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Efficient Cross-Priming of Antiviral CD8$^+$ T Cells by Antigen Donor Cells Is GRP94 Independent[1]

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Cross-priming, the activation of naive CD8$^+$ T cells by dendritic cells presenting Ags synthesized by other cells, is believed to play an important role in the generation of antiviral and antitumor responses. The molecular mechanism(s) underlying cross-priming remain poorly defined and highly controversial. GRP94 (gp96), an abundant endoplasmic reticulum chaperone with innate immune-activating capacity, has been widely reported to play a major role in cross-priming. In this study, we show that cells whose expression of GRP94 is silenced via transient or stable transfection with GRP94-directed small interfering RNAs demonstrate no reduction in their abilities to generate class I peptide complexes in cultured cells or to prime antiviral CD8$^+$ T cell responses in vivo. In demonstrating the dispensability of GRP94, our finding points to the importance of alternative mechanisms for generation of class I peptide complexes from endogenous and exogenous Ags and immunogens. *The Journal of Immunology,* 2009, 183: 4205–4210.

A ntiviral CD8$^+$ T cells recognize viral oligopeptides bound to cell surface MHC class I molecules. CD8$^+$ T cells can play critical roles in antiviral immunity, where they limit viral replication by either lysing infecting cells or releasing cytokines with direct or indirect antiviral activities. For a number of viruses where traditional vaccines that elicit neutralizing Abs are ineffective, there is great interest in developing vaccines that elicit effective antiviral CD8$^+$ T cell responses.

The mechanism of naive CD8$^+$ T cell activation is a critical issue for rationally designing CD8$^+$ T cell-inducing vaccines. There is compelling evidence that naive CD8$^+$ T cells are activated by dendritic cells (DCs)[3] located in lymphatic tissues (lymph nodes and spleen) with access to active areas of viral infection (1). It is uncertain, however, to what extent CD8$^+$ T cell activation follows DC presentation of peptides derived from de novo protein synthesis in the presenting DC itself (direct priming) vs peptides derived from extracellular sources, i.e., acquired from virions or infected cells (cross-priming) (2).

The origin of peptides derived from de novo protein synthesis is reasonably well established; a fraction of all protein synthesis is subject to rapid degradation and the resulting peptides provide the relevant Ag source for direct priming (3). Debate continues,

however, over how Ags are transferred from infected cells (or tumor cells) to DCs during the process of cross-priming. Evidence from in vitro models supports two major cross-priming routes: 1) Ag internalization, transfer to the cytosol, degradation by proteasomes, and subsequent peptide transfer/loading on MHC class I molecules (4–7) and 2) receptor-mediated uptake of molecular chaperone-peptide complexes, in particular GRP94, and transfer of the peptide, by an as yet undetermined pathway, to MHC class I molecules in the endoplasmic reticulum (ER) (8, 9). Evidence for the post-ER transfer of GRP94-associated peptides to recycling MHC class I molecules has also been presented (10).

Glucose-regulated protein 94 (GRP94, also known as gp96), is a highly abundant ER chaperone that participates in the biogenesis of numerous cell surface and secreted proteins, including a subset of integrins and Toll-like receptors (11–14). A number of reports conclude that GRP94-associated peptides are efficiently acquired by DCs, with CD91, SRA, Lox-1, or CD36 serving as the relevant endocytic receptors (15, 16). GRP94 is also reported to exhibit adjuvant activity by inducing proinflammatory cytokines and chemokines that promote DC maturation (17, 18). The molecular origin and basis of the adjuvant activity attributed to GRP94 is uncertain. The capacity of GRP94 to tightly bind endotoxin in conjunction with the technical hurdle of preparing GRP94 with biologically insignificant levels of endotoxins and purification-derived contaminants (i.e., Con A, a lectin frequently used for GRP94 purification), has lent a significant element of uncertainty to this function (19–21). Also, it has not yet been demonstrated that GRP94 functions in vivo as a peptide-binding protein, and the biochemical basis for GRP94 function as a bona fide (i.e., specific, regulated, and reversible) peptide-binding protein has been questioned (19, 22–25).

None of these studies is conclusive, however, and GRP94 is being actively pursued as a CD8$^+$ T cell vaccine in human clinical trials (26, 27). Since it is of obvious interest and importance to explore its role in cross-priming, we have examined the effect of GRP94 gene knockdown on the cross-priming capacity of cells infected with vaccinia virus (VV), influenza A virus (IAV), or vesicular stomatitis virus (VSV).

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[3] Abbreviations used in this paper: DC, dendritic cell; ER, endoplasmic reticulum; GRP94, glucose-regulated protein 94; IAV, influenza A virus; neo, aminoglycoside phosphotransferase; NP, nucleoprotein; VV, vaccinia virus; VSV, vesicular stomatitis virus; siRNA, small interfering RNA; PEC, peritoneal exudate cell; shRNA, short hairpin RNA.

Table I. *Peptides used*

Name	Sequence	Protein/Virus
OVA$_{257}$	SIINFEKL	Chicken OVA
B8R	TSYKFESV	VV
K3L	YSLPNAGDVI	VV
B2R	YSQVNKRYI	VV
A3L	KSYNYMLL	VV
A42R	YAPVSPIVI	VV
L2R	VIYIFTVRL	VV
A23R	IGMFNLTFI	VV
J3R	SIFRFLNI	VV
A8R	ITYRFYLI	VV
VSV-N52	RGYVYQGL	VSV
PA224	SSLENFRAYV	IAV
NP366	ASNENMDAM	IAV
PB1-F2–62	LSLRNPILV	IAV
NP55	RLIQNSLTI	IAV
NS1–114	RTFSFQLI	IAV

Materials and Methods

Mice

All experiments used 6- to 10-wk-old female C57BL/6J mice purchased from Taconic Farms. Mice were housed in the animal care facility at the National Institute of Allergy and Infectious Diseases (NIAID) under specific pathogen-free conditions, maintained on standard mouse chow and water provided ad libitum, and cared for with strict adherence to animal regulations and all studies were performed on an Institutional Animal Care and Use Committee-approved animal study proposal.

Peptides and Abs

Peptides were synthesized and characterized by the Biologic Resource Branch, National Institute of Allergy and Infectious Diseases. Peptides used in this article are described in Table I. For each synthetic peptide, mass spectrometric analysis indicated that the predicted mass form constituted >95% of the material analyzed. Peptides were dissolved in DMSO at 10 mM and stored at −20°C. Abs to GRP94 (rat IgG2a Ab 9G10) were from StressGen. Rabbit anti-peptide Abs were specific for ER resident proteins.

Cell culture and transfections

L-K^b (L929 stably transfected with K^b molecule), HEK-293–94KD, and HEL293-SCRM (HEK-293 stably transfected with plasmids encoding a GRP94-directed or sequence scrambled siRNA were cultured in DMEM containing 10% FBS and DC-like DC2.4 cells (H-2^b) were cultured in IMDM containing 10% FBS. NP-S-EGFP plasmid transient transfections were performed with Lipofectamine 1 day before injections. Transient siRNA transfections were conducted by transfecting 1–2 × 10^6 L-K^b cells in solution V using Amaxa nucleofector and the T-24 program. siRNA specific for GRP94 and neo (5′- pGGCUCAAGGACAGAUGAUGtt and pAAUGAACUGCAGGACGAGGCAtt, respectively) were used at 10 nM. As indicated, cells were infected 3 to 5 days after siRNA transfection. All siRNAs were purchased from Dharmacon. siRNA knockdown efficacy was determined by immunoblotting using GRP94-specific Ab.

Viral infections

rVVs or rVSVs containing the fusion protein mCherry-ub-OVA$_{257}$, NP-SIINFEKL-EGFP, NP-S-mCherry, or OVA were used at a multiplicity of infection of 10. Cells were incubated with the viruses for 20 min at 37°C, with occasional mixing, in balanced salt solution containing 0.1% BSA. For the PR8-OVA infections (multiplicity of infection = 10), cells were washed and resuspended in autoclavable minimal essential medium with 0.1% BSA at pH 6.8 and incubated at 37°C for 1 h. Cells were then incubated at 37°C in growth medium for the remainder of the assay.

Flow cytometry

K^b-OVA$_{257}$ levels were determined by incubating cells for 30 min on ice with 25-D.1.16 Ab conjugated with Alexa Fluor 647. K^b levels were determined by FITC-conjugated anti-K^b Ab (AF6-88.5; BD, Pharmingen). Cellular FITC and Alexa Fluor 647 levels were determined by an LSR II

(BD Biosciences) using FACSDiva (BD Biosciences) software and data were analyzed using FlowJo (Tree Star) software.

Cross-priming experiments with intracellular cytokine staining

HEK-293–94KD and HEL293-SCRM cells were infected with rVVs or rVSV at 10 PFU/cell, incubated for 6 h at 37°C to allow for viral gene expression, and then exposed to 254 nM light at 4 mW/cm^2 for 1 h on ice. This treatment suffices to reduce the PFUs to below detection and abrogate the cross-priming capacity of cells infected with rVVs expressing short-lived minigene products (28). Alternatively, cells were infected for 5 h with A/Puerto Rico/8/34 influenza virus (PR8) with SIINFEKL inserted into the neuraminidase (29). To prevent infection with PR8, cells were coinjected with a sufficient amount of the neutralizing mAb H28-E23 to block the immunogenicity of a substantial dose of PR8 (30). For each condition, 1 × 10^7 cells were injected i.p. per mouse. Six days subsequent, for the vaccinia model, and 7 days subsequent, for the influenza model, splenic and peritoneal cells were harvested from primed animals, suspended in RPMI 1640 medium plus 10% FBS, and seeded at 2 × 10^6 cells/well in U-bottom 96-well plates. Peptides were added to wells to a final concentration of 0.5 μM. Cells were incubated initially for 2 h at 37°C and then for 3 h with brefeldin A (Sigma-Aldrich) at 10 μg/ml. Dead cells were first stained with ethidium monoazide at 5 μg/ml in balanced salt solution/BSA for 15 min in the dark on ice followed by 15 min on ice in light. Cells were then stained with FITC anti-CD8α mAb on ice for 30 min, washed, and fixed with 1% paraformaldehyde in PBS at room temperature for 20 min, then further stained with Alexa Fluor 647-conjugated anti-IFN-γ in PBS containing 0.1% saponin (Sigma-Aldrich). Stained cells were analyzed on an LSR II flow cytometer with live-gate on the CD8$^+$ cells. A total of 150,000 cells were normally acquired and analyzed with FlowJo software (Tree Star).

OT-I proliferation experiments

OT-I cells (TCR-transgenic CD8$^+$ T cells specific for K^b-OVA$_{257}$) were purified from spleen and lymph nodes of OT-I mice by positive selection with magnetic beads (Miltenyi Biotec). Cells were labeled with CFSE (1.7 mM for 10 min) and injected via the tail vein (2.5 × 10^6/mouse) into CD45.1 C57BL/6 mice. One × 10^7 HEK-293 cells transfected with DNA or infected with different viruses as described above were injected i.p. 3 h after injection of OT-I cells. Spleens were harvested ~72 h after OT-I injection and OT-I proliferation was determined by staining for CD8 and CD45.1 and gating for CFSE-labeled CD8$^+$CD45.1$^+$ cells by an LSR II flow cytometer. A total of 2 million events were normally acquired and analyzed with FlowJo software (Tree Star).

Results

Suppression of GRP94 expression does not diminish Ag processing and presentation

GRP94 has been proposed to play at least two critical roles in Ag processing and presentation: acceptor of peptides transported into the ER by the TAP and aminopeptidase functioning in the proteolytic trimming of TAP-transported peptides to a length optimal for class I binding (31). As peptide acceptor, GRP94 is thought to bind the antigenic peptide repertoire of the cell, thereby enabling it to serve as an ideal peptide vector in Ag cross-presentation (32).

To identify a role(s) for GRP94 in the cross-presentation of viral Ags, we first examined the Ag presentation capacity of viral protein-expressing cells using control and GRP94-siRNA knockdown cell lines. We silenced GRP94 expression by transfecting L-K^b cells with siRNA specific for either GRP94 or, as a negative control, aminoglycoside phosphotransferase (neo). GRP94-siRNA reduced GRP94 expression to near undetectable levels (>95% reduction, relative to untransfected, mock-transfected, or neo-siRNA transfected cells) as measured by immunoblotting (Fig. 1A). siRNA-transfected cells were subsequently infected with either a rVV expressing a reporter fusion protein, NP-S-mCherry, consisting of IAV nucleoprotein (NP) containing in-frame OVA$_{257}$ peptide (SIINFEKL) followed by mCherry fluorescent protein, or a rVV expressing mCherry-Ub-S, consisting of mCherry fused to ubiquitin and terminating in the OVA$_{257}$ peptide. Previous studies have demonstrated that ubiquitin hydrolases cleave the fusion protein from the ubiquitin COOH terminus, releasing the peptide for
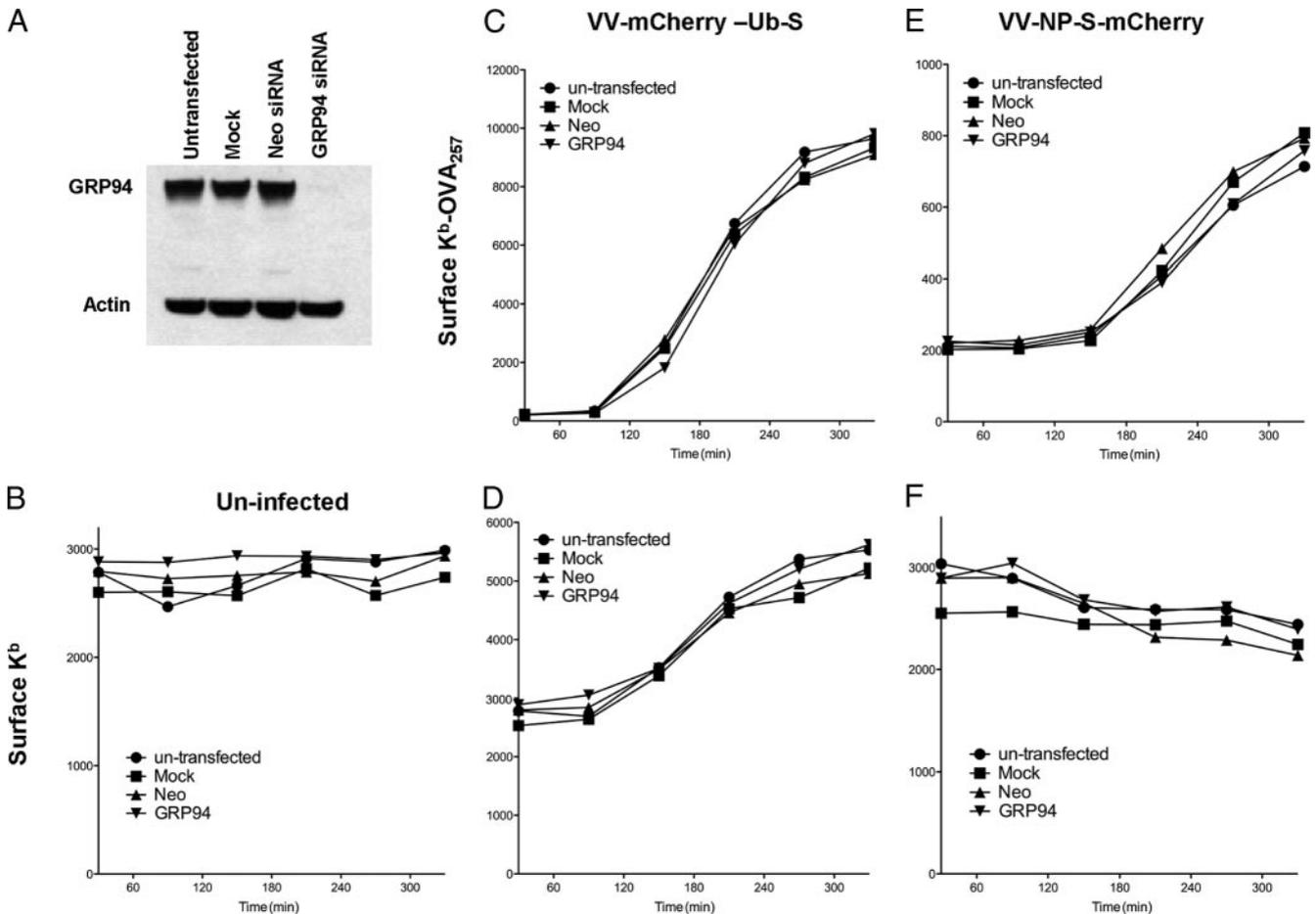
FIGURE 1. GRP94 has no essential role in Ag processing and presentation. *A*, Knockdown of GRP94 was confirmed by Western blot analysis. L-K^b cells were untransfected (●), mock transfected (■), transfected with control Neo siRNA (▲), or transfected with GRP94 siRNA (▼). Cells were infected 3–5 days later with VV-mCherry-Ub- S (*C* and *D*) or VV-NP-S-mCherry (*E* and *F*) or uninfected as a control (*B*). Samples were taken every 1h up to 5.5 h after infection. Levels of surface K^b-OVA$_{257}$ were then measured by staining with Alexa Fluor 647-labeled 25-D.1.16 (*C* and *E*), and levels of the surface K^b molecule (*B, D,* and *F*) were determined by staining with anti-K^b Ab (HB176) followed by a secondary FITC-conjugated anti-mouse Ab. The experiment was repeated two times.

presentation on MHC molecules (33) Following rVV infection, cell aliquots were sampled every hour for 5.5 h, and cell surface K^b-SIINFEKL complex levels were quantitated via flow cytometry using the 25-D.1.16 TCR-like mAb (34).

As seen in Fig. 1, GRP94 knockdown had no discernible effect on the capacity of cells to generate K^b-SIINFEKL complexes from NP-S-mCherry (Fig. 1*E*) or mCherry-Ub-S (Fig. 1*C*), which is a >10-fold efficient source of K^b-SIINFEKL due to the direct liberation of SIINFEKL from the nascent gene. The four-cell populations examined expressed the indicator fluorescent protein at nearly identical levels (data not shown). Silencing GRP94 expression did not diminish the total amount of conformed total cell surface K^b molecules in uninfected cells (Fig. 1*B*) or cells infected with either rVV (Fig. 1, *D* and *F*).

Together, these data indicate that GRP94 does not contribute to a rate-limiting step(s) in the generation of either viral or self-peptide-class I complexes or the biogenesis of MHC class I molecules.

In vivo analysis of GRP94 function in cross-priming

Next, we evaluated GRP94 function in cross-priming, by immunizing B6 mice with HEK-293 cells expressing NP-S-GFP from different vectors. Because they are of human origin, HEK-293 cells cannot directly present Ags to murine CD8$^+$ T cells (or donate K^b-SIINFEKL complexes to DCs via "cross-dressing" (35)).

Therefore, assuming cell- associated virus is completely inactivated, activation of naive CD8$^+$ T cells by injected HEK-293 cells must occur via cross-priming.

HEK293 cells were stably transfected with GRP94-targeting siRNA or siRNA encoding a scrambled version of the GRP94-targeting sequence. Via immunoblotting, GRP94 is undetectable in the knockdown cells, while the levels of TRAM, BiP, TRAPα, and Sec61 are similar between control and knockdown cells, indicating that ER resident chaperones and integral membrane proteins are expressed at normal levels in the absence of GRP94 expression (Fig. 2*A*). The lack of functional GRP94 was confirmed by the intracellular accumulation of TLR2 (Fig. 2, *B–D*), which requires GRP94 for export from the ER (36). In addition, silencing of GRP94 expression was not accompanied by discernible changes in cell doubling time or morphology (data not shown), consistent with previous studies where GRP94 expression was knocked out via chemical mutagenesis (37) (Fig. 2).

HEK-293 cells were infected with rVV or rVSV expressing NP-S-GFP or transfected with a plasmid expressing NP-S-GFP under the control of the CMV promoter and injected into B6 mice that had received OT-I TCR-transgenic CD8$^+$ T cells. OT-I cells are specific for K^b-SIINFEKL complexes and their division (monitored by CFSE fluorescence) following adoptive transfer provides a sensitive measure of cross-priming (38). If GRP94 contributes
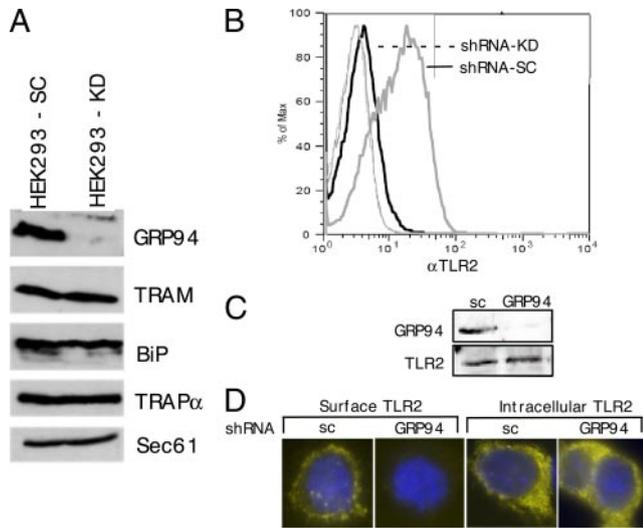
FIGURE 2. Knockdown of GRP94 from HEK-293 cells. *A*, Western blot analysis of HEK-293 cells stably transfected with GRP94 or control scramble siRNA to determined expression levels of GRP94 and other ER proteins indicated. *B–D*, Stable HEK293 cell lines expressing short hairpin RNA (shRNA) directed against GRP94 (shRNA-GRP) or scrambled shRNA control (shRNA-sc) were transfected with a plasmid encoding FLAG-tagged TLR2. Forty-eight hours posttransfection, TLR2 surface expression was examined by flow cytometry using FLAG epitope-specific mAb (*B*). Histograms for the shRNA-GRP94-expressing cells are represented by the dark line; the gray line represents the shRNA-sc-expressing cells. Immunoblot confirmed equal amounts of TLR2 expression in total cell extracts (*C*). The presence of TLR2 in the ER of GRP94 knockdown cells was shown by confocal microscopy of fixed, permeabilized cells (*D*). Examination of fixed, unpermeabilized cells confirmed the lack of surface expression in GRP knockdown cells.

significantly to cross-priming in an in vivo viral infection model, OT-I division would be predicted to be lower in the knockdown vs the control cell injection animals. As seen in Fig. 3, OT-I division was identical in all scenarios examined, indicating that suppression of GRP94 expression did not affect the cross-priming capacity of HEK-293 expressing NP-S-GFP via either infection or DNA transfection.

Efficient cross-priming of peptides derived from vaccinia and influenza viruses expressed in GRP94-silenced cells

We next determined the GRP94 contribution to cross-priming by measuring the ability of HEK-293 cells to induce primary CD8^{+} T cell responses to peptides encoded by rVVs expressing full-length OVA or recombinant IAV expressing SIINFEKL in the stalk region of the neuraminidase (39). In these experiments, mice were immunized by i.p. injection of rVV-infected cells and CD8^{+} T cell responses were measured at day 6. Analyses were performed on both peritoneal exudate cells (PECs) and splenocytes by ex vivo intracellular cytokine staining using SIINFEKL or immunodominant viral peptides or DMSO as a negative control. Total antiviral responses were estimated using APCs infected with VV or IAV. As seen in Fig. 4, GRP94 knockdown cells were capable of eliciting responses to SIINFEKL, eight VV, and five IAV determinants. Consistent with the results reported above, the overall in vivo antiviral immunological responses to GRP94 knockdown cells were equal or even greater than the control cells.

Discussion

Our findings demonstrate that for multiple Ags and viral systems, GRP94 is dispensable for the generation of both peptide class I complexes in cultured cells and immunogens in donor cells with cross-priming activity. In the latter case, although these findings do
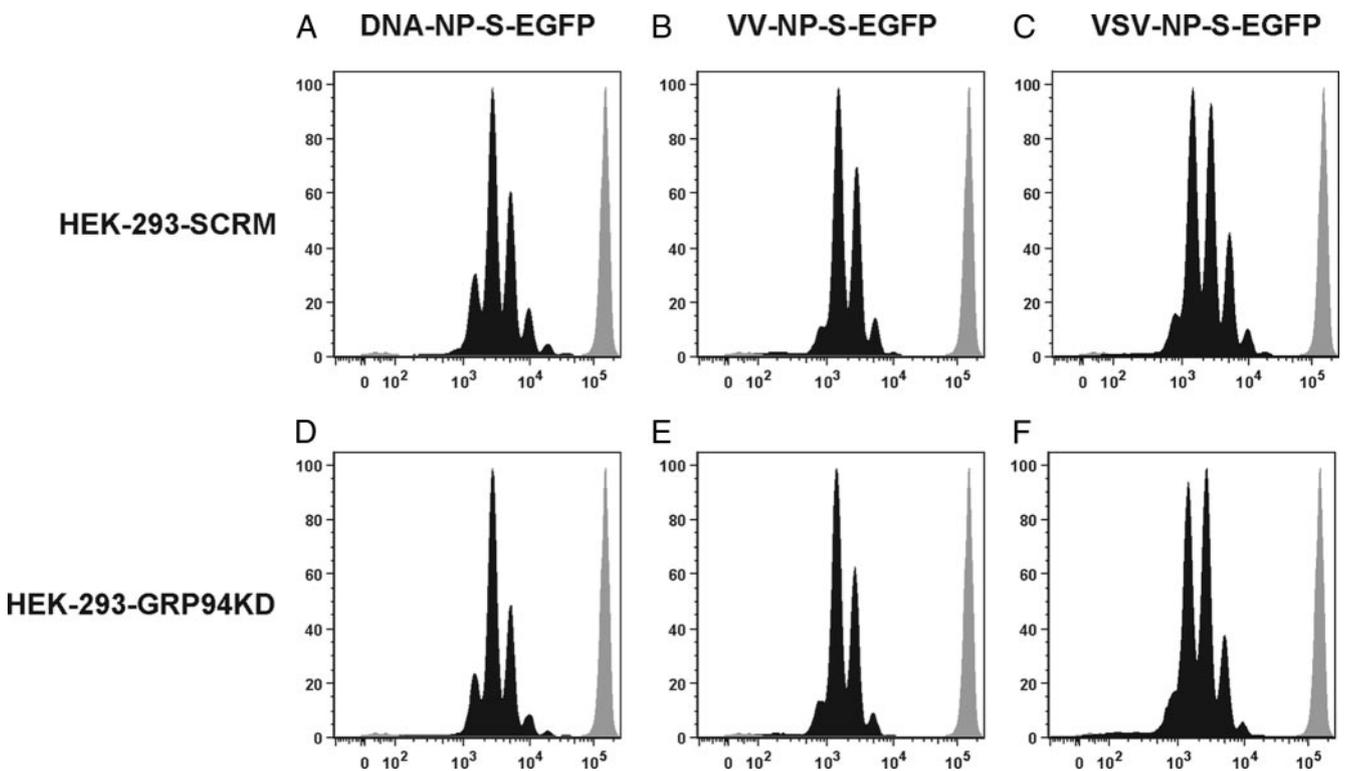
FIGURE 3. GRP94 is not required for in vivo OVA$_{257}$ cross-priming measured by OT-I CD8^{+} T cell division. Donor HEK-293 cells expressing GRP94 (*A–C*) or knockdown of GRP94 (*D–F*) were transfected with NP-S-EGFP DNA (*A* and *D*) or infected with VV-NP-S-EGFP (*B* and *E*) or VSV-NP-S-EGFP (*C* and *F*) and injected into B6 mice. The proliferation of CFSE-labeled OT-I CD8^{+} T cells was measured 3 days after adaptive transfer. In each graph, the black histogram represents the infected or transfected cells and the gray histogram represents control cells without infection or transfection. Each experiment was repeated two times.

FIGURE 4. GRP94 show no effect on cross-priming of viral peptides measured by intracellular staining for INF-γ. Mice were immunized i.p. with infected HEK-293 cells expressing GRP94 or GRP94 knocked down. Six days later, splenocytes and PECs were tested for IFN-γ accumulation following restimulation with the indicated peptides or infected DC2.4. DMSO was used as a negative no-peptide control. A, Cross-priming of cells infected with rVV-OVA. B, Cross-priming of cells infected with PR8-OVA$_{257}$. Values were subtracted from background obtained from wells receiving no peptides and are expressed as mean ± SE of three individual mice in each group for splenic responses. For peritoneal responses, PECs were pooled. Each experiment was repeated two times.
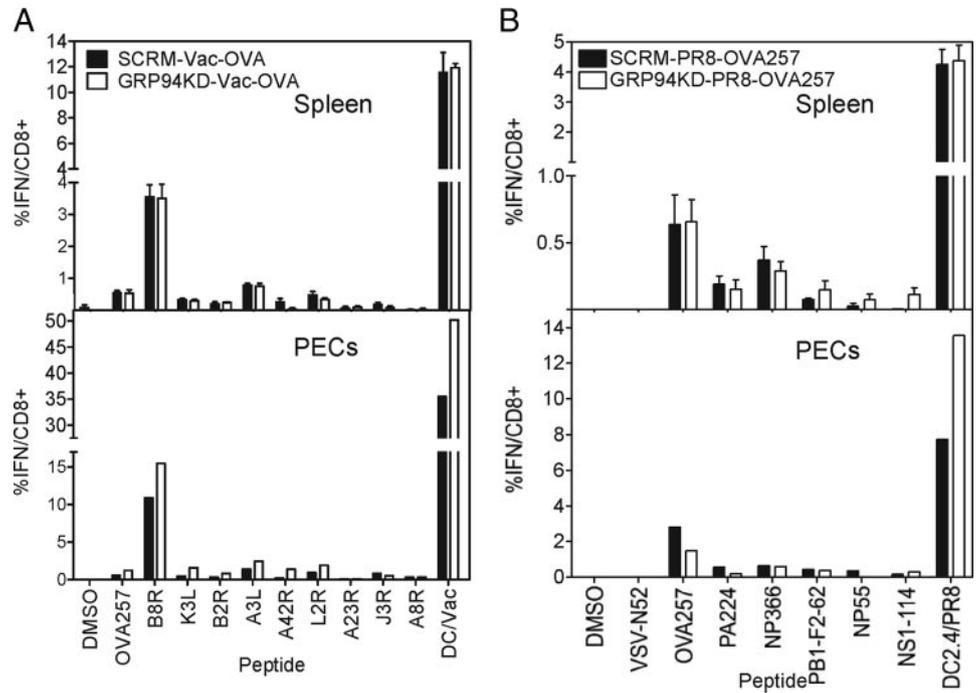
not address the possibility that other chaperones function in Ag presentation and cross-priming, they are consistent with previous studies that indicate that protein-based cross-priming is the rule rather than the exception (5–7, 40), and also from the original findings of Rammensee and colleagues (41), extended now to many systems, that naturally processed antigenic peptides are, with few exceptions (28), extremely short-lived in cells unless protected by binding to MHC class I molecules.

The view that chaperones and heat shock proteins could serve as universal immunogens, with application to human cancer immunotherapy, has generated tremendous interest among clinicians, immunologists, and chaperone biologists. In the case of GRP94, the available clinical trial data have not, however, convincingly demonstrated substantial utility for this application (42, 43).

Beyond the complexities that befall interpretation of clinical immunotherapy data, a key question remains unanswered, does GRP94 associate stably with antigenic peptides in vivo? Arguing against such a function are the findings of this study, the inability to detect a substantial and diverse population of associated peptides in biochemically purified GRP94 (18, 21–23) and, to a lesser degree, the kinetic/structural characteristics of the in vitro peptide complexation reaction, where associated synthetic peptides are stable to SDS (17, 44).

In contrast, in models using cells engineered to secrete GRP94, specific CD8$^+$ T cell responses to cell-derived Ags have been clearly demonstrated (45–48). Some measure of caution, however, is necessary in concluding that such data identify an in vivo peptide-binding function for GRP94. Because GRP94 elicits substantial innate immune responses, it is possible that GRP94-secreting (tumor) cells are de facto high-priority immunosurveillance targets and as a result are efficiently processed for in vivo cell-based cross-priming reactions.

From a clinical standpoint, whether the efficacy of tumor cell-secreted GRP94 derives from GRP94-peptide complexes or APC-dependent processing of GRP94-secreting (tumor) cells is of somewhat limited significance. Defining an essential role for GRP94 in Ag processing and/or peptide cross-priming does require, however, an unequivocal demonstration that GRP94 can sta-

bly associate with a diverse peptide pool and that such an activity is an intrinsic function of the native protein. Nearly two decades after the initial proposal of such an intrinsic activity (49), such evidence does not exist.

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Disclosures

The authors have no financial conflict of interest.

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Corrections

Lev, A., P. Dimberu, S. R. Das, J. C. Maynard, C. V. Nicchitta, J. R. Bennink, and J. W. Yewdell. 2009. Efficient cross-priming of antiviral CD8$^+$ T cells by antigen donor cells is GRP94 independent. *J. Immunol.* 183: 4205–4210.

Ref. 49 was not cited in the paper. At the end of the first section under *Results* (*Suppression of GRP94 expression does not diminish Ag processing and presentation*), the text should read as follows: "Together, these data indicate that GRP94 does not contribute to a rate-limiting step(s) in the generation of either viral or self-peptide-class I complexes or the biogenesis of MHC class I molecules, extending the previous findings of Lammert et al. (49) that GRP94 expression is not required for normal class I expression or CD8$^+$ T cell recognition of allo- or minor H Ags."

In *References*, Ref. 49 should be added as follows:

49. Lammert, E., D. Arnold, H. G. Rammensee, and H. Schild. 1996. Expression levels of stress protein gp96 are not limiting for major histocompatibility complex class I-restricted antigen presentation. *Eur. J. Immunol.* 26: 875–879.

In addition, during copyediting, a series of errors were introduced to the numbering of references. Hence, where the text of the article refers to any reference numbered 37 through 49, the corresponding reference in the *References* section is lower by one digit. For example, when the text refers to Ref. 39, the reference in the *References* section is 38.

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