig. 4A, heated or LyUV). The effect of these inhibitors on intact cells was not apparent, as no significant differences were detected (Fig. 4A, intact). The possibility that the effect of HSP90 inhibitors was due to a direct inhibition of the responder T cell lines was ruled out as peptide-loaded APCs incubated with either untreated or inhibitor-treated ADCs induced similar activation of the T cell lines (activation was almost 85%, data not shown). In addition, we ruled out possible negative effects of these inhibitors on the NP levels in ADCs by intracellular NP staining and flow cytometry (Fig. 4B).

**Analysis of LCMV-NP uptake, processing, and degradation in APCs**

The capacity of the APC to present Ags is dependent on its ability to internalize the Ags where receptors such as the scavenger receptor A (SR-A) may be playing a role. We therefore examined the contribution of SR-A in the uptake of Ags by incubating APCs with the SR-A inhibitor fucoidin. The ADCs encoding the LCMV-NP were treated as before (intact, heated, or LyUV), followed by incubation with fucoidin-treated or untreated ADCs induced similar activation of the T cell lines (activation was almost 85%, data not shown). In addition, we ruled out possible negative effects of these inhibitors on the NP levels in ADCs by intracellular NP staining and flow cytometry (Fig. 4B).

**FIGURE 2.** Cross-presentation of LCMV-NP in vivo is mediated primarily via the long-lived form of NP. The stable transfectants HEK-NP and HEK-Ub-NP were injected i.p. (1 × 107/mouse), and spleen and peritoneal exudates cells (PECs) were recovered after 8 days to examine direct ex vivo TCD8+ activation after addition of the NP396 peptide (396) with an IFN-y ICS assay; as a negative control, either DMSO or the gp33 peptide was added to the cells (A). T cell frequencies were estimated in TCD8+ percentage of activated T cells by IFN-

5% in both conditions. Moreover, we tested for the NP expression in ADCs at the same time point (intact cells conditions) and found no significant differences between ADCs incubated with untreated or treated BMCs (data not shown).

After examining LCMV-NP uptake, we studied the contribution of the processing pathways involved in its cross-presentation. By using BFA, which inhibits the export of proteins from the endoplasmic reticulum (ER), we tested whether the cross-presentation of LCMV-NP epitopes requires transport through the ER-Golgi complex. BFA blocked the presentation in all three conditions to variable degrees (Fig. 5B, □). Although cross-presentation of intact or heat shocked but viable cells were inhibited to nearly background levels, presentation of LyUV-treated cells showed possible minor contributions of other pathways, e.g., the vacuolar or the recently discovered new ER-phagosome fusion pathway (30, 32, 33). Nevertheless, the strong inhibition by BFA suggests that Ag processing is occurring mainly through the classical cytosolic route in this system. There were no significant differences in NP expression in ADCs incubated with untreated or treated BMCs (data not shown) when cells were examined 24 h after treatment.

To assess the relative contribution of the proteasome-independent vacuolar processing pathway, we made use of the proteasome inhibitor LC. When we used LC-treated APCs, the presentation of LCMV-NP was significantly diminished in all three conditions (Fig. 5B, □), suggesting that the NP presentation pathway involves translocation to the cytosol and degradation by the proteasome. Flow cytometric analysis of NP expression revealed no adverse effect of these inhibitors (data not shown). Peptide-loaded APCs incubated with the inhibitors (BFA or LC) induced typical T cell stimulation, showing that these inhibitors did not interfere with presentation by surface expressed MHC class I molecules (data not shown).
Thus far, we have examined the processing routes involved in the presentation of LCMV-NP; however, one essential issue was to delineate the crucial factors responsible for the initial degradation of the long-lived protein during cross-presentation. To address this, we incubated the APCs with chloroquine, an inhibitor of endo/lysosomal acidification. Presentation was significantly inhibited in either the heated or LyUV conditions (Fig. 5C, hatched bars#), showing that acidification is required. To investigate whether endosomal enzymes were also required, we treated APCs with two different protease inhibitors, leupeptin and pepstatin. Incubation of APCs in the presence of leupeptin reduced presentation, whereas pepstatin slightly increased the presentation signals (Fig. 5C, filled or dotted bars). However, the increase was found not to be significant (p > 0.05 as calculated using the paired Student’s t test). Accordingly, endosomal acidification and serine and cysteine proteases (cathepsin B, L, or S) play an important role in LCMV-NP processing, whereas aspartate proteases (cathepsin D or E) do not significantly affect this epitope generation during cross-presentation. In all of these inhibition studies, presentation by peptide-loaded APCs treated in the same manner yielded T cell responses similar to the untreated APCs (data not shown).

**Defective ribosomal products are not the mediators of cross-presentation**

A fundamental issue regarding the presentation of LCMV-NP through the cross-presentation pathway centers on whether DRiPs per se are a source of Ag for cross-presentation. To address this point, we tried two different approaches: the first one was based on inhibition of de novo protein synthesis before injecting the ADCs in vivo. As shown in Fig. 6A, we could not detect any significant differences if ADCs were treated with cycloheximide, suggesting that the mature folded protein is responsible for the cross-presentation activity. In vitro, when de novo protein synthesis in ADCs was inhibited by cycloheximide, an inhibitory effect on cross-presentation in vitro was observed (data not shown). Unfortunately, using this approach is inadequate because translation inhibitors can affect a variety of factors in the cell.

To resolve these issues, we used the tight tetracycline (tet)-repressor system where systemic effects of inhibiting protein neosynthesis are ruled out. This system relies on the tet-repressible expression of LCMV-NP in the fibroblast transfectant B8tNP64. In these cells, NP neosynthesis can be switched on and off without using inhibitors, which has been instrumental in identifying LCMV-NP as a DRiP substrate (5). First, we checked whether
LCMV-NP induced for 24–72 h in B8tNP64 cells was cross-presented in vivo. This occurred to a similar degree as we have observed for HEK-NP cells (Fig. 2 and data not shown). We have thus chosen the 24-h time point for further in vitro analyses. To assess the control of LCMV-NP expression and the specificity of cross-presentation in vitro, we used either B8 recipient cells (B8-wt) or B8tNP64 cells (NP64) that were tet suppressed and confirmed that cross-presentation was undetectable when LCMV-NP was not expressed (Fig. 6B). To examine the effect of neo-synthesis, we removed tet for 5 h (Fig. 6B, −5 h) so that we could analyze the role of DRiPs as an Ag source, but we failed to detect NP396 presentation after exogenous processing, unlike direct presentation (data not shown). We could recover the activity if tet had been removed for a period of 24 h, thus allowing LCMV-NP accumulation (Fig. 6B, fourth column, tet−), strongly indicating that the accumulated mature protein rather than DRiPs or nascent proteins is responsible for cross-presentation. The LCMV-NP expression levels detected by FACS and immunoblot analyses in either B8tNP64 or HEK-NP cells support this proposition (Fig. 6C and data not shown). The measurements obtained by flow cytometry reflect the nature of the fully folded protein since this Ab does not detect native forms of NP in Western blots. Importantly, as indicated before with the HEK-NP cells, when we awaited NP expression to build up for 24 h and then applied the chaperone inhibitors, no significant negative effects on NP expressions were found (Fig. 6C, histogram overlays).

The ability to cross-present LCMV-NP was significantly reduced when chaperones of the HSP90 family were inhibited in B8tNP64 cells with geldanamycin and herbimycin that were added to cells after tet removal for 24 h (Fig. 6B, columns 5 and 6). To exclude that HSP90 inhibitors negatively affected NP396 presentation by APCs, we used peptide-pulsed APCs as a control (Fig. 6B, columns 8–10). To further scrutinize the requirement for neo-synthesis in cross-presentation, we allowed protein synthesis to proceed for 24 h and then added tet for 5 h to block any further neo-synthesis (Fig. 6B, −5 h). If DRiPs were playing a role, then the signal would be drastically reduced. This experiment revealed that, when only the mature LCMV-NP protein was present but no DRiPs, cross-presentation was as efficient as if LCMV-NP synthesis was permitted over 24 h. Taken together, it is evident from our results that NP neosynthesis is not required as an Ag source in LCMV-NP cross-presentation.

**Discussion**

Better understanding of factors contributing to the cross-presentation pathway is an important issue at present in the field of Ag presentation and immune surveillance. Recently, a novel phagosomal pathway involved in cross-presentation, which merits further assessment, has been discovered (30, 32, 33). Furthermore, the involvement of DRiPs as a source of Ags is a new paradigm in direct presentation (4). These new concepts have prompted us to examine whether cross-presentation of the long-lived LCMV-NP (5) is attainable in APCs after its uptake, since it is not readily degraded in ADCs. Consequently, we set our goals to scrutinize the parameters involved in its degradation in APCs and investigated the relative contribution of proteins vs DRiPs or peptides as Ag sources and the role of HSPs therein.

We tested the ability of this long-lived protein and its degradable products to cross-present in vitro and in vivo to investigate whether it conforms with the recent findings regarding the role of proteins in cross-priming (20–22) or its stability does not allow it to access the cross-presentation pathway (19). Although the in vitro assays proved to be a more sensitive readout system compared with in vivo, the overall conclusion reached was similar in both systems. Accordingly, and contrary to previously reported data (19), we found that NP transfectants (xeno- or alloresponses) can prime T_{CD8+} cells in vivo via the exogenous pathway. Perhaps, our system was more sensitive to detect such activity because our transfectants expressed sufficient amounts of NP. Cross-priming and presentation of this long-lived protein was considerably reduced when it was targeted for rapid degradation by the proteasome. This conclusion was confirmed by different means; T cell frequencies were estimated using tetramer analysis and their activation was examined directly ex vivo or after expansion in vitro. Considering all of the data combined, it is clear that less efficient T cell activation, at least four times less, occurred when the NP was targeted for degradation. We hypothesized at this stage that the long-lived form of NP is a better source for cross-priming than...
the unstable ubiquitin (Ub)-NP fusion protein. This finding concurs with and further extends the concept that proteasomal substrates in general rather than proteasomal products are the main source of cross-priming in vivo (20–22).

Although the Ub-NP transfectants generate more peptides or polypeptides than full-length protein in the donor cells, they nevertheless were poor inducers of T<sub>CD8</sub><sup>H11001</sup> cells via cross-priming. We confirmed these findings with rVV encoding either the full-length protein or the minigene, where the full-length protein was superior in its ability to cross-present compared with the cytosolic peptide. These conclusions are supported by earlier reports showing that cytosolic peptides, unless bound to chromatin, are unstable with a very short half-life due to cytosolic proteases (34). Furthermore, cross-priming of T cell responses in vivo does not require antigenic peptides in the ER (35). According to our results, degraded proteins are unlikely to play a major role in cross-priming of T cell responses in agreement with recently published data (20–22). Yet, HSPs bound to antigenic peptides were suggested to be a potent source of Ag for cross-presentation (24, 36). In contrast, the circumstances under which chaperone-peptide interactions play a
specific role in immunity has been put in question recently and need to be thoroughly investigated (36, 37). Our findings reconcile the proposed role of HSPs in cross-presentation on the one hand and the inability of peptides to cross-prime on the other hand. Based on our results, the ability of HSPs to enhance cross-presentation is evidently biased toward proteins interactions and not proteasomal products.

To examine conditions where possible danger signals may affect the activation of pAPCs (36–38), we compared intact HEK-NP cells with other stress settings where induction or release of chaperones is envisaged. Either LyUV or heat shock protocols led to a marked enhancement of cross-presentation. This was not the case if we just UV treated intact ADCs (data not shown), indicating that the differential cell treatment can influence immunogenicity (39, 37). Our findings reconcile the proposed role of HSPs in cross-presentation on the one hand and the inability of peptides to cross-prime on the other hand. Based on our results, the ability of HSPs to enhance cross-presentation is evidently biased toward proteins interactions and not proteasomal products.

There are several reasons, currently under investigation by us, why these chaperones could have led to increased presentation. It is possible that the effect is indirect due to stimulation of APCs via cell surface signaling receptors like TLRs or increased uptake of ADCs via HSP receptors (36–38). The chaperones may be required to maintain the proper folding or function of other unknown interacting factors that are generally needed for the cross-presentation pathway. Alternatively, if HSPs are tightly bound to NP, either LyUV or heat shock protocols led to a marked enhancement of cross-presentation. This was not the case if we just UV treated intact ADCs (data not shown), indicating that the differential cell treatment can influence immunogenicity (39, 40). Our results show that the use of HSP90 and gp96 inhibitors impeded HSP90 function (43), which is blocked in cells treated with geldanamycin and herbimycin A because they bind to the ATP binding site of HSP90 according to x-ray crystallographic analysis (44). In addition, a very recent finding reported on reduced cross-prime in mice that were defective in heat shock factor 1 (Hsf1). These mice have a decreased expression of several HSPs including HSP90 and HSP70 (45).
Hitherto, three pathways have been described for MHC class I presentation of exogenous Ags by pAPCs. In addition to the phagosome-cytosol-ER pathway, and the vacuolar TAP-independent pathway, a new ER-phagosome fusion pathway has recently been discovered (7). It was therefore interesting for us to examine which pathway may be involved in LCMV-NP cross-presentation. NP cross-presentation was markedly reduced in LC- or BFA-treated APCs, indicating that the classical phagosome-cytosol-ER pathway is the major route for the presentation of this Ag. Furthermore, we made use of several inhibitors to examine the proteolytic pathways involved in NP396 cross-presentation (Fig. 7). This was an important point in our study, since this long-lived protein is not degraded in ADCs.

Accordingly, we examined what enzymes or organelles during cross-presentation are facilitating the processing before proteasomal degradation. Cross-presentation was affected by lowering the endolysosomal pH and cysteine and serine proteases, but not aspartate proteases. It appears that acid-dependent endolysosomal proteases are preprocessed with the aid of endosomal acidification (i) and cysteine proteases (ii) before their release from endosomes and degradation via acid-dependent endolysosomal pathways involved in NP396 cross-presentation (Fig. 7).

Ultimately, we addressed the role of de novo protein synthesis in ADCs in cross-presentation. Using the tight tet-repressor system wherein newly synthesized Ags are made by the cell’s own translation machinery. It is also possible that their findings reflect an alternative mechanism when fully folded newly synthesized proteins can directly access the cytosol in bacterially infected cells.

In this study, we highlight the extent of DRiPs’ contributions to the presentation of a stable virus protein via the exogenous Ag-processing pathway. Our study clearly shows that although proteasomal substrates are important in cross-presentation as previously reported (20–22), neither DRiPs per se nor de novo synthesized proteins can contribute to this pathway. It was the accumulated LCMV-NP protein possibly aided by a specific class of molecular chaperones, which was capable of providing Ags.

Since newly synthesized DRiPs constitute a large fraction of the proteasome’s substrates (50), it makes sense that DRiPs are crucial for the immediate direct NP presentation while cross-presentation awaits the build up of stable proteins that are likely to reflect heavily infected cells. Thus, cross-presentation can make long-lived viral proteins accessible for T cell priming, even if protein neosynthesis is curtailed in infected cells after the onset of the IFN response. Our data provide further insights into significant aspects regarding generation of viral immunity (10–15) and highlight how the direct and cross-presentation pathways complement each other during viral infection (Fig. 7).

**FIGURE 7.** A model describing the different presentation pathways for long-lived viral proteins. In an infected APC that primes T<sub>cells</sub> cells, virus proteins are translated by ribosomes soon after infection (1 h). A significant portion of newly synthesized proteins that are DRiPs (I) get degraded by the proteasome and contribute to the direct presentation of LCMV-NP within 3 h (2). The mature full-length protein (i) can still interact with HSPs and accumulates for at least 24 h (ii) before it can contribute Ags to noninfected cross-presenting APCs via SR-A (iii). In the acceptor APC, these long-lived proteins are preprocessed with the aid of endosomal acidification (iv) and cysteine proteases (v) before their release from endosomes and degradation via the proteasome in the cytosol and transport via the classical cytosol-ER-MHC I presentation route (vi). We propose, based on our data, that components of the DRiP presentation pathway (1 and 2 depicted here by the interrupted dotted arrows) fail to supply Ags to the cross-presenting cells and suggest that direct and cross-presentation pathways are mutually exclusive concerning DRiPs processing and presentation.
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Disclosures
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