

# A lymphocytic choriomeningitis virus glycoprotein variant that is retained in the endoplasmic reticulum efficiently cross-primes CD8<sup>+</sup> T cell responses

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Recent studies indicate that T cell cross-priming preferentially occurs against long-lived, stable proteins. We have studied cross-priming by using the glycoprotein (GP) of lymphocytic choriomeningitis virus (LCMV), a protein that normally is not MHC class I cross-presented. This study shows that a C-terminally truncated, noncleavable variant of LCMV-GP led to the accumulation of stable, soluble GP trimers in the endoplasmic reticulum (ER) of the antigen donor cell, and thereby converted LCMV-GP into a potent immunogen for cytotoxic T lymphocyte cross-priming. Immunization of mice with tumor cells expressing an ER-retained LCMV-GP variant cross-primed protective antiviral cytotoxic T lymphocyte responses *in vivo* at least 10,000-fold better than immunization with cells expressing the cross-presentation-“resistant” wild-type LCMV-GP. Thus the ER is a cellular compartment that can provide antigen for cross-presentation, and modifications affecting stability and subcellular localization of the antigen significantly increase its availability for MHC class I cross-presentation. These findings impinge on vaccine strategies.

cross-presentation | cytotoxic T lymphocytes | vaccination | antigen presentation

CD8<sup>+</sup> T cells specifically recognize and eliminate infected or transformed cells that display antigenic peptides on their surface MHC class I molecules. Induction of strong CD8<sup>+</sup> T cell responses is, therefore, a major goal in the development of preventive and therapeutic vaccines against persistent viruses and tumors. Most MHC class I positive cells exclusively present peptides generated during the degradation of endogenously synthesized proteins. In contrast, professional antigen-presenting cells (APCs), the key initiators of CD8<sup>+</sup> T cell responses (1), can additionally present peptides derived from exogenous antigens taken up from the extracellular milieu via a pathway termed cross-presentation (2–4). Cross-presentation is most efficient for particulate or cell-associated antigens that target phagocytic uptake by APCs. Antigenic peptides may be directly generated by endosomal proteases; however, the main cross-presentation pathway *in vivo* appears to involve antigen translocation from the phagosome into the cytosol and degradation by the proteasome (2, 3). Although the mechanisms governing the uptake and processing of exogenous antigen are now being elucidated at the molecular level (5–14), much less is known about whether the nature or subcellular localization of the antigen influence its ability to enter the cross-presentation pathway.

One critical parameter limiting the ability of a given antigen to be cross-presented very likely is its half-life. Indeed, several recent studies indicate that mature, stable protein represents a major source of antigen for cross-presentation *in vivo*. For instance, cytotoxic T lymphocyte (CTL) responses were found to be preferentially cross-primed against peptide determinants of

long-lived proteins but not of rapidly degraded signal sequences (15). Conversely, cross-priming is enhanced by inhibition of proteasomal degradation of proteins (16). Cross-presentation was also found not to require antigen neosynthesis by the antigen donor cell (17). Last, it was shown that the cross-priming antigen can be removed from cell lysates by depletion of the intact protein, further suggesting that cross-presented peptides were derived from stable protein (18). Taken together, these studies imply that effective vaccines for cross-priming CTL responses should aim at maximal metabolic stability of the antigen.

We have tested whether this concept could be exploited to augment cross-presentation of antigens that normally do not enter the cross-presentation pathway. Previous studies investigating CTL priming against lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) as a cell-associated antigen (19–21) have consistently failed to demonstrate a role for cross-presentation in these responses. Therefore, we attempted to increase the stability of LCMV-GP and generated a soluble, noncleavable, and trimer-stabilized GP variant. Our findings indicate that these modifications not only led to an unexpected retention and accumulation of the variant protein within the endoplasmic reticulum (ER) of the antigen donor cell but also drastically enhanced its potential to enter the cross-presentation pathway and to induce protective CTL responses *in vivo*.

## Results

**A Soluble, Noncleavable Variant of LCMV-GP Is Retained in the ER.** Wild-type (WT) LCMV-GP (Fig. 1A) is translated as a membrane-bound single precursor protein (GPc) into the ER, where it undergoes folding, glycosylation, and trimerization before ER export (22, 23). Within the Golgi compartment, the GPc trimers are proteolytically processed by S1P/SKI-1 (24) into a peripheral receptor-binding subunit GP-1 and a transmembrane subunit GP-2 (Fig. 1A). Both subunits stay noncovalently associated and

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Abbreviations: APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; Endo H, endoglycosidase H; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GP, glycoprotein; GPc, GP precursor; GPER, ER-retained LCMV-GP; LCMV, lymphocytic choriomeningitis virus; MC-GP, murine MC57 fibrosarcoma cells expressing LCMV-GP; MC-GPER, MC57 cells expressing LCMV-GPER; RIP-GP, LCMV-GP expressed under control of the rat insulin promoter; VV-G2, recombinant vaccinia virus expressing LCMV-GP.

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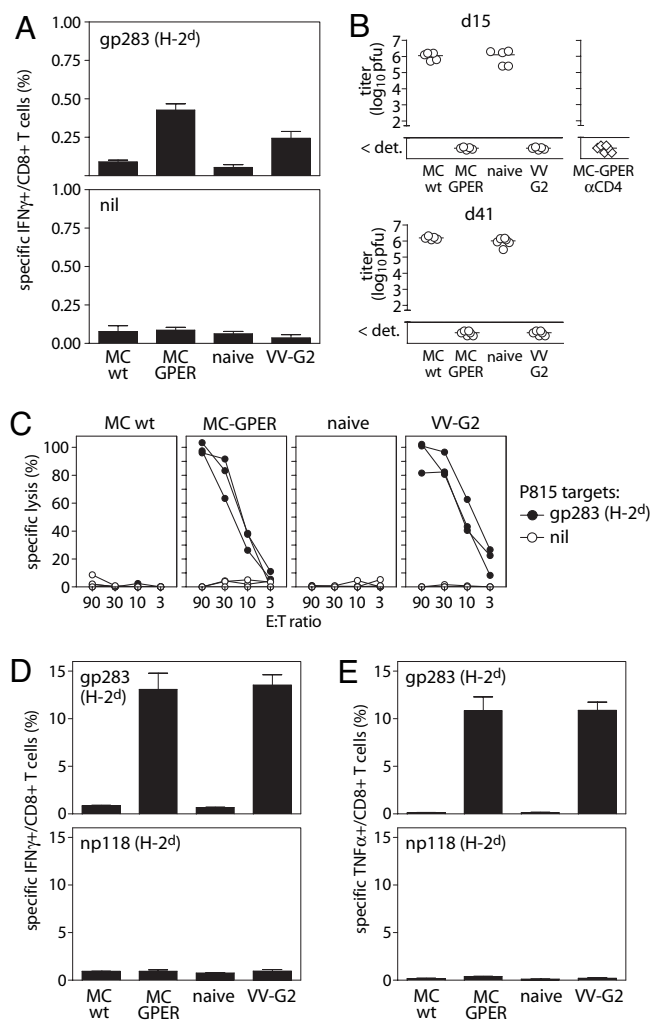
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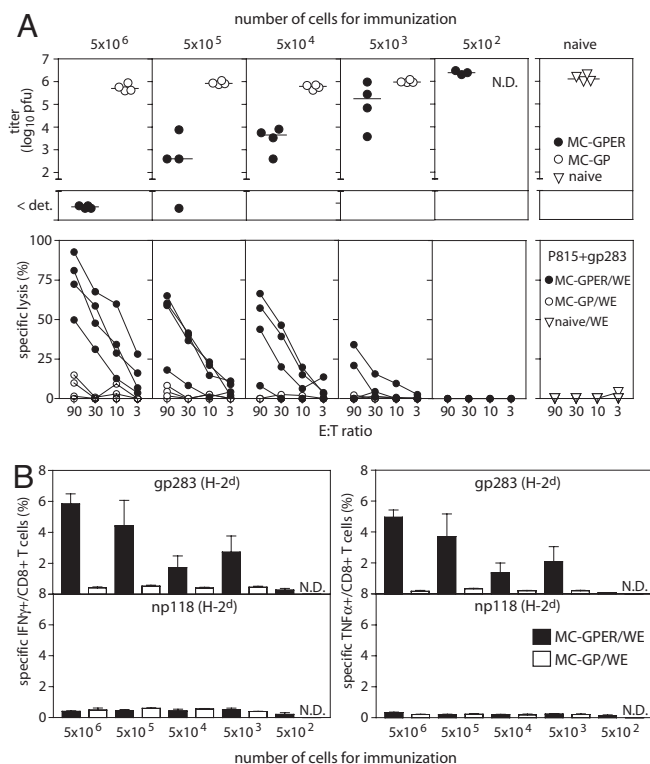






**Fig. 4.** Protective CTL responses induced by GPER-expressing tumor cells. BALB/c mice were i.p. immunized with parental MC57 (MCwt), MC-GPER, GP-recombinant vaccinia virus (VV-G2), or were left naïve as controls, and challenged 15 or 41 days later with 200 pfu of LCMV-WE. Virus titers (days 15 and 41 are shown) and T cell responses (day 15 is shown) were assessed in spleens on day 4 after LCMV challenge. (A) gp283-specific CD8<sup>+</sup> T cell responses on day 15 after immunization with MC-GPER. (B) Virus titers in mice that received the LCMV challenge either 15 days (Upper) or 41 days (Lower) after being immunized as indicated (open circles). CD4-depleted (open diamonds) animals were immunized with MC-GPER and challenged 15 days later with LCMV-WE. (C–E) *Ex vivo* cytotoxicity (C) and frequency of IFN $\gamma$ -producing (D) or TNF $\alpha$ -producing (E) gp283 and np118-specific CD8<sup>+</sup> T cells in spleens on day 4 after virus challenge.

priming by the tumor cell itself, we injected the H-2<sup>b</sup> MC-GPER into H-2<sup>d</sup> BALB/c recipient mice. Immunization of BALB/c mice with MC-GPER primed gp283-specific CTL responses, to a comparable extent as that observed after infection with a recombinant vaccinia virus expressing LCMV-GP (VV-G2; Fig. 4A). Although low in frequency at 15 days after immunization, the gp283-restricted CTLs expanded drastically after challenge infection with LCMV-WE and were able to confer full protection against this virus (Fig. 4B–E). We could exclude that the gp283-specific CTL response reflected a primary response induced by the LCMV challenge infection rather than a secondary response of cross-primed CTL induced by tumor cells. Immunization of the mice showed no CTL responses against the LCMV nucleoprotein-derived np118 epitope, which is the immunodominant H-2<sup>d</sup> epitope. In addition, specific CTL were not detected at this time point in mice receiving virus



**Fig. 5.** ER-retained GP, but not WT GP, is a potent immunogen for cross-priming of protective antiviral CTLs. BALB/c (H-2<sup>d</sup>) mice were primed with titrated numbers of MC-GPER (filled circles) or MC-GP (open circles) and challenged with LCMV-WE on day 15 after immunization. Four days later, virus titers (A Upper), *ex vivo* cytotoxicity (A Lower), and IFN $\gamma$ -producing (B Left) or TNF $\alpha$ -producing (B Right) virus-specific CD8<sup>+</sup> T cells were assessed using spleen cells. Filled bars, MC-GPER; open bars, MC-GP. N.D., not determined. Symbols represent individual mice in A, the mean  $\pm$  SEM of groups of 3–4 mice in B.

challenge alone (Fig. 4D and E). Four days after infection, spleens of MC-GPER primed mice were free of virus and contained high numbers of cytokine producing gp283-specific CD8<sup>+</sup> T cells that lysed gp283-labeled target cells directly *ex vivo*. Furthermore, cross-primed gp283-specific CTL induced by MC-GPER immunization protected against LCMV infection not only during the effector phase (day 15 after immunization) but also during the memory phase (day 41 after immunization) of the immune response (Fig. 4B). A contribution of CD4<sup>+</sup> T cell responses to antiviral protection could be excluded because tumor cells and virus replication were controlled in CD4<sup>+</sup> T cell-depleted animals (Fig. 4B). Thus, immunization with cells expressing ER-retained GPER cross-primed strong CTL responses that protected against infection with LCMV-WE.

**Modifications of GPER Determine Cross-Priming Potential.** Given that other studies failed to demonstrate cross-priming after immunization with tumor cells expressing full-length WT LCMV-GP (20, 21), we set out to determine whether it was the altered stability and subcellular localization, or the total quantity of GPER, that enabled cross-presentation. To address this question, BALB/c mice were immunized with titrated numbers of MC-GP or MC-GPER. CTL responses were analyzed by their ability to produce cytokines, to lyse target cells directly *ex vivo*, and to protect against *in vivo* challenge with a low-dose infection of LCMV-WE (Fig. 5). Consistent with the observations of others (20, 21), we found that MC-GP cells expressing WT LCMV-GP did not cross-prime CTL responses, and consequently failed to provide protection against challenge infection.

In contrast, antiviral protection after immunization with MC-GPER was consistently observed at a priming dose of as few as 50,000 cells, and could be detected even in a subset of the animals immunized with 5,000 cells (Fig. 5A). Spleens of these mice also contained LCMV-GP-specific cross-primed CTLs capable of cytokine production and of lysing gp283-labeled targets directly *ex vivo* (Fig. 5). These data demonstrate that GPER-expressing cells were at least 1,000- to 10,000-fold more potent at inducing CTL cross-priming than WT GP-expressing cells. Our earlier data showed that GPER is expressed at  $\approx 10$ -fold greater levels than WT LCMV-GP in murine fibrosarcoma cells (Fig. 1P). Therefore, the enhanced cross-presentation potential of GPER not only was determined by the increased antigen dose but very likely resulted from the modifications affecting its solubility, stability, and subcellular localization.

## Discussion

We have previously performed extensive studies of CTL priming against cell-associated LCMV-GP without detecting a contribution of cross-presentation to the induction of GP-specific CD8<sup>+</sup> T cells in response to GP-expressing fibroblasts (20, 21) or splenocytes derived from GP-transgenic mice (21). In addition, and in contrast to similar studies employing other model antigens, such as ovalbumin (27, 28), LCMV-GP is also not cross-presented when expressed in pancreatic islet cells (19, 29). In the present study we show that the normally cross-presentation-“resistant” LCMV-GP can be converted into a potent antigen for CTL cross-priming by several modifications that affect the solubility and stability of the trimeric GP complex, its subcellular localization, and the quantity expressed on a per-cell basis.

The variant GPER differed from the WT GP by its lack of a transmembrane domain, the addition of a phage-derived trimerization-promoting domain, and by its resistance to processing into metastable GP-1/GP-2 complexes because of a mutated protease cleavage site. Although all of these modifications very likely have increased the stability of the GP trimeric complex, they also led to the retention and accumulation of the variant protein within the ER. Because the capacity of both proteins to cross-prime CTL represented a difference of  $>3$ – $4$  orders of magnitude, the increased CTL cross-priming efficiency of GPER cannot be attributed to the accumulation of  $\approx 10$ -fold higher protein levels alone. Instead, both the presence of soluble, noncleavable, and trimer-stabilized GP complexes and the protein's retention in the ER are likely to be responsible for its acquired ability to be cross-presented.

Cross-presented antigens are preferentially long-lived proteins (15–18). Presumably GPER formed stable, soluble trimeric complexes that might survive longer in the endosomal pathway of the APC, which in turn could increase the chance of translocation of sufficient amounts into the cytosol for antigen processing and cross-presentation. That degradation in the APC phagosome critically limits the availability of protein for cross-presentation is illustrated by the observation that blocking phagosome acidification enhances CTL cross-priming *in vivo* (30). Importantly, the proteolytically activated GP-1/GP-2 complexes of WT GP dissociate on endosomal acidification (25) and expose the N-terminal sequences of GP-2 containing the gp283 epitope. Premature degradation of this CTL epitope may therefore be limited or prevented in noncleavable GPER where gp283 remains buried in the interior of the GP-1/GP-2 complex. In addition to its increased stability, the ER retention of GPER may have also promoted cross-presentation. Although both cell membrane-bound WT GP and ER-retained GPER would be phagocytosed as cell-associated antigens, accumulation of GPER within the ER could enhance cross-presentation by providing the antigen in a more concentrated manner. Furthermore, it is conceivable that, on cell death, the GPER might be enclosed within ER-derived membranes or vesicles that could

further protect the protein from rapid degradation within the endocytic pathway. Yet another aspect of ER retention should be considered. Although GPER had trimerized and folded overall correctly to generate the conformational GP-1 epitope detected by the antibody KL25 (see Fig. 1C), the observed ER-retention phenotype was likely caused by some minor folding and/or export defect. This phenotype, in turn, would lead to reassociation with chaperones, which has been reported to enhance delivery of proteins and peptides to APCs for cross-presentation (12, 17). In addition, misfolded proteins are retrotranslocated into the cytosol and destroyed via the ER-associated degradation pathway (ERAD) (31). Thus, GPER-expressing donor cells may contain higher numbers of antigenic peptides derived from continuous degradation of misfolded and accumulated protein.

Finally, our observations may also signify a more general phenomenon. Although CTL cross-priming was not observed for LCMV under WT conditions, other virus infections may well induce overexpression and accumulation of viral proteins in the ER, and thus trigger the ER-stress response (32). While this cellular response to unfolded proteins is known to assist protein folding by up-regulating molecular chaperones, it may also promote apoptosis of infected cells after prolonged exposure to ER stress (33). In view of our data, it is tempting to speculate that viral proteins accumulating in the ER of infected cells undergoing ER stress-triggered apoptosis could be used by the immune system as a source of antigen for cross-presentation. Thus, the ER quality control might be linked to antigen cross-presentation via the ER stress response, in a manner similar to that already recognized for direct MHC I presentation via the ERAD.

In summary, our findings identify the ER as a cellular compartment that efficiently provides antigen for CTL cross-presentation, and they show that modifications affecting the stability and subcellular localization of an antigen can drastically enhance its delivery into the cross-presentation pathway.

## Materials and Methods

**Cell Lines and Constructs.** All cell lines were originally obtained from the American Type Culture Collection (ATCC) (Manassas, VA). LCMV-GP constructs were prepared by using a full-length human codon-optimized GP sequence provided by D. von Laer (34). GPER is composed of amino acids 1–353 of LCMV-GP with a mutated SKI-1/S1P cleavage site (R262A; ref. 24) fused to the phage T4-derived fibrin sequence GYIPEA-PRDGQAYVRKDGWVLLSTFL (35).

**Immunofluorescence Microscopy.** The monoclonal antibody KL25 (36), the polyclonal  $\alpha$ -calnexin rabbit serum (37), and the GFP-KDEL expression plasmid (38) have been described. Cells were fixed with 2% formaldehyde, blocked and permeabilized for 20 min with 10% goat serum/5% FCS/10 mM glycine/0.05% saponin in PBS, followed by staining with primary antibodies (KL25 at 10  $\mu$ g/ml and anti-calnexin serum at 1:1,000) for 2 h. Cells were washed, and stained with secondary antibodies (anti-rabbit IgG AlexaFluor 647, anti-rabbit IgG AlexaFluor 488, and anti-mouse IgG AlexaFluor 594; all from Molecular Probes, Invitrogen, Basel, Switzerland; at 1:400) for 45 min. Coverslips were rinsed with water and mounted with Immuno-Mount (Thermo Shandon, Pittsburgh, PA). Images were recorded with a confocal microscope (model LSM510 m; Zeiss, Feldbach, Switzerland) equipped with a  $\times 100/1.40$  Plan-Apochromat objective.

**Pulse-Chase.** Transfected HeLa cells were starved for 2 h with DMEM lacking methionine and cysteine, labeled with 72  $\mu$ Ci/ml [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine (1 Ci = 37 GBq) for 30 min, washed, and then chased for 3 h with normal medium. Cells were lysed (1% Triton X-100), nonsoluble material was removed by centrifugation,

and supernatants were incubated overnight with protein A-Sepharose and KL25 at 4°C. Peptide-N-glycosidase F and Endo H were used as described by the supplier (New England Biolabs, Frankfurt am Main, Germany). Samples were reduced and analyzed by SDS/PAGE on 10–16% polyacrylamide gels, followed by phosphorimaging using a BAS 1500 (Fuji, Tokyo, Japan) for <sup>35</sup>S-labeled proteins.

**Mice and Viruses.** C57BL/6 mice, 318 TCR-transgenic mice (39), RIP-GP mice (19), and H8 mice (40) were bred at the Institut für Labortierkunde, University of Zurich. BALB/c (H-2<sup>d</sup>) mice and CB6F1 (H-2<sup>bxd</sup>) mice were from Harlan (Horst, The Netherlands). BALB/c and H8 mice were crossed to obtain B/c × H8 F<sub>1</sub> (H-2<sup>bxd</sup>) mice. All animal experiments were performed according to institutional guidelines and Swiss federal regulations, and were approved by the veterinary office of the Kanton of Zurich. T cell responses of tumor-immunized mice were assessed in peritoneal exudate cells and spleens. For challenge experiments, tumor-primed mice were infected intravenously with 200 pfu of LCMV-WE as indicated. After 4 days, spleens were collected for analysis of T cell responses and virus titers (41). RIP-GP mice (19) were injected with tumor cells or LCMV, and blood glucose concentration was monitored by using a Glucometer Elite (Bayer, Zurich, Switzerland). LCMV-WE was propagated on L929 cells. Vaccinia virus expressing the full-length LCMV-GP (VV-G2) was propagated on BSC40 cells.

**Native Western Blotting.** GPER complexes were assessed in MC-GPER cell lysates and in 40-fold concentrated cell supernatant. Cells were disrupted in lysis buffer (1% octyl glucopyranoside/0.15 M NaCl in PBS) by Dounce homogenization. Samples were not heated and were separated by SDS/PAGE under nonreducing conditions, blotted onto nitrocellulose membranes, and detected by using KL25 (5 μg/ml) followed by secondary HRP-coupled goat anti-mouse IgG1 (Zymed, San Francisco, CA).

**Flow Cytometry Staining.** Lymphocytes were stained with H-2D<sup>b</sup>/gp33 or H-2D<sup>b</sup>/gp276 tetramers in FACS buffer (FB = PBS/2% FCS/20 mM EDTA) for 10 min at 37°C. Then anti-CD62L-FITC and anti-CD8a-APC (both BD Pharmingen, Basel, Switzerland) were added, and staining continued at 4°C for 30 min. Cells were washed, fixed (FACS Lysis Solution, BD Pharmingen), and analyzed on a FACSCalibur (BD Biosciences, Basel, Switzerland) by using the CellQuest software. To assess GP expression, 10<sup>6</sup> fixed tumor cells were permeabilized in permeabilization buffer (PB = FB containing 0.1% wt/vol saponin) for 5 min at 4°C, washed, and stained with KL25 in PB at 4°C for 60 min. Cells were then washed, and bound antibody was detected by FITC-labeled goat anti-mouse IgG (BD Pharmingen).

**Intracellular Cytokine Staining.** Spleen cells or peritoneal exudate cells were cultured in IMDM containing 5 μg/ml brefeldin A, 50 units/ml IL-2, 10% FCS, and antibiotics for 6 h at 37°C, either with or without specific peptide (10<sup>-6</sup> M). Cells were first stained with anti-CD8a-PE (BD Pharmingen) for 20 min at 4°C, then fixed with 4% paraformaldehyde for 5 min at 4°C, permeabilized with PB for 5 min at 4°C, and stained with anti-mouse IFNγ-APC and anti-mouse TNFα-FITC (both BD Pharmingen) in PB for 60 min at 4°C. After three washes, cells were resuspended in FB and analyzed by flow cytometry as stated above.

**CTL Assay.** Cytotoxic T cell responses in spleens of tumor- and virus-primed mice were tested against peptide-pulsed and non-pulsed <sup>51</sup>Cr-labeled P815 (H-2<sup>d</sup>) cells in a standard chromium-release assay directly *ex vivo*. Direct MHC class I presentation of GP-derived CTL epitopes by GP-expressing cells was tested by using LCMV-specific CTLs as effectors and <sup>51</sup>Cr-labeled tumor cells as targets either with or without pulse with gp33 peptide.

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