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

The Journal of Immunology

Satoshi Oizumi, Natasa Strbo, Savita Pahwa, Vadim Deyev and Eckhard R. Podack

References   This article **cites 44 articles**, 23 of which you can access for free at:
http://www.jimmunol.org/content/179/4/2310.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription   Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions   Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts   Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Molecular and Cellular Requirements for Enhanced Antigen Cross-Presentation to CD8 Cytotoxic T Lymphocytes[1]

Satoshi Oizumi, Natasa Strbo, Savita Pahwa, Vadim Deyev, and Eckhard R. Podack[2]

MHC class I-mediated cross-priming 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-priming of CD8 CTL by several million-fold over the cross-priming activity of unchaperoned protein alone. Gp96 also acts as adjuvant for cross-priming 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-priming 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-priming 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.

C ross-priming 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 physical nature of the cross-priming 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 on dendritic cells (DC) (16–18) and elicit peptide-specific immune responses (19) by delivering the chaperoned peptide to the MHC class I pathway to be cross-presented to CD8$^+$ CTL (7, 15, 20, 21). Cross-priming by HSP-gp96 is associated with TLR2 and TLR4 stimulation and DC maturation resulting in a CD8 CTL-biased response (15, 22–24).

We have previously developed a model of tumor cell-secreted gp96 for use as tumor vaccine. Immunization with gp96-secreting tumor cells generated tumor-specific and surrogate Ag-specific immunity that was independent of CD4 cells (25, 26). Using this immunization method to quantitate CD8 responses, we report here that minute, femtomolar amounts of gp96-chaperoned Ag are sufficient for cognate CD8 cross-priming locally at the site of gp96 release independent of lymph nodes and CD4 cells.

Department of Microbiology and Immunology, University of Miami, Miller School of Medicine, Miami, FL 33136

[2] Address correspondence and reprint requests to Dr. Eckhard R. Podack, Department of Microbiology and Immunology, University of Miami, Miller School of Medicine, 1600 NW 10th Avenue, Miami, FL 33136. E-mail address: epodack@miami.edu

[3] Abbreviations used in this paper: HSP, heat shock protein; DC, dendritic cell; LTα, lymphotoxin α; PEC, peritoneal exudate cell; ko, knockout; wt, wild type; PC, peritoneal cavity.

Materials and Methods

Mice

Wild-type (wt) and B7.1, B7.2, B7.1/2, CD40L, lymphotoxin α (LTα), and CD4-deficient mice in the C57BL/6 (B6) background were obtained from The Jackson Laboratory. B6.Jα281$^{-/-}$ mice (NKT deficient, renamed Jα18 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 using magnetic separation (>95% pure; Miltenyi Biotec). One million 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 × 10^6 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-priming of OT-I

Groups of B6 wt mice were i.p. immunized with 2 × 10^6 3T3-OVA-gp96-Ig or 3T3-gp96-Ig. After 3 days peritoneal exudate cells (PEC) were collected and 10^5 PEC were cocultured with purified, naive 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
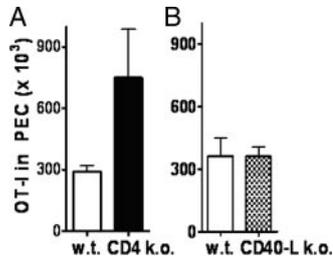
FIGURE 1. Gp96-mediated cross-priming of CD8 T cells is enhanced in the absence of CD4 cells but not affected by the absence of CD40L. Mice received 1 million GFP-OT-I i.v. and were immunized 2 days later i.p. with 4 million EG7-gp96-Ig. Cells were harvested from PC after 5 additional days and analyzed 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.

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-priming of CD8 cells by gp96. We used adoptively transferred K^{b-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-myc 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 readout than measuring tumor rejection.

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. 1*A*). In contrast, in CD40L ko mice OT-I expansion was similar to the expansion in wt mice (Fig. 1*B*). 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-
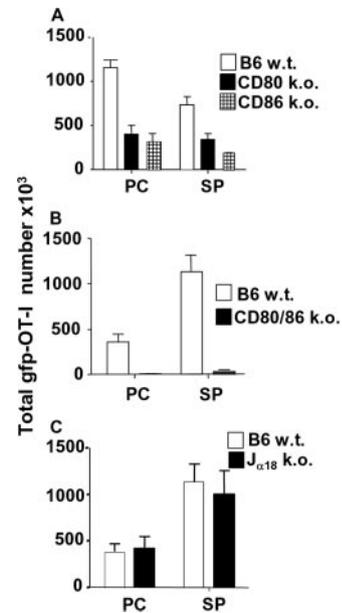

FIGURE 2. Gp96-mediated cross-priming of CD8 T cells requires CD80 and CD86 and is independent of NKT cells. Mice received 1 million GFP-OT-I i.v. and were immunized 2 days later i.p. with 4 million EG7-gp96-Ig (*A* and *C*) or 2 million 3T3-OVA-gp96-Ig (*B*). Cells were harvested from the spleen (SP) 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 (J$_{\alpha18}$ ko).

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. 2*A*) were able to co-stimulate 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. 2*B*), gp96-mediated cross-priming of OT-I was completely abolished.

NKT cells are frequently involved in antitumor immunity (29–31); their role in gp96-mediated priming of CD8 T cells is not known. J$_{\alpha18}$ ko mice lack NKT cells due to their inability to generate the invariant TCR v$_{\alpha14}$ chain characteristic for CD1d-restricted invariant NKT cells (29). The ability of J$_{\alpha18}$ ko mice to support undiminished OT-I expansion (Fig. 2*C*) 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
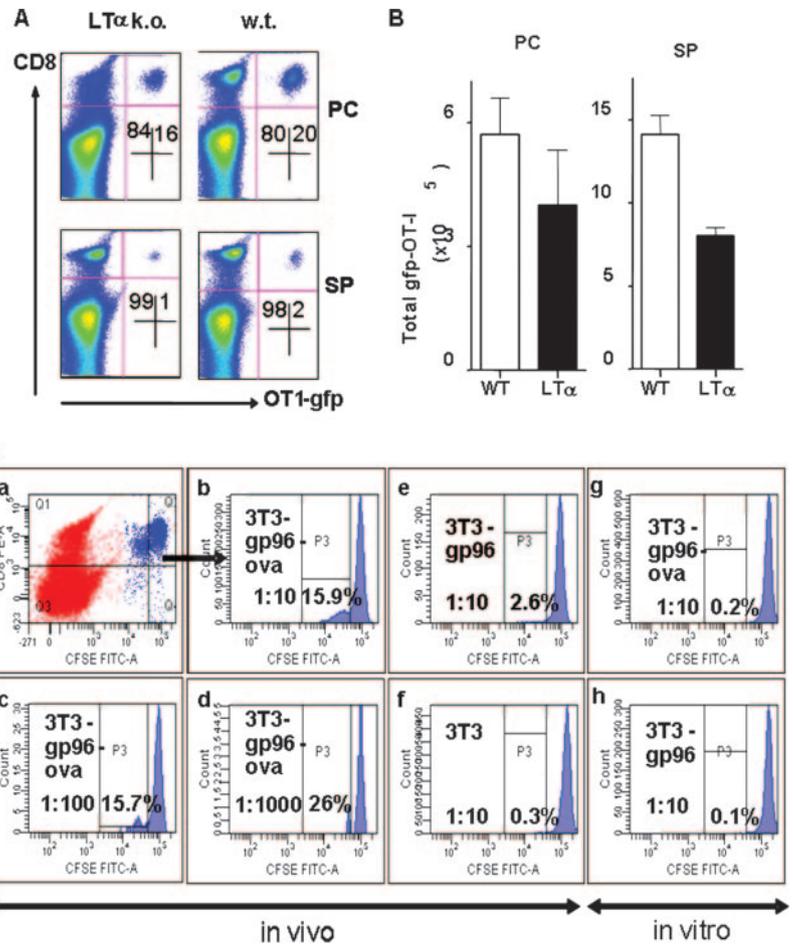
FIGURE 3. Efficient cross-priming by gp96 in the absence of lymph nodes. *A*, LTα deficiency, representative FACS data. Mice received 1 million GFP-OT-I i.v. and were immunized 2 days later i.p. with 2 million 3T3-OVA-gp96-Ig. Cells were harvested from the indicated sites after 5 additional days and 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*.

nodes, including Peyer's patches, and are impaired in antiviral responses (32). However, when analyzed for gp96-OVA-mediated OT-I expansion, LTα-deficient mice showed almost normal OT-I expansion in the peritoneal cavity (PC) when compared with wt mice (Fig. 3, *A* and *B*). In the spleen, the accumulation of GFP OT-I was diminished by ~50%, reflecting the absence of lymph node-based OT-I clonal expansion. This finding suggested that lymph nodes are not essential for gp96-mediated peptide cross-priming and that local cross-priming takes place at the site of gp96 release.

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 F4/80^{dim} monocyte/macrophages. Resident peritoneal macrophages present before immunization were F4/80^{bright} 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
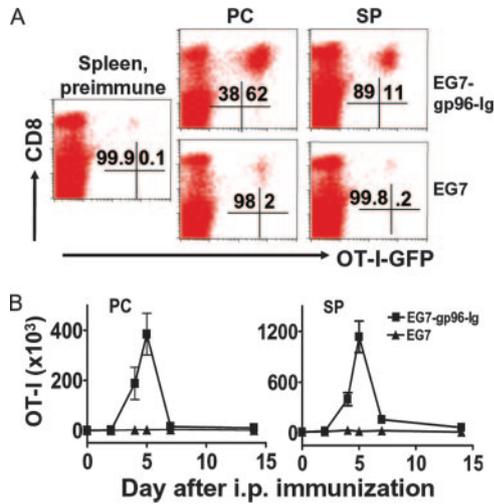
FIGURE 4. Cross-priming of CD8 T cells by gp96-OVA is more efficient than direct priming by Ag presentation through EG7-K^{b-OVA}. Mice received 1 million GFP-OT-I i.v. and were immunized 2 days later i.p. with 2 million EG7-gp96-Ig or EG7. *A*, Cells were harvested from the PC before immunization (preimmune) and 5 days after immunization and analyzed for GFP-OT-I frequency in the CD8 gate by FACS. *B*, Kinetics of GFP-OT-I expansion in the PC and spleen after EG7-gp96-Ig and EG7 immunization; total number of GFP-OT-1 at the given site is plotted.

of EG7-gp96-Ig doubled the number of total cells recruited into the PC within the first 2 days (Fig. 5*A*). 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 F4/80^{dim} cells and tripled the number of DC and NK cells over EG7 not secreting gp96-Ig (Fig. 5*B*). DC
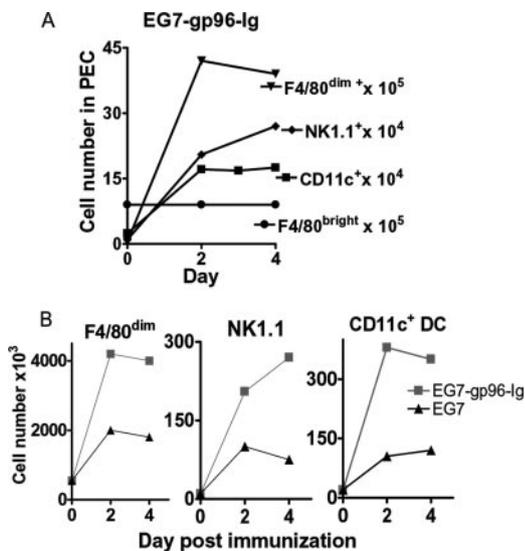

FIGURE 5. Increased recruitment of innate immune cells into the PC by gp96. One million OT-1 were transferred i.v. on day −2 and 2 days later 4 million EG7 or EG7gp96-Ig cells were injected i.p. Cells were harvested from the PC on the days indicated and phenotyped by flow cytometry. *A*, Recruitment of CD11c$^+$, NK1.1$^+$, and F4/80^{dim} cells by injection of EG7-gp96-Ig. F4/80^{bright} cells are present in the PC before immunization and do not change in number after immunization. *B*, Comparison of cell recruitment into the PC by EG7 and EG7-gp96-Ig.
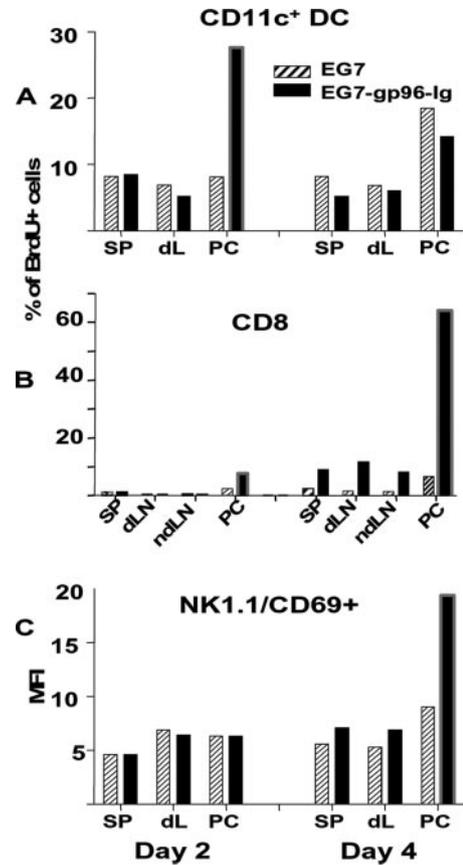

FIGURE 6. Gp96 secretion mediates proliferation of DC and CD8 cells and activates NK cells in the PC. Mice received 1 million GFP-OT-I i.v. and were immunized 2 days later i.p. with 4 million EG7-gp96-Ig or EG7. *A*, Proliferation of CD11c$^+$ cells measured by BrdU staining in the PC, mesenteric, and para-aortic lymph nodes (dL) and spleen (SP) 2 and 4 days after immunization with 4×10^6 EG7 or EG7-gp96-Ig. CD11c$^+$ cells were gated and analyzed for BrdU by intracellular staining. Note CD11c$^+$ proliferation (red lined) on day 2 only in the PC and only after EG7-gp96-Ig administration. Mice received BrdU in 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, nondraining 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.

recruited into the PC by gp96 within the first 2 days incorporated significant amounts of BrdU, indicating their activation. DC isolated from draining para-aortic lymph nodes, mesenteric lymph nodes, and spleen in contrast were BrdU negative (Fig. 6*A*). This finding strongly suggests that DC are activated and proliferate locally of at the site of gp96 secretion. Only later are BrdU-positive DC also found in lymph nodes and spleen. EG7, not secreting gp96, did not cause BrdU uptake by DC in the PC within the first 2 days. Interestingly however, EG7 recruited DC were weakly BrdU positive on day 4 (Fig. 6*A*), indicating delayed and weak activation by EG7 in contrast to early and strong activation by EG7-gp96. The delayed DC activation by the wt tumor EG7 is associated with only minimal CD8 expansion.

CD8 cells present in the PC by day 2 in the EG7-gp96-Ig group showed significant BrdU uptake, while at the same time CD8 cells in draining lymph nodes and spleen remained BrdU negative (Fig. 6*B*). This finding is consistent with local, peritoneal initiation of

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. 6*B*).

NK cells in the gp96 group but not in the EG7 group were activated by day 4 as indicated by CD69 (Fig. 6*C*) and 2B4 (data not shown) up-regulation. NK activation, as measured by CD69 up-regulation, only occurred in PEC (Fig. 6*C*) 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 × 10^6 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^{b-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 mg 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.
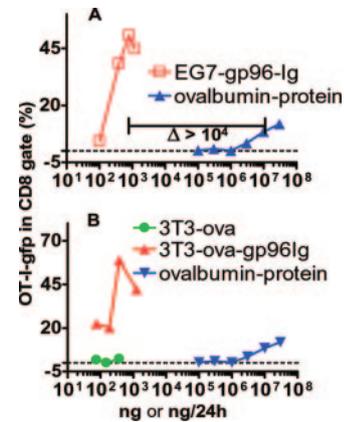

FIGURE 7. Enhanced CD8 T cells cross-priming by gp96-chaperoned OVA compared with free OVA protein. C57BL/6 mice received 1 million GFP-OT-I i.v.; 2 days later they were immunized i.p. with different numbers of syngeneic EG7-gp96-Ig or allogeneic 3T3-OVA or 3T3-OVA-gp96-Ig or with OVA protein in PBS. The number of cells for i.p. injection was adjusted to yield the quantity of secreted gp96-Ig or OVA in 24 h as indicated on the *x*-axis. OVA and gp96-Ig secretion, respectively, was determined in vitro by ELISA. GFP-OT-I expansion was determined on day 5 after immunization in the PC by flow cytometry. *A*, GFP-OT-I expansion in response to EG7-gp96-Ig and to OVA protein. *B*, GFP-OT-I expansion in response to 3T3-OVA, to 3T3-OVA-gp96-Ig and to OVA protein.

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. 8*A*, 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 coinjected 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 coinjected 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.
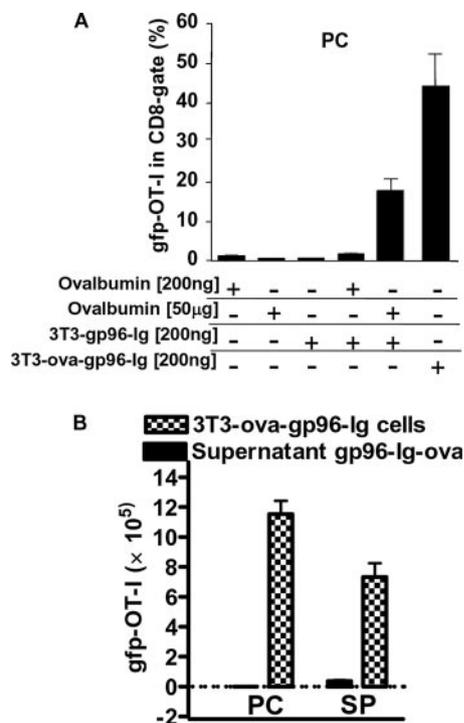
FIGURE 8. Gp96 is an adjuvant for protein cross-priming and acts most efficiently when continuously released. *A*, Mice received 1 million GFP-OT-I i.v. and 2 days later were i.p. immunized as indicated. In vivo GFP-OT-I expansion was measured by FACS on day 4 after immunization. Secreted products from 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.

Continuous secretion of gp96-Ig provides maximal CD8 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 with 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. 8*B*). 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

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.p. 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.

References

1. Bevan, M. J. 1976. Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. *J. Immunol.* 117: 2233–2238.
2. den Haan, J. M., and M. J. Bevan. 2001. Antigen presentation to CD8+ T cells: cross-priming in infectious diseases. *Curr. Opin. Immunol.* 13: 437–441.
3. Ackerman, A. L., and P. Cresswell. 2004. Cellular mechanisms governing cross-presentation of exogenous antigens. *Nat. Immunol.* 5: 678–684.
4. Melief, C. J. 2003. Mini-review: regulation of cytotoxic T lymphocyte responses by dendritic cells: peaceful coexistence of cross-priming and direct priming? *Eur. J. Immunol.* 33: 2645–2654.
5. Moron, G., G. Dadaglio, and C. Leclerc. 2004. New tools for antigen delivery to the MHC class I pathway. *Trends Immunol.* 25: 92–97.
6. Zanetti, M., P. Castiglioni, S. Schoenberger, and M. Gerloni. 2003. The role of relB in regulating the adaptive immune response. *Ann. NY Acad. Sci.* 987: 249–257.
7. Binder, R. J., and P. K. Srivastava. 2005. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8+ T cells. *Nat. Immunol.* 6: 593–599.
8. Norbury, C. C., S. Basta, K. B. Donohue, D. C. Tscharke, M. F. Princiotta, P. Berglund, J. Gibbs, J. R. Bennink, and J. W. Yewdell. 2004. CD8+ T cell cross-priming via transfer of proteasome substrates. *Science* 304: 1318–1321.
9. Serna, A., M. C. Ramirez, A. Soukhanova, and L. J. Sigal. 2003. Cutting edge: efficient MHC class I cross-presentation during early vaccinia infection requires the transfer of proteasomal intermediates between antigen donor and presenting cells. *J. Immunol.* 171: 5668–5672.
10. Shen, L., and K. L. Rock. 2004. Cellular protein is the source of cross-priming antigen in vivo. *Proc. Natl. Acad. Sci. USA* 101: 3035–3040.
11. Udono, H., D. L. Levey, and P. K. Srivastava. 1994. Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes CD8+ T cells in vivo. *Proc. Natl. Acad. Sci. USA* 91: 3077–3081.
12. Udono, H., and P. K. Srivastava. 1993. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J. Exp. Med.* 178: 1391–1396.
13. Li, Z., and P. K. Srivastava. 1993. Tumor rejection antigen gp96/grp94 is an ATPase: implications for protein folding and antigen presentation. *EMBO J.* 12: 3143–3151.
14. Srivastava, P. K., and M. Heike. 1991. Tumor-specific immunogenicity of stress-induced proteins: convergence of two evolutionary pathways of antigen presentation? *Semin. Immunol.* 3: 57–64.
15. Arnold, D., S. Faath, H. Rammensee, and H. Schild. 1995. Cross-priming of minor histocompatibility antigen-specific cytotoxic T cells upon immunization with the heat shock protein gp96. *J. Exp. Med.* 182: 885–889.
16. Binder, R. J., D. K. Han, and P. K. Srivastava. 2000. CD91: a receptor for heat shock protein gp96. *Nat. Immunol.* 1: 151–155.
17. Theriault, J. R., S. S. Mambula, T. Sawamura, M. A. Stevenson, and S. K. Calderwood. 2005. Extracellular HSP70 binding to surface receptors present on antigen presenting cells and endothelial/epithelial cells. *FEBS Lett.* 579: 1951–1960.
18. Delneste, Y., G. Magistrelli, J. Gauchat, J. Haeuw, J. Aubry, K. Nakamura, N. Kawakami-Honda, L. Goetsch, T. Sawamura, J. Bonnefoy, and P. Jeannin. 2002. Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity* 17: 353–362.
19. Blachere, N. E., Z. Li, R. Y. Chandawarkar, R. Suto, N. S. Jaikaria, S. Basu, H. Udono, and P. K. Srivastava. 1997. Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. *J. Exp. Med.* 186: 1315–1322.
20. Suto, R., and P. K. Srivastava. 1995. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* 269: 1585–1588.
21. Arnold-Schild, D., D. Hanau, D. Spehner, C. Schmid, H. G. Rammensee, H. de la Salle, and H. Schild. 1999. Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. *J. Immunol.* 162: 3757–3760.
22. Singh-Jasuja, H., H. U. Scherer, N. Hilf, D. Arnold-Schild, H. G. Rammensee, R. E. Toes, and H. Schild. 2000. The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. *Eur. J. Immunol.* 30: 2211–2215.
23. Vabulas, R. M., S. Braedel, N. Hilf, H. Singh-Jasuja, S. Herter, P. Ahmad-Nejad, C. J. Kirschning, C. Da Costa, H. G. Rammensee, H. Wagner, and H. Schild. 2002. The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway. *J. Biol. Chem.* 277: 20847–20853.
24. Ramirez, S. R., H. Singh-Jasuja, T. Warger, S. Braedel-Ruoff, N. Hilf, K. Wiemann, H. G. Rammensee, and H. Schild. 2005. Glycoprotein 96-activated dendritic cells induce a CD8-biased T cell response. *Cell Stress Chaperones* 10: 221–229.
25. Strbo, N., S. Oizumi, V. Sotosek-Tokmadzic, and E. R. Podack. 2003. Perforin is required for innate and adaptive immunity induced by heat shock protein gp96. *Immunity* 18: 381–390.
26. Yamazaki, K., T. Nguyen, and E. R. Podack. 1999. Cutting edge: tumor secreted heat shock-fusion protein elicits CD8 cells for rejection. *J. Immunol.* 163: 5178–5182.
27. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76: 17–27.
28. Ikawa, M., S. Yamada, T. Nakanishi, and M. Okabe. 1998. 'Green mice' and their potential usage in biological research. *FEBS Lett.* 430: 83–87.
29. Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278: 1623–1626.
30. Subleski, J. J., V. L. Hall, T. C. Back, J. R. Ortaldo, and R. H. Wiltrout. 2006. Enhanced antitumor response by divergent modulation of natural killer and natural killer T cells in the liver. *Cancer Res.* 66: 11005–11012.
31. Motohashi, S., A. Ishikawa, E. Ishikawa, M. Otsuji, T. Iizasa, T. Hanaoka, N. Shimizu, S. Horiguchi, Y. Okamoto, S. Fujii, et al. 2006. A phase I study of in vitro expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. *Clin. Cancer Res.* 12: 6079–6086.
32. Suresh, M., G. Lanier, M. K. Large, J. K. Whitmire, J. D. Altman, N. H. Ruddle, and R. Ahmed. 2002. Role of lymphotoxin α in T-cell responses during an acute viral infection. *J. Virol.* 76: 3943–3951.
33. Shrikant, P., and M. F. Mescher. 1999. Control of syngeneic tumor growth by activation of CD8+ T cells: efficacy is limited by migration away from the site and induction of nonresponsiveness. *J. Immunol.* 162: 2858–2866.
34. Singh-Jasuja, H., R. E. Toes, P. Spee, C. Munz, N. Hilf, S. P. Schoenberger, P. Ricciardi-Castagnoli, J. Neefjes, H. G. Rammensee, D. Arnold-Schild, and H. Schild. 2000. Cross-presentation of glycoprotein 96-associated antigens on

major histocompatibility complex class I molecules requires receptor-mediated endocytosis. *J. Exp. Med.* 191: 1965–1974.

35. Baker-LePain, J. C., M. Sarzotti, T. A. Fields, C. Y. Li, and C. V. Nicchitta. 2002. GRP94 (gp96) and GRP94 N-terminal geldanamycin binding domain elicit tissue nonrestricted tumor suppression. *J. Exp. Med.* 196: 1447–1459.

36. Janetzki, S., D. Palla, V. Rosenhauer, H. Lochs, J. J. Lewis, and P. K. Srivastava. 2000. Immunization of cancer patients with autologous cancer-derived heat shock protein gp96 preparations: a pilot study. *Int. J. Cancer.* 88: 232–238.

37. Kang, S. S., and P. M. Allen. 2005. Priming in the presence of IL-10 results in direct enhancement of CD8$^+$ T cell primary responses and inhibition of secondary responses. *J. Immunol.* 174: 5382–5389.

38. Mueller, K. L., M. A. Daniels, A. Felthauser, C. Kao, S. C. Jameson, and Y. Shimizu. 2004. Cutting edge: LFA-1 integrin-dependent T cell adhesion is regulated by both Ag specificity and sensitivity. *J. Immunol.* 173: 2222–2226.

39. Teague, R. M., R. M. Tempero, S. Thomas, K. Murali-Krishna, and B. H. Nelson. 2004. Proliferation and differentiation of CD8$^+$ T cells in the

absence of IL-2/15 receptor β-chain expression or STAT5 activation. *J. Immunol.* 173: 3131–3139.

40. Carrio, R., O. F. Bathe, and T. R. Malek. 2004. Initial antigen encounter programs CD8+ T cells competent to develop into memory cells that are activated in an antigen-free, IL-7- and IL-15-rich environment. *J. Immunol.* 172: 7315–7323.

41. Yajima, T., K. Yoshihara, K. Nakazato, S. Kumabe, S. Koyasu, S. Sad, H. Shen, H. Kuwano, and Y. Yoshikai. 2006. IL-15 regulates CD8$^+$ T cell contraction during primary infection. *J. Immunol.* 176: 507–515.

42. Goyos, A., N. Cohen, J. Gantress, and J. Robert. 2004. Anti-tumor MHC class Ia-unrestricted CD8 T cell cytotoxicity elicited by the heat shock protein gp96. *Eur. J. Immunol.* 34: 2449–2458.

43. Maniero, G. D., and J. Robert. 2004. Phylogenetic conservation of gp96-mediated antigen-specific cellular immunity: new evidence from adoptive cell transfer in *Xenopus. Transplantation* 78: 1415–1421.

44. Morales, H., A. Muharemagic, J. Gantress, N. Cohen, and J. Robert. 2003. Bacterial stimulation upregulates the surface expression of the stress protein gp96 on B cells in the frog Xenopus. *Cell Stress Chaperones* 8: 265–271.