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Molecular and Cellular Requirements for Enhanced Antigen Cross-Presentation to CD8 Cytotoxic T Lymphocytes

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MHC class I-mediated cross-presentation of CD8 T cells by APCs is critical for CTL-based immunity to viral infections and tumors. We have shown previously that tumor-secreted heat shock protein gp96-chaperoned peptides cross prime CD8 CTL that are specific for genuine tumor Ags and for the surrogate Ag OVA. We now show that tumor-secreted heat shock protein gp96-chaperoned peptides enhance the efficiency of Ag cross-presentation of CD8 CTL by several million-fold over the cross-presentation activity of unchaperoned protein alone. Gp96 also acts as adjuvant for cross-presentation by unchaperoned proteins, but in this capacity gp96 is 1000-fold less active than as a peptide chaperone. Mechanistically, the in situ secretion of gp96-Ig by transfected tumor cells recruits and activates dendritic cells and NK cells to the site of gp96 release and promotes CD8 CTL expansion locally. Gp96-mediated cross-presentation of CD8 T cells requires B7.1/2 costimulation but proceeds unimpeded in lymph node-deficient mice, in the absence of NKT and CD4 cells and without CD40L. Gp96-driven MHC I cross-presentation of CD8 CTL in the absence of lymph nodes provides a novel mechanism for local, tissue-based CTL generation at the site of gp96 release. This pathway may constitute a critically important, early detection, and rapid response mechanism that is operative in parenchymal tissues for effective defense against tissue damaging antigenic agents. The Journal of Immunology, 2007, 179: 2310–2317.

Cross-presentation of CD8 cells (1) by bone marrow-derived APCs is critical for the induction of cytotoxicity against intracellular pathogens and tumors (2–6), but the physiological nature of the cross-presentation Ag taken up by APCs and cross-presented by MHC I is controversial (7–10).

Heat shock proteins chaperone peptides that can be taken up by APCs and cross-presented to CD8 cells (11–15). Exogenous heat shock protein (HSP)3 are actively captured by CD91 and LOX-1 APCs and cross-presented to CD8 cells (11–15). Exogenous heat presented by MHC I is controversial (7–10).


Materials and Methods

Mice

Wild-type (wt) and B7.1, B7.2, B7.1/2, CD40L, lymphoxygenin α (LTα), and CD4-deficient mice in the C57BL/6 (B6) background were obtained from The Jackson Laboratory, B6.Js1218 (NKT deficient, renamed Js1218 knockout (ko)) were provided by Dr. M. Lotze (University of Pittsburgh Medical Center, Pittsburgh, PA) with permission from Dr. Taniguchi (Chiba University, Chiba, Japan) (29). GFP-transgenic mice were obtained by permission of the producers (28). C57BL/6 OT-I mice were obtained from Dr. M. Bevan (University of Washington School of Medicine, Seattle, WA) (27). All mice were used at 6–12 wk of age.

Cell lines

EG7, the OVA-transfected EL4 lymphoma line, generously provided by Dr. M. Bevan, was further transfected with the vector pCMG-His containing gp96-Ig as described previously (26). NIH 3T3 cells were transfected with OVA in pAC-neo-OVA (generously provided by Dr. M. Bevan) and with pCMG-His containing gp96-Ig.

Antibodies

Fluorescent Abs were purchased from BD Pharmingen and eBioscience.

Purification and adoptive transfer of OT-I cells

GFP-marked OT-I cells were purified by positive selection with anti-CD8 antibody (BD Biosciences) and used for adoptive transfer at an average of 1 × 106 per mouse. GFP-OT-I cells were adoptively transferred through tail veins of C57BL/6 mice in a volume of 0.3 ml of PBS.

Immunization

Two days after adoptive transfer of GFP-OT-I, 2–4 × 106 nonirradiated EG7-gp96-Ig cells or control EG7 cells were injected i.p. in a volume of 0.5 ml of PBS. For some experiments, mice were immunized i.p. with 3T3-OVA-gp96-Ig, 3T3-OVA, or intact OVA (Sigma-Aldrich) dissolved in PBS.

Ex vivo Ag cross-presentation and cross-presentation of OT-I

Groups of B6 wt mice were i.p. immunized with 2 × 106 3T3-OVA-gp96-Ig or 3T3-gp96-Ig. After 3 days peritoneal exudate cells (PEC) were collected and 107 PEC were cocultured with naïve CFSE-labeled OT-I at different ratios (5:1, 10:1, 100:1, and 1000:1) for 48 and 72 h in round-bottom 96-well microtiter plates in 200 μl of tissue culture medium. CFSE-labeled OT-I were also cocultured directly with 3T3, 3T3-OVA-gp96-Ig and 3T3-gp96-Ig. After the indicated period of time, cells were...
collected and stained with anti-CD8-PE. OT-I expansion was measured by CFSE dilution as analyzed in an LSR II flow cytometer (BD Biosciences). Cell division was analyzed by CFSE dilution in gated lymphocytes or in total CFSE+ cells and expressed as the percentage of total CFSE+ cells.

BrdU labeling and analysis
Mice were administered BrdU (Sigma-Aldrich) in their drinking water (0.8 mg/ml) at the time of immunization. Cell samples were analyzed by staining for BrdU (eBioscience) after fixation and permeabilization.

Results
CD4 cells inhibit Ag cross-presentation to CD8 CTL by HSP gp96-peptide
Previous data indicated that tumor cells transfected with a secretable form of gp96, gp96-Ig, become immunogenic and induce tumor-specific immunity in mice (26). Tumor immunity required CD8 cells but was independent of CD4 cells in either the afferent or efferent arm of the immune response.

Because CD4 cells express both helper cell and regulatory cell activity, we were interested to determine whether any of these opposing functions regulated cross-presentation of CD8 cells by gp96. We used adoptively transferred Kb-OVA-specific TCR-transgenic CD8 cells, OT-I (27), to quantitate CTL expansion in response to EG7-gp96-Ig or EG7 immunization i.p. (25). EG7 is the OVA-transfected EL4 lymphoma. OT-I expansion in this system has previously been shown to be dependent on gp96-chaperoned OVA peptides and not to be influenced by the Ig-Fc-tag because gp96-myec was equally active. The tumor-secreted gp96-Ig immunization system also generates immunity to genuine tumor Ags. However, measuring OT-I expansion provides a more precise and rapid read-out than measuring tumor regression.

In CD4 ko, all CD4 functions (helper and regulatory) are deleted, whereas in CD40L ko primarily the helper cell function of CD4 cells is missing. We therefore compared OT-I expansion in response to gp96-OVA secreted by EG7-gp96 in CD4 ko and in CD40L ko vs wt mice (Fig. 1). To facilitate analysis, OT-I TCR-transgenic cells were also GFP-marked (GFP-OT-I) by breeding OT-I mice with GFP-transgenic mice (28). In comparison to wt mice, OT-I expansion in CD4 ko mice was increased by 100% in response to EG7-gp96-Ig, suggesting that the presence of CD4 cells interferes with CD8 clonal expansion (Fig. 1A). In contrast, in CD40L ko mice OT-I expansion was similar to the expansion in wt mice (Fig. 1B). The data indicate that CD4 helper function mediated via CD40L is not required for gp96-mediated Ag cross-presentation to CD8 CTL. CD4+ T regulatory cells in contrast, absent in CD4 ko, normally down-regulate OT-I cross-priming by gp96-

FIGURE 1. Gp96-mediated cross-priming of CD8 T cells is enhanced in the absence of CD4 cells but not affected by the absence of CD40L. Mice received 1 million GFP-OT-I i.v. and were immunized 2 days later i.p. with 4 million EG7-gp96-Ig. Cells were harvested from PC after 5 additional days and analyzed for GFP-OT-I frequency in the CD8 gate by FACS. Values are expressed as absolute numbers of GFP-OT-I in the PC. A, CD4-deficient mice compared with wt mice. B, CD40L-deficient mice.

OVA. Whether inhibition is achieved at the level of Ag cross-presentation by APCs or at the level of the OT-I effector cell is under study.

CD8 cross-priming by gp96 is dependent on B7.1 and B7.2 and independent of NKT cells
Efficient T cell priming requires DC maturation and up-regulation of MHC and costimulatory molecules, frequently mediated through CD40 signals. However, CD4 help via the CD40L/CD40 axis clearly is not needed for gp96-mediated OT-I priming (Fig. 1). Gp96 binds to CD91 and to TLR2 and TLR4 (16, 23) and thereby may be able to activate DC independent of CD40. We determined whether this mechanism of cross-priming of CD8 cells in vivo relied on B7.1 (CD80) and B7.2 (CD86) costimulation. Mice deficient either for B7.1 or B7.2 alone (Fig. 2A) were able to costimulate gp96-mediated cross-priming of OT-I at ~50% efficiency of wt mice. However, in the complete absence of both, B7.1 and B7.2, in double-deficient mice (Fig. 2B), gp96-mediated cross-priming of OT-I was completely abrogated.

NKT cells are frequently involved in antitumor immunity (29–31); their role in gp96-mediated priming of CD8 T cells is not known. Jα18 ko mice lack NKT cells due to their inability to generate the invariant TCR vα14 chain characteristic for CD1d-restricted invariant NKT cells (29). The ability of Jα18 ko mice to support undiminished OT-I expansion (Fig. 2C) suggests that NKT cells are not essential for gp96-mediated OT-I cross-priming.

Ag cross presentation by gp96 does not require lymph nodes
Draining lymph nodes bring together APCs, CD4 helper cells, CD8 CTL precursors and NK cells, promote cellular interactions, and enhance CTL priming and expansion. Because gp96-mediated Ag cross-priming is independent of CD4 help, we raised the question of the requirement for draining lymph nodes for OT-I expansion. LTα-deficient mice lack peripheral and mesenteric lymph

FIGURE 2. Gp96-mediated cross-priming of CD8 T cells requires CD80 and CD86 and is independent of NKT cells. Mice received 1 million GFP-OT-I i.v. and were immunized 2 days later i.p. with 4 million EG7-gp96-Ig (A and C) or 2 million 3T3-OVA-gp96-Ig (B). Cells were harvested from the spleen (SP) or the PC after 5 additional days and analyzed for GFP-OT-I frequency in the CD8 gate by FACS. A, CD80 or CD86 single deficiency; B, CD80/CD86 double deficiency; and C, NKT deficiency (Jα18 ko).
nodes, including Peyer’s patches, and are impaired in antiviral responses (32). However, when analyzed for gp96-OVA-mediated OT-I expansion, LTα deficiency, representative FACS data. Mice received 1 million GFP-OT-I i.v. and were immunized 2 days later i.p. with 2 million 3T3-OVA-gp96-Ig. Cells were harvested from the indicated sites after 5 additional days and were analyzed for GFP-OT-I frequency in the CD8 gate by FACS. A. Same data presented as histograms. Data are representative of two independent experiments, each bar represents the mean ± SE of two mice. C. Ex vivo cross-priming of OT-I by 3T3-OVA-gp96-Ig. PEC harvested from mice injected i.p. 3 days earlier with 3T3-OVA-gp96-Ig, 3T3-gp96-Ig, or 3T3 were incubated with CFSE-labeled OT-I for 72 h at ratios of 1:10, 1:100, and 1:1000 OT-I:PEC (a–f). As additional controls, 3T3 transfectants were also incubated directly with OT-I in vitro. Cells were stained with anti-CD8-PE and analyzed for CFSE dilution, which is plotted in b–h.

FIGURE 3. Efficient cross-priming by gp96 in the absence of lymph nodes. A, LTα deficiency, representative FACS data. Mice received 1 million GFP-OT-I i.v. and were immunized 2 days later i.p. with 2 million 3T3-OVA-gp96-Ig. Cells were harvested from the indicated sites after 5 additional days and were analyzed for GFP-OT-I frequency in the CD8 gate by FACS. B. Same data presented as histograms. Data are representative of two independent experiments, each bar represents the mean ± SE of two mice. C. Ex vivo cross-priming of OT-I by 3T3-OVA-gp96-Ig. PEC harvested from mice injected i.p. 3 days earlier with 3T3-OVA-gp96-Ig, 3T3-gp96-Ig, or 3T3 were incubated with CFSE-labeled OT-I for 72 h at ratios of 1:10, 1:100, and 1:1000 OT-I:PEC (a–f). As additional controls, 3T3 transfectants were also incubated directly with OT-I in vitro. Cells were stained with anti-CD8-PE and analyzed for CFSE dilution, which is plotted in b–h.

To test lymph node-independent cross-priming of OT-I directly, we isolated PEC from B6 mice on day 3 after i.p. immunization with allogeneic 3T3 cells, 3T3-OVA cells, or 3T3-OVA-gp96-Ig cells. The PEC were mixed at various ratios with CFSE-labeled OT-I and CFSE dilution determined 48 and 72 h later. PEC isolated from mice injected with 3T3-OVA-gp96-Ig were able to cross-prime OT-I in vitro (Fig. 3, C, a–d) as indicated by CFSE dilution. In contrast, PEC isolated from mice injected with 3T3-gp96 or untransfected 3T3 were unable to stimulate OT-I proliferation (Fig. 3 c, e, f). Likewise, direct in vitro incubation of CSFE-labeled OT-I with 3T3-OVA-gp96-Ig or with 3T3-gp96-Ig was unable to cause CSFE dilution. The data support the model of gp96-OVA-induced Ag cross-presentation to cognate CD8 cells in the absence of lymph nodes in the PC.

Gp96 recruits DC and NK cells to the site of its release and causes their activation

Cross-priming at minimum requires the bringing together of APCs and CD8 cells, while CD4 cells are not essential in our model system. We determined whether the local release of gp96 in the PC caused local recruitment and activation of APCs and OT-I, thereby bypassing the need for lymph nodes.

OT-I expansion upon gp96-Ig immunization is maximal by days 4 and 5 and is most pronounced in the PC. Starting from essentially 0, ~0.5 million OT-I accumulate on days 4 and 5 in the PC, representing up to 60% of the recruited CD8 cells. Strong OT-I expansion is highly dependent on gp96-Ig secretion (Fig. 4) and is minimal in response to EG7, as also observed by others (33). The ability of gp96 to cross-prime CD8 cells within 4 days in wt mice suggests early activation of APCs and other innate cells. It is known that gp96 is able to activate and mature DC in vitro and that gp96-chaperoned peptides are cross-presented by MHC I on DC and macrophages in vitro and in vivo (15, 22, 34). It has also been reported that gp96 is able to activate NK cells (25, 35, 36). It is not known, however, whether gp96 in vivo recruits and activates innate cells and whether they can be found locally at the injection site. The fact that unimpaired OT-I activation takes place in LTα ko mice suggested that cell recruitment and activation must take place locally at the site of gp96 release.

Following i.p. injection of EG7-gp96-Ig, or EG7 as control, PEC were harvested on days 1–4 and analyzed for activation by phenotype and by the uptake of BrdU. The largest fraction of EG7-gp96-Ig-recruited cells, ~80–90%, were F4/80high monocyte/macrophages. Resident peritoneal macrophages present before immunization were F4/80low and did not change in number following EG7-gp96-Ig injection. CD11c+ DC and NK1.1+ NK cells each constituted ~5–10% of the cells recruited within the first 2 days into the PC. B cells and CD4 T cells were found in increasing numbers in the PC beginning on day 3 and further increased on days 4 and 5 (data not shown). In comparison to EG7, i.p. injection...
of EG7-gp96-Ig doubled the number of total cells recruited into the PC within the first 2 days (Fig. 5A). This effect required the secretion of at least 60 ng of gp96-Ig by the injected cells within 24 h, as measured by ELISA (26). If lower amounts of gp96-Ig were secreted by the number of injected cells, the effect on cell recruitment and CD8 cross-priming quickly tapered off, suggesting that there is a threshold level of sensitivity for stimulation of cross-priming (data not shown). Gp96 secretion by EG7 doubled the total number of recruited F4/80dim cells and tripled the number of DC and NK cells over EG7 not secreting gp96-Ig (Fig. 5B). DC recruited into the PC by gp96 within the first 2 days incorporated the drinking water from the day of immunization. B, CD8 proliferation measured by BrdU uptake is detectable only in the PC on day 2 (red lined) and only after gp96 priming. Strong CD8 proliferation on day 4 in the PC after EG7-gp96-Ig immunization; dLN, draining lymph node (para-aortic, mesenteric); ndLN, non-draining lymph node (inguinal). C, Activation of NK1.1 cells in the PC by EG7-gp96-Ig immunization as measured by CD69 up-regulation. A–C are representative of three independent experiments.
CD8 proliferation rather than in lymph nodes. By day 4, gp96-dependent BrdU uptake by CD8 cells in the PC was very pronounced and still significantly higher than in lymph nodes or spleen (Fig. 6B).

NK cells in the gp96 group but not in the EG7 group were activated by day 4 as indicated by CD69 (Fig. 6C) and 2B4 (data not shown) up-regulation. NK activation, as measured by CD69 up-regulation, only occurred in PEC (Fig. 6C) and not in lymph nodes or spleen (data not shown), again suggesting local activation.

These data demonstrate that local gp96 release in the PC is able to transmit signals that result in the local recruitment and activation of innate and adaptive immune cells, providing a cellular mechanism for CD8 cross-priming independent of lymph nodes and CD4 cells. This cross-priming mechanism is not dependent on the specific anatomy of the PC, because s.c. administration of EG7-gp96-Ig or 3T3-OVA-gp96-Ig is equally effective in OT-I cross-priming (data not shown).

**Highly efficient CD8 CTL cross-priming by gp96-chaperoned peptides**

Secretion of gp96-Ig by EG7-gp96-Ig results in a dramatic increase in OT-I expansion when compared with EG7 even though both cell lines secrete comparable quantities of OVA (~80 ng/24 h × 10⁶ cells) (Fig. 4). Similar differences in OT-I expansion are seen when OT-I expansion is compared in response to allogeneic 3T3-OVA and 3T3-OVA-gp96-Ig (25). Gp96-Ig secreted from OVA-transfected cells contains a small fraction (~0.1% or less) of gp96 molecules that chaperone OVA peptides (gp96-OVA) and these are thought to be responsible for OT-I cross-priming (19, 20). However, secreted gp96 may also act as nonspecific adjuvant for the recruitment and activation of DC and thereby enhance uptake and cross-priming of OVA protein. Finally, it is possible that gp96-Ig and OVA protein are secreted as separate molecules and form gp96-Ig-OVA complexes extracellularly. Several experiments were conducted to distinguish between these possibilities.

First, we compared dose-response profiles of the efficiency of OT-I expansion in the PC and spleen after i.p. injection of 3T3-OVA, 3T3-OVA-gp96-Ig, or EG7, EG7gp96-Ig and pure OVA protein (Fig. 7). The rate of secretion of OVA and of gp96-Ig, respectively, was determined in vitro by ELISA as nanograms secreted per 24 h. By injecting different cell numbers, a dose range of secreted OVA and gp96-Ig was achieved as shown in Fig. 7. OT-I expansion was measured 4 days after stimulation. 3T3-OVA cells, secreting only OVA at a rate of 80–800 ng per 24 h, were unable to expand OT-I. Clearly, this quantity of OVA is unable to cross-prime OT-I even in the presence of allogeneic activation of the immune system. Similarly, syngeneic EG7 cells secreting OVA alone expand OT-I only minimally (Fig. 4) even though EG7 cells express K⁺,OVA, suggesting that direct priming of OT-I is very inefficient. In contrast, when gp96 is secreted from OVA-containing tumor cells, 80–800 ng per 24 h of gp96 efficiently cross-prime OT-I and result in their expansion locally and in the spleen. Efficient OT-I cross-priming by OVA-protein in contrast required 3–10 μg protein. The difference in sensitivity of OT-I expansion in response to OVA protein vs gp96 secreted from OVA-containing cells is ~10,000-fold (Fig. 7) in terms of weight. Taking into account molecular weights and the fact that maximally 0.1% of secreted gp96 molecules are associated with OVA peptides, the difference in OT-I cross-priming activity by gp96-OVA vs OVA protein is about 20 million-fold in molar terms.

**Adjuvant activity of gp96 for CD8-CTL cross-priming by nonchaperoned protein**

The data presented in Fig. 7 leaves open the possibility that gp96-Ig and OVA secreted as separate molecules rather than as a gp96-OVA complex are responsible for the efficient cross-priming of OT-I. To examine this possibility, OT-I expansion was studied under conditions where gp96 and OVA were deliberately administered as separate molecules. 3T3-gp96 cells secreting only gp96, but not OVA, were i.p. injected alone or coinjected with OVA protein and OT-I expansion was quantitated as usual. As shown in Fig. 8A, allogeneic 3T3-gp96-Ig cells secreting 200 ng per 24 h gp96-Ig did not cause unspecific OT-I expansion. Likewise, 200 ng and 50 μg OVA injected alone was unable to mediate OT-I expansion. In contrast, when 50 μg OVA was cojected with 3T3-gp96-Ig cells secreting 200 ng per 24 h gp96-Ig, almost optimal OT-I expansion was observed, indicating that gp96 acts as adjuvant for OVA cross-priming of OT-I. The action of gp96 acting in trans with OVA increases OT-I cross-priming by a factor of a 100–1000 over OVA alone, while gp96 chaperoning OVA (in cis) increases cross-priming by a factor of more than 1 million (relative to OVA alone). As negative control, 3T3-gp96-Ig in the absence of OVA had no effect on OT-I expansion despite their allogenicity. Moreover, 3T3-gp96-Ig secreting 200 ng gp96-Ig in combination with cojected 200 ng OVA protein was unable to cross-prime OT-I, ruling out the possibility of extracellular complex formation gp96-Ig and OVA.

The data suggest that the adjuvant effect of gp96 is mediated by activation of DC and stimulation of pinocytosis, resulting in increased uptake of OVA protein and cross-presentation by MHC I to OT-I. Although gp96 shows considerable adjuvanticity for cross-priming of nonchaperoned OVA, internalization of gp96-OVA complexes via the CD91 receptor is even more efficient in procuring the gp96-chaperoned peptides for class I MHC presentation and thereby further enhancing cross-priming efficiency.
GP96–Ig-OVA harvested from 3T3–OVA–gp96–Ig cultures with the cells. Functions with endogenous tumor-specific and OVA-specific CD8 shown. Together, these data indicate that gp96-mediated cross-expansion as OT-I, starting from a lower frequency (data not quantified to a frequency of 1–3% in the CD8 gate, indicating similar cross-priming against endogenous Ags. Endogenous, non–GFP-OT-I, compared with preimmune mice, suggesting gp96-dependent increased, CD8-dependent protection against subsequent challenge and for endogenous CD8 cells cross-priming activity for adoptively transferred transgenic and for endogenous CD8 cells

The model system of secretion of gp96 from tumor cells raises the question how continuous secretion of gp96 compares to bolus injection of gp96 in its effect on OT-I cross-priming. Because OVA and OT-I are artificial test systems, it was also important to ensure that data obtained from OT-I are applicable to endogenous, nontransgenic CD8 cells. Importantly, in data published previously, EG7-gp96-Ig immunization of B6 mice provided 50- to 100-fold increased, CD8-dependent protection against subsequent challenge with parental EL4 cells, but not against Lewis lung carcinoma (26), compared with preimmune mice, suggesting gp96-dependent cross-priming against endogenous tumor Ags. Endogenous, nontransgenic OVA-specific CD8 cells occurring at low frequency of ~1 in 20,000 CD8 cells (0.005%) expand to EG7-gp96-Ig immunization to a frequency of 1–3% in the CD8 gate, indicating similar expansion as OT-I, starting from a lower frequency (data not shown). Together, these data indicate that gp96-mediated cross-priming is not restricted to the TCR-transgenic OT-I cells but also functions with endogenous tumor-specific and OVA-specific CD8 cells.

Comparing the effect of i.p. injection of 200 ng serum-free gp96-Ig-OVA harvested from 3T3-OVA-gp96-Ig cultures with the effect of injecting 3T3-OVA-gp96-Ig cells secreting 200 ng within 24 h in vivo, a dramatic increase of OT-I expansion was observed when gp96-OVA was secreted continuously over a bolus of gp96-OVA (Fig. 8B). This observation indicates that continuous release of gp96 that may occur, e.g., as a consequence of ongoing cell death by infection, is an optimal stimulus for cognate CD8 cross-priming without CD4 help and without need for lymph nodes.

**Discussion**

This study reveals an astonishing enhancement of cross-priming activity by gp96-chaperoned peptides by >1 million-fold in comparison to pure protein alone. This finding is significant because it provides a highly sensitive mechanism for the generation of CD8 CTL to antigenic peptides released by dying cells.

It may be questioned whether the OVA/OT-I TCR-transgenic model system is representative for other TCR specificities. The OT-I system without exception has been representative for the behavior of nontransgenic CD8 cells with regard to thymic selection (27), activation (33), expansion, differentiation (37–39), generation of memory (40), and apoptosis (41). In our analysis of the efficiency of Ag cross-presentation, OT-I expansion served as a sensitive and quantitative readout for Ag cross-presentation mediated by gp96-OVA, by gp96 plus OVA, or by OVA alone. The observed differences in OT-I expansion can only be explained by the efficiency of cross-presenting activity of the different forms of OVA. Gp96-chaperoned-OVA clearly is most active in cross-presentation, followed by OVA plus gp96 as adjuvant and then OVA alone, which is more than 1 million-fold less active in cross-priming than chaperoned OVA.

We suggest that this mechanism of gp96-mediated cross-priming may be physiologically important when cells die due to infection or necrosis, a process that may be accompanied by the release of gp96-chaperoning antigenic peptides derived from the infectious agent that caused cell death. The attraction and activation of DC and NK cells to the site of infection, cell death, and gp96 release provides an efficient pathway for cross-presentation of antigenic, gp96-chaperoned peptides to CD8 cells and for the generation of CTL in situ independent of lymph nodes. These CTL then serve to eliminate neighboring infected cells, thereby limiting the spread of the infectious agent.

A defense system based on stimulation of the innate immune system by heat shock proteins clearly has been in existence already in early vertebrate phylogeny in amphibians (42–44). With the evolution of adaptive immunity, it appears that the role of gp96 expanded from its adjuvant function to that of a carrier of specific Ags for efficient MHC class I cross-presentation and cross-priming of CD8 CTL.

In support of this model and hypothesis, we provide evidence that gp96 secretion in situ results in local recruitment and activation of large numbers of DC and NK cells that are able to activate cognate CD8 cells locally. DC in response to gp96 secretion proliferate in the PC but not at other sites; similarly, NK cells become activated only in the PC. Cognate CD8 cells show earliest and most active proliferation in the PC; later however, CD8 proliferation also spreads to other sites including the spleen. The interpretation of local cross-priming of CD8 cells by gp96, as suggested in our model in the PC, predicted and required that the cross-priming process should be able to function in the absence of lymph nodes. This prediction was confirmed in LTα ko mice. Importantly, efficient CD8 cross-priming by gp96-Ig is not restricted to the PC. Equally efficient CD8 cross-priming and generation of systemic immunity (26) was also observed upon s.c. immunization with gp96-Ig-secreting tumors (data not shown). The peritoneal

**FIGURE 8.** Gp96 is an adjuvant for protein cross-priming and acts most efficiently when continuously released. A, Mice received 1 million GFP-OT-I i.v. and 2 days later were i.p. immunized as indicated. In vivo GFP-OT-I expansion was measured by FACS on day 4 after immunization. Secreted products from injected cells were quantitated by ELISA in vitro. The amount of secreted product indicated refers to the amount secreted in culture within 24 h by the number of cells injected. Note that 50 μg OVA along with 200 ng gp96 secreted from 3T3-gp96-Ig cells cause less GFP-OT-I expansion than 200 ng gp96-Ig secreted from 3T3-OVA-gp96-Ig containing ~0.1% gp96-OVA. B, Mice received 1 million GFP-OT-I i.v. Two days later, they were i.p. immunized with 200 ng soluble gp96-Ig harvested from supernatant 3T3-OVA gp96-Ig cultures or with the number of 3T3-gp96-Ig cells secreting 200 ng gp96-Ig within the succeeding 24 h. GFP-OT-I expansion in the PC was determined on day 4 after immunization by flow cytometry.
site was chosen for analysis due to its easy access and absence of confounding cell populations found at other sites.

Lymph node-independent cross-priming of CD8 cells by gp96-chaperoned peptides is in accord with its independence of CD40L, and CD4 help. DC activation instead appears to be mediated by gp96 binding to CD91 and TLR2/4 as shown previously by others (23). In preliminary experiments, we were able to demonstrate that anti-CD91 Abs completely blocked gp96-mediated CD8 cross-priming. Costimulation of CD8 cells by CD80 and CD86, however, is absolutely required for CD8 cross-priming by gp96.

Our studies also show that gp96 can act as adjuvant for CTL generation by enhancing cross-priming of antigenic proteins residing in the extracellular milieu. Release of heat shock proteins from dying cells may act as a “danger signal” activating the innate immune response by activating DC, stimulation of pinocytosis of extracellular proteins by DC and their MHC I cross-presentation. The adjuvant activity of gp96 also activates NK cells, thereby triggering Th1 responses and enhancing the clearance of extracellular infectious agents.

An important factor for the extraordinary cross-priming activity of gp96 is its continuous, sustained release by secretion. In our model system, allogeneic or syngeneic tumor cells secrete gp96, allowing the analysis of a single variable, gp96 secretion vs non-secretion, in an in vivo system. This methodology does not require cell fractionation and purification of Ag or gp96, thereby avoiding potential problems associated with biochemical purification procedures. Our data show that sustained (24 h) release (secretion) of small quantities of gp96-peptide complexes (~200 ng/24 h) is much more efficient in CD8 cross-priming than the same amount of gp96-peptide complex injected as a bolus. Apparently, continuous stimulation of the immune system over a period of time, similar to what would be observed in an ongoing infection, is a much stronger immune stimulus than a bolus that is quickly diluted or taken up by phagocytic cells. Preliminary data suggest that the live, i.e. injected allogeneic 3T3 fibroblasts secreting gp96 survive for 5–7 days before they are eliminated. Irradiation of gp96-secreting tumor cells, or treatment with mitomycin C, does not diminish their gp96 secretion nor their in vivo cross-priming activity (data not shown), indicating that cell replication is not required for enhanced CD8 cross-priming.

In addition to revealing a potentially important lymph node-and CD4-independent immune defense mechanism, these studies provide the basis for the design of efficient cellular vaccine strategies. Studies are ongoing to determine whether gp96-secreting tumor cells will be effective as antitumor and anti-HIV vaccines.

Disclosures
The authors have no financial conflict of interest.

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