# Perforin Is Required for Innate and Adaptive Immunity Induced by Heat Shock Protein Gp96

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## Summary

Tumor-secreted gp96-Ig is highly immunogenic and triggers CD8 T cell-mediated tumor rejection. In vivo secreted gp96-Ig and gp96-myc cause NK activation and clonal expansion of specific CD8$^+$ CTL in wild-type and in Fas-ligand-deficient (gld) mice but not in perforin- (PKO) or IFN-$\gamma$-deficient (GKO) mice. Transfer of perforin-competent NK cells restores the ability of PKO mice to clonally expand CD8 CTL in response to gp96-Ig. The data demonstrate an essential role for perforin-mediated functions in the activation of innate and adaptive immunity by heat shock protein gp96-peptide complexes. Crosspresentation of antigens by heat shock proteins seems to require a perforin-dependent positive feedback loop between NK and DC for both sustained NK activation and clonal CTL expansion. The studies also explain how depressed NK activity in patients with tumors or after viral infections could diminish CTL responses.

## Introduction

CD8$^+$ CTL and NK cells are critical effectors for the elimination of intracellular parasites and for tumor rejection. Perforin is a pore-forming molecule related to complement component C9 (Henkart and Henkart, 1982; Podack and Dennert, 1983); it is expressed constitutively in NK cells and inducibly in CTL and has been shown to be an important molecule for the elimination of virus-infected and tumor cells (Kagi et al., 1994; Lichtenheld et al., 1988; Smyth et al., 1990, 1999). Perforin has also been shown to participate in the clonal contraction of CD8 CTL, although the mechanism of this effect has remained controversial (Kagi et al., 1999; Matloubian et al., 1999; Spielman et al., 1998; Stepp et al., 2000). Interferon-$\gamma$ (IFN) is well recognized for its anti-infectious and antitumor function by activating antigen-presenting cells (Schreiber, 1984, 1988). A role for IFN-$\gamma$ in clonal contraction of CD8 CTL has recently been described (Badovinac and Harty, 2000).

Perforin is one of the primary cytolytic molecules expressed by activated lymphocytes and deployed during the effector phase of the immune response. A role for NK cytotoxicity in inducing a CTL response to CD70-expressing tumors was recently reported (Kelly et al., 2002) suggesting that NK cell-mediated lysis can trigger T cell-mediated immunity. As demonstrated in this report, perforin expression in addition to IFN-$\gamma$ was essential for NK cell activation and for the clonal expansion

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of CD8 CTL in response to heat shock protein gp96-peptide comlexes, indicating an essential function for perforin-mediated lysis in the nascent NK and CD8 CTL response.

Heat shock proteins are potent activators of the cellular immune response (Palliser, 2001; Singh-Jasuja et al., 2001; Srivastava and Amato, 2001). Heat shock proteins including gp96 are released by cell death caused by trauma, infection, or necrosis (Basu et al., 2000; Berwin et al., 2001). Gp96-peptide complexes bind to CD91 and other receptors on dendritic cells (Basu et al., 2001; Berwin et al., 2002; Binder et al., 2000), mediate endocytosis and dendritic cell maturation (Singh-Jasuja et al., 2000), and chaperone peptides into class I MHC of dendritic cells and macrophages (Arnold et al., 1995; Udono and Srivastava, 1993). It has been postulated that activation of APC by gp96 is responsible for the subsequent CTL response in vivo.

In previous studies we generated a secreted form of gp96, gp96-Ig, by deletion of the endoplasmic reticulum retention signal and replacement with the Fc portion of murine IgG$_1$. When transfected into tumor cells and used for immunization of mice, secreted gp96-Ig generated a protective and specific immune response against subsequent challenge with the same tumor (Yamazaki et al., 1999). Immune rejection was dependent on CD8 cells and did not require CD4 help, supporting the concept that heat shock proteins could activate CD8 CTL responses in vivo. Similar results were obtained more recently with membrane anchored gp96 generated by replacement of KDEL with a transmembrane domain (Zheng et al., 2001).

Here we are using adoptively transferred TCR transgenic CD8 cells (Kearney et al., 1994) to quantitate and study the molecular and cellular mechanisms of CTL expansion by tumor-secreted gp96-Ig in vivo. The studies led to the discovery of an unexpected function for perforin and an unexpected mechanism by which NK cells enable the clonal expansion of CD8 CTL.

## Results

### Expansion of Cognate CD8 Cells and NK Cells in Response to Tumor-Secreted gp96-Ig

OT-1 cells are CD8$^+$ TCR transgenic T cells expressing a clonal T cell receptor recognized by antibodies to v$_{\alpha 2}$ and v$_{\beta 5.1-2}$ and by K$^b$-tetramers associated with the ovalbumin-derived peptide SIINFEKL (Hogquist et al., 1994). CD8 cells in the spleen cells of OT-1 transgenic mice are largely TCR transgenic and stain with anti v$_{\alpha 2}$ (Figures 1A and 1B). Upon purification of OT-1 by magnetic selection with anti-CD8, 93% of the purified gated lymphocyte population are CD8 positive. About 90% of these CD8 cells stain with tetramer (Figures 1C and 1D) or the specific v-region antibodies (data not shown). To facilitate detection of OT-1, we have also bred the transgene into gfp transgenic mice (Ikawa et al., 1998) to generate gfp-OT-1 that can be detected by FACS even without antibody staining.
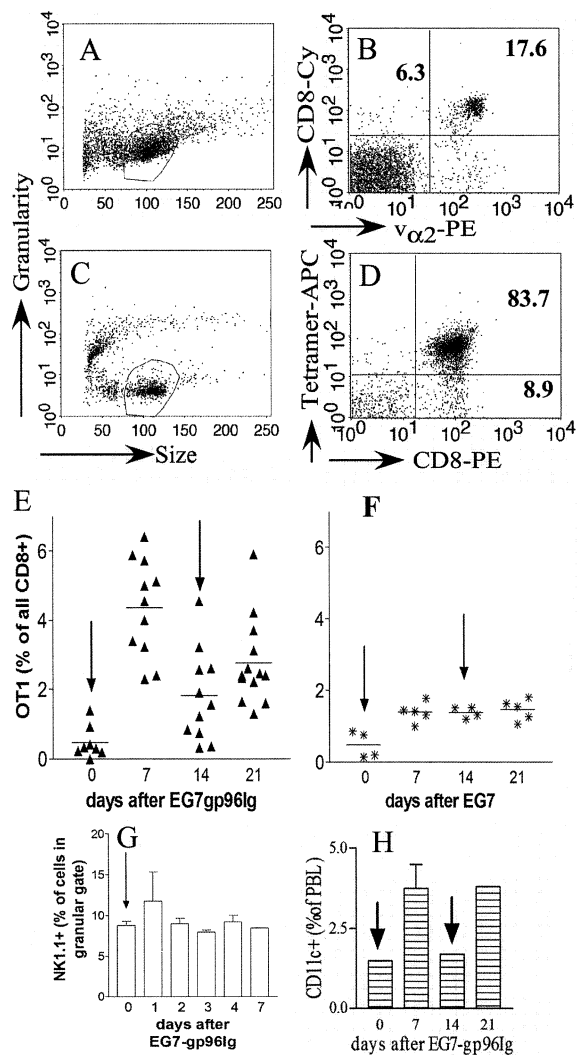
In order to measure expansion of OT-1 in vivo, approx-

Figure 1. Detection and Clonal Expansion of CD8 TCR Transgenic T Cells (OT-1) in Response to Secreted gp96-Ig

(A–D) Characterization of OT-1 isolated from OT-1 transgenic mice. (A and B) Unpurified spleen cells from OT-1 mice were stained with anti-$v_{\alpha2}$-PE specific for the $\alpha$ chain of the OT-1-TCR and with anti-CD8-Cy. (C and D) Purified CD8 cells from OT-1 transgenic mice. The purified cells were stained with anti-CD8-PE and K[b-SIINFEKL]-tetramer-APC (tetramer) (NIAID Tetramer Facility supported by the NIH AIDS Research and Reference Reagent Program). The lymphocyte gate was analyzed by flow cytometry on a FACS Vantage (Becton Dickinson). The numbers represent the percentage of gated cells in the respective quadrant.

(E–H) Clonal expansion of OT-1 cells, NK cells, and CD11c^{+} cells in response to EG7-gp9Ig and EG7 (control). C57Bl/6 mice received one million purified OT-1 i.v. Two and fourteen days later (arrows in [E] and [F]), they were injected i.p. with one million live EG7-gp96Ig (E, G, and H) or with one million live EG7 (F). Mice were bled on days 0, 7, 14, and 21 and the PBL were analyzed for tetramer positive cells by staining with anti-CD8-PE and tetramer-APC or with anti-NK1.1-FITC or anti-CD11c-FITC. (E) Individual data from 11 mice immunized with EG7-gp96Ig (arrows) analyzed in three separate experiments for CD8/tetramer double-positive cells or gfp-OT-1-positive cells on the days indicated. Frequencies of OT-1 are expressed as a percentage of total CD8 cells. (F) Same as in (E) except that mice were immunized with EG7 that were not secreting gp96-Ig (arrows). (G) NK1.1 cell frequency in peripheral blood of two mice after primary immunization with EG7-gp96Ig. Frequencies are

expressed as a percentage of NK1.1-positive cells in the gate for large granular cells. (H) The frequency of CD11c-positive cells in blood after immunization with EG7-gp96Ig (arrows), expressed as a percentage of PBL.

imately one million of the purified cells were intravenously transferred to syngeneic B6 mice (Kearney et al., 1994). After 2 days resting, the mice were injected with one million live EG7-gp96Ig cells intraperitoneally. EG7-gp96Ig is a tumor derived from the EL4 lymphoma by transfection with ovalbumin and with gp96-Ig. One million EG7-gp96Ig secrete 50 ng gp96-Ig within 24 hr as measured by ELISA (Yamazaki et al., 1999) (data not shown). The secreted gp96-Ig chaperones ovalbumin peptides via the CD91 receptor or other receptors (Berwin et al., 2002) into antigen-presenting cells, where the ovalbumin peptides are further processed, presented as K[b-SIINFEKL], and then trigger the specific clonal expansion of cognate CD8 CTL including adoptively transferred OT-1. By increasing the frequency of ovalbumin-specific CD8 cells to about 0.2%–0.5% by adoptive transfer of OT-1, we hoped to create a sensitive in vivo assay for CD8 CTL expansion in response to secreted heat shock protein gp96-Ig.

The expansion of OT-1 after i.p. immunization with live EG7-gp96Ig was determined in peripheral blood by flow cytometry on days 0, 7, 14, and 21 by electronically gating on CD8-positive lymphocytes and determining the number of the K[b-SIINFEKL]-tetramer positive cells (Figure 1E) or $v_{\alpha2}$ positive cells (data not shown). In some experiments spleen cells were analyzed and showed essentially the same results as PBL. Within 1 week of EG7-gp96Ig immunization, TCR transgenic OT-1 expanded from a starting frequency of 0.5% to a mean frequency of 4.5% with a range of 2% to 6.5% within the CD8 pool (Figure 1E). During the second week after primary immunization OT-1 CTL contracted to a mean frequency of 2%. A booster immunization with EG7-gp96Ig on day 14 reexpanded the OT-1 frequency to more than 3% of the CD8 cells by day 21. The expansion of OT-1 was dependent on secreted gp96-Ig. Vector (mock) transfected EG-7 or untransfected EG-7 caused little OT-1 expansion (Figure 1F) in agreement with studies by others (Dalyot-Herman et al., 2000). In addition, mice injected with EG7 developed tumors and died while mice injected with live EG7-gp96Ig rejected the tumors (Yamazaki et al., 1999). Particularly noteworthy is the finding that the limited OT-1 expansion mediated by EG7 (without gp96-Ig secretion) is not followed by clonal contraction and secondary expansion upon boosting.

To exclude the possibility that the expansion of CD8^{+} CTL was an artifact of the cell transfer or of the peculiar properties of OT-1 transgenic cells, we determined the expansion of endogenous K[b-SIINFEKL]-tetramer positive cells in the absence of OT-1 transfer. Endogenous tetramer positive cells also expanded strongly after two immunizations (data not shown). However, due to the larger expansion of the adoptively transferred OT-1, improving signal to noise ratio, that system was preferred in subsequent studies. Expanded OT-1 exhibited effector function as measured by ELI-spot assay for IFN-$\gamma$. The frequency of IFN-$\gamma$-producing CD8 cells expanded in parallel with tetramer positive cells (data not shown).
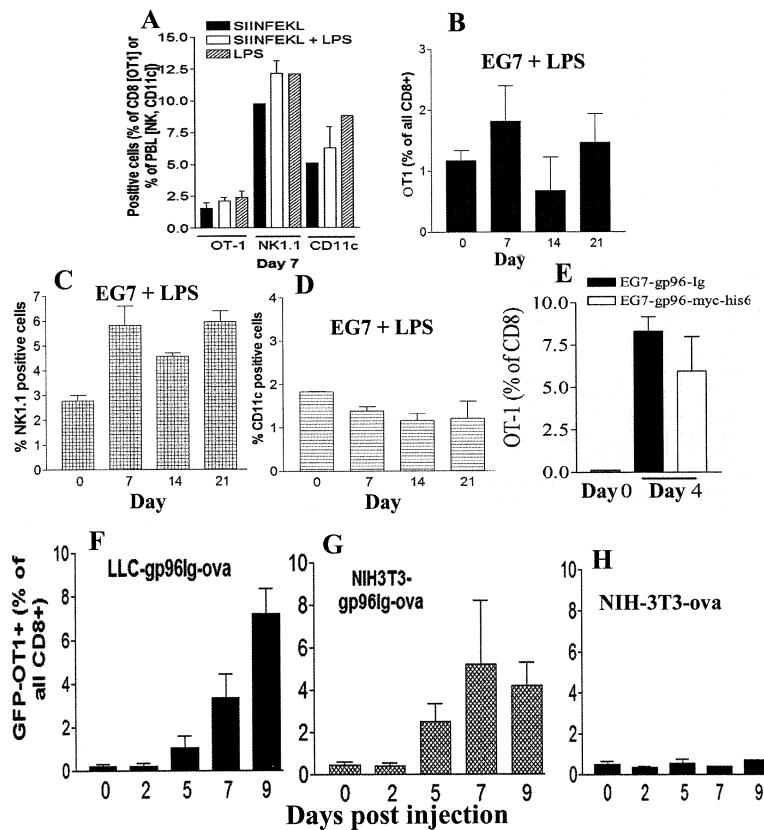
Figure 2. LPS Cannot Substitute for gp96-Ig Secretion; the Ig Portion of gp96-Ig Is Not Required; gp96-Ig Release Allows Cross-priming of OT-1

(A) Effect of SIINFEKL peptide and LPS on OT-1, NK, and CD11c expansion. Groups of three C57Bl/6 mice received one million OT-1. Two days later they were injected i.p. with 50 μg SIINFEKL with or without 50 μg LPS as indicated. Seven days later cell frequencies were analyzed.

(B–D) Effect of EG7 and LPS. Groups of three C57Bl/6 mice received one million OT-1 on day −2. On days 0 and 14 they were injected i.p. with one million EG7 plus 50 μg LPS. OT-1, NK1.1, and CD11c frequencies were determined in PBL on the days indicated.

(E) The Ig-tail of gp96-Ig is not required; C57Bl/6 mice received one million purified OT-1 i.v. and 2 days later (day 0) either one million EG7-gp96Ig or EG7-gp96myc-his₆. Four days later OT-1 expansion was measured by flow cytometry. Each group contained three mice. Mean and SE of tetramer positive cells in the CD8 gate are shown.

(F and G) One million purified gfp-OT-1 cells were used as detection system. (F) Lewis lung carcinoma cells were transfected with ovalbumin and gp96-Ig and used for immunization. One million cells producing 60 ng gp96-Ig in 24 hr were injected i.p.

(G and H) Crosspriming of gfp-OT-1 expansion by gp96-Ig-secretion. C57Bl/6 mice received gfp-OT-1 as above and 2 days later were injected i.p. with one million NIH-3T3-gp96Ig-ova (G) or with one million NIH-3T3-ova (H).

CTL expansion in response to viral infection is frequently preceded by NK cell activation and expansion (Biron et al., 1986, 1999). Since cell damage by infection or trauma is expected to release heat shock proteins including gp96 (Basu et al., 2000; Kotera et al., 2001), we were interested to determine whether secreted gp96-Ig was able to stimulate NK cell activation and expansion. NK cell frequencies in peripheral blood were determined with the NK1.1 (Figure 1G) and DX5 antibody (data not shown). Gating on large granular cells using forward and side scatter revealed that the frequency of NK1.1⁺ cells was about 9% among the large granular cells in peripheral blood (PBL) of untreated mice. Following EG7-gp96Ig injection, NK1.1⁺ cells expanded to 12% (in the granular gate) within 24 hr in response to secreted gp96-Ig and then contracted to the starting level. Just as untransfected EG-7 failed to support OT-1 expansion, EG7 also did not mediate NK1.1 expansion (data not shown).

Since gp96 has been reported to activate antigen-presenting cells, we also determined the mobilization of CD11c-positive cells in blood and in the spleen. CD11c⁺ cells increased 2-fold within 1 week after EG7-gp96Ig injection (Figure 1H) and returned to normal levels by 2 weeks. Boosting again doubled the frequency of CD11c⁺ cells in PBL. These data suggest that secreted gp96-Ig is able to expand CTL and NK cells and mobilize CD11c positive antigen-presenting cells (APC).

It was possible that gp96-Ig functioned as activator of APC and that SIINFEKL, the antigenic peptide, was provided directly by Kᵇ on EG7. In order to evaluate this possibility, we tested immune stimulation by the combination of synthetic SIINFEKL peptide with LPS and the combination of EG7 with LPS. C57Bl/6 mice that had received adoptively transferred OT-1 were immunized with SIINFEKL peptide in the presence or absence of LPS, and 1 week later OT-1, NK1.1, and CD11c frequencies were evaluated in blood (Figure 2A). SIINFEKL alone had a limited capacity to expand OT-1 to a frequency of about 2% of all CD8 cells. Addition of LPS to SIINFEKL did not significantly alter this effect. SIINFEKL had no effect on NK frequency while LPS with or without peptide caused moderate NK expansion (from 9% to 12% in the gate for granular cells). CD11c mobilization was more strongly triggered by LPS, and this effect was independent of peptide. We also evaluated the combination of EG7 with LPS on OT-1, NK1.1, and CD11c expansion (Figures 2B–2D). EG7 even in combination with LPS was unable to expand and mobilize any of the populations to an extent similar to EG7-gp96Ig. The data clearly indicate that SIINFEKL plus LPS or EG7 plus LPS cannot synergize and substitute for gp96-Ig-ovalbumin-peptide complexes in NK and CTL expansion, even though LPS and SIINFEKL individually have effects on OT-1, NK, and CD11c cells.

Although the Fc portion of IgG₁ used in gp96-Ig has low affinity for Fc receptors, we wanted to ascertain that the gp96 domain and not the Ig domain of gp96-Ig was

responsible for CTL and NK expansion. We therefore constructed a gp96-myc fusion protein by substituting the Ig domain with the myc epitope followed by six histidine residues (his$_6$). Secretion of gp96-myc upon transfection into EG7 was verified by Western blots and was comparable to gp96-Ig using anti gp96 antibodies. EG7-gp96Ig and EG7-gp96-myc were compared in their ability to expand OT-1 in vivo (Figure 2E). Within 4 days after immunization OT-1 had expanded similarly from 0.5% to about 7% frequency, regardless of which form of gp96 was secreted by EG7. Therefore, the Ig part in gp96-Ig is not required for clonal expansion.

To exclude the possibility that the EG7 tumor has unique properties in this system of gp96-Ig secretion, we transfected the Lewis lung carcinoma (LLC, C57Bl/6) with ovalbumin alone or with ovalbumin and gp96-Ig (LLC-gp96Ig-ova). In this experiment gfp-OT-1 were used, and they expanded to about 7% within 9 days in response to LLC-gp96Ig-ova immunization (Figure 2F); LLC-ova had no effect (data not shown). OT-1 expansion could have been effected by direct stimulation through K$^{b\text{-SIINFEKL}}$ expressed by EG7-gp96Ig or LLC-gp96Ig-ova; secreted gp96-Ig could have acted as a costimulus for TCR stimulation of OT-1. Direct OT-1 TCR stimulation is not possible by allogeneic cells that do not express K$^b$; therefore, OT-1 expansion, if observed, has to proceed through crosspresentation of ova-peptides by APC. We used NIH-3T3 cells as fully allogeneic cells. One million NIH-3T3 cells transfected with ovalbumin and gp96-Ig secreted 250 ng gp96-Ig within 24 hr. OT-1 expansion was similar (Figure 2G) when mice were injected with syngeneic LLC-gp96Ig-ova or with allogeneic NIH-3T3-gp96Ig-ova. NIH-3T3-ova not secreting gp96-Ig had no effect on OT-1 expansion (Figure 2H) despite its allogenicity, excluding OT-1 expansion by unspecific bystander activation. The data implicate dendritic cells or other antigen-presenting cells in CTL expansion by secreted gp96 fusion proteins.

## NK Activation by gp96-Ig Precedes OT-1 Activation

The activation of NK cells and of CD8 CTL by secreted gp96-Ig raised the question of the interdependence of the two phenomena and of the kinetics of the events. OT1 expansion does not begin significantly until day 3 (72 hr) after injection of EG7-gp96Ig but then rises continuously until day 7 (Figure 3A). In contrast, NK1.1 activation and expansion is detectable and maximal already after 24 hr followed by contraction (Figure 3B). On day 7 after immunization 5% of the NK1.1$^+$ cells are tetramer positive, suggesting that they are derived from OT-1 cells that express NK1.1. It has been reported that CD8-positive T cells upon activation can express NK markers including NK1.1 (Lan et al., 2001; Stremmel et al., 2001). We find that after 7 days up to 5% of the NK1.1-positive cells are tetramer positive, corresponding to about 10% of the expanded OT-1 cells. Taken together the data indicate that secreted heat shock protein gp96-Ig-peptide complexes mediate rapid NK cell activation during the first 2 days, followed by CD8 CTL (OT-1) expansion during the next 5 days. About 10% of the expanded OT-1 cells express NK markers and may have NK-like specificity (Stremmel et al., 2001).

## Perforin Deficiency Abrogates OT-1 Expansion by Tumor-Secreted gp96-Ig

The data indicate that CTL expansion caused by tumor-secreted gp96-Ig is preceded by NK activation. While this information indicates that heat shock proteins have an effect on innate immunity through NK activation (Multhoff et al., 2000), the data do not answer the question as to whether NK activation by gp96-Ig is related to CD8 CTL expansion. To address this question, NK cells were depleted in wild-type C57Bl/6 mice with anti-asialo-GM2 or with anti-NK1.1. Depletion was checked by flow cytometric analysis of PBL. The levels of NK marker positive cells were 0.3% after depletion indicating a better than 90% in vivo removal (Figure 3D). NK-depleted mice received one million purified OT-1 cells and 2 days later were immunized with EG7-gp96Ig. Depletion of 90% of the NK cells resulted in a 50% reduction of OT-1 expansion when compared to fully NK-competent mice (Figure 3E). These data suggested that NK activation enhanced CTL expansion by secreted heat shock protein gp96.

The participation of NK cells in gp96-Ig-mediated CTL expansion raised the question as to which molecular components of NK cells were required in this model system. NK cells mediate cytotoxicity by constitutively expressing perforin and Fas-ligand and produce IFN-γ upon stimulation. To determine whether any of these NK mediators are required for CTL expansion by tumor-secreted gp96-Ig, perforin knockout (PKO), IFN-γ knockout (GKO), and Fas-ligand-defective (gld) mice were analyzed for their ability to support OT-1 expansion in response to EG7-gp96Ig. The absence of perforin in PKO mice completely abolished OT-1 expansion in response to EG7-gp96Ig (Figures 4A and 4B) after both primary and secondary immunization. The absence of Ifn-γ (GKO) strongly diminished OT-1 expansion (Figure 4B). In contrast to perforin and Ifn-γ, Fas-ligand is dispensable for OT-1 expansion following EG7-gp96Ig immunization. OT-1 expanded in Fas-L-defective mice almost as well as in wild-type mice after primary immunization. Clonal contraction and secondary expansion, however, were diminished in gld mice. Perforin/Fas-ligand double-deficient mice (cdd), as expected, did not support OT-1 expansion after EG7-gp96Ig immunization.

The activation of endogenous NK1.1 cells after EG7-gp96Ig immunization was also impaired in PKO mice (Figure 5A), even though the adoptively transferred OT-1 used in the experiment are perforin sufficient. Similarly, endogenous NK1.1$^+$ cells failed to be activated in GKO mice after EG7-gp96Ig injection (Figure 5B) indicating that perforin and Ifn-γ produced by adoptively transferred normal OT-1 is not sufficient for NK and CTL expansion to gp96 in PKO or GKO mice. The findings therefore indicate that perforin and Ifn-γ are necessary for both NK activation and CTL expansion in response to gp96-Ig-peptide complexes, but that these two mediators are not derived from cognate CD8 CTL. Likely cellular candidates for releasing perforin and Ifn-γ are NK cells constitutively expressing perforin and secreting Ifn-γ upon stimulation.

Since wild-type OT-1 after differentiation to CTL express perforin, it was possible that an immune response to adoptively transferred, perforin-expressing OT-1 in
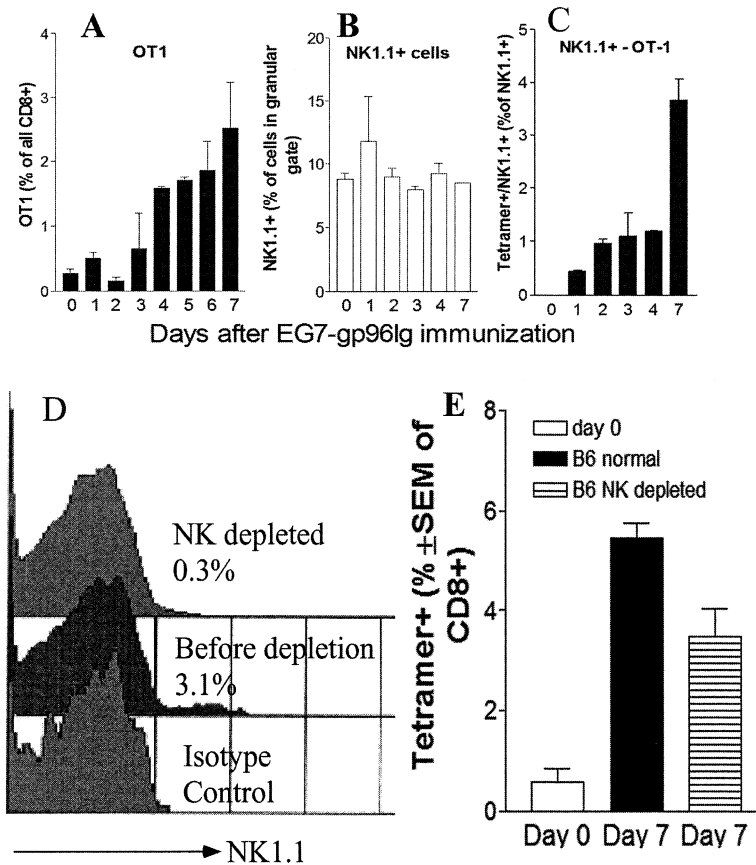
Figure 3. NK Activation Precedes OT-1 Expansion, and NK Depletion Abrogates OT-1 Expansion

(A–C) C57Bl/6 mice received one million purified OT-1 i.v. and 2 days later (day 0) one million EG7-gp96Ig i.p. Blood samples were obtained daily for 7 days and analyzed for expression of tetramer^{+} cells in the CD8 gate (A), for NK1.1 expression in a gate containing large lymphocytes (B), and for tetramer/NK1.1 double-positive cells (C). Data are from four mice per group, showing the mean and standard error.

(D and E) Effect of NK depletion on OT-1 expansion. For NK depletion anti-NK1.1 or anti-asialo-GM2 (100 μg) was injected twice 2 days apart to deplete NK cells. Control mice received control IgG. (D) The frequency of NK1.1 cells was analyzed 2 days after the last injection by flow cytometry of PBL stained with anti-NK1.1. (E) NK-depleted and control mice (three in each group) received one million purified OT-1 i.v. and 2 days later (day 0) one million EG7-gp96Ig i.p. Tetramer positive cells in the CD8 gate were analyzed in PBL on days 0 and 7. The mean and SEM are shown.

PKO mice abolished OT-1 expansion. We therefore bred the OT-1 TCR transgene into the PKO background, generating perforin-deficient OT-1. Adoptive transfer of perforin-deficient OT-1 into PKO mice did not restore their ability to expand in response to EG7-gp96Ig immunization (Figure 5C, third column). Therefore, the deficiency of perforin, rather than an immune response to perforin, is responsible for the lack of OT-1 expansion in PKO mice. As expected, OT-1 expansion (Figure 5D) and NK1.1 activation (data not shown) are also not supported in PKO by EG7.

### Perforin-Competent NK Cells Restore OT-1 Expansion in PKO Mice in Response to gp96-Ig

Since normal OT-1 (not deficient in perforin or IFN-γ) do not sustain their own expansion in the PKO or GKO background in response to EG7-gp96Ig immunization, it seemed clear that perforin and IFN-γ needed to be provided by non-CD8 cells. This hypothesis was tested by adoptively transferring wt syngeneic spleen cells into PKO mice and assessing OT-1 expansion in response to EG7-gp96Ig. Partial reconstitution of PKO mice with five million wt spleen cells containing approximately 200,000 NK1.1 cells largely restored OT-1 expansion upon EG7-gp96Ig injection (Figure 6A). To further define the perforin-containing cell type responsible for restoration of OT-1 expansion in the PKO background, we purified wt, syngeneic C57Bl/6 NK cells. Transfer of 500,000 perforin-sufficient NK1.1^{+} cells of 75% to 80% purity

into PKO mice restored the ability of EG7-gp96Ig immunization to expand OT-1 even better than 5 million spleen cells containing 200,000 NK cells (Figure 6B). The purified NK1.1 cells contained 2% CD8, 4% CD4 T cells, and 20% CD19/B220^{+} B cells. The reconstitution data of five mice in Figure 6 are consistent with the interpretation that perforin produced by wt NK cells is essential and sufficient to restore OT-1 expansion in the PKO background in response to gp96-Ig. The data also indicate that there is no defect other than perforin deficiency in PKO mice responsible for the failure of the CTL response following gp96-Ig peptide complex immunization.

### Discussion

Heat shock proteins isolated from or secreted by tumor cells are powerful tumor vaccines causing tumor rejection and generating specific memory (Palliser, 2001; Singh-Jasuja et al., 2001; Srivastava and Amato, 2001; Yamazaki et al., 1999). Using tumor-secreted gp96 or tumor membrane-associated gp96, tumor rejection was found to be independent of CD4 cells but required the participation of NK and CD8 cells. The current study was designed to quantitate the CD8 and NK cell response in vivo and to define the molecular and cellular mechanisms for the induction of the CTL response by gp96.

Tumor-secreted gp96 triggers tetramer-specific CD8 CTL expansion in our model system to a high frequency in vivo. Expansion of tetramer positive cells correlated with the frequency of IFN-γ ELI-spot forming cells (data
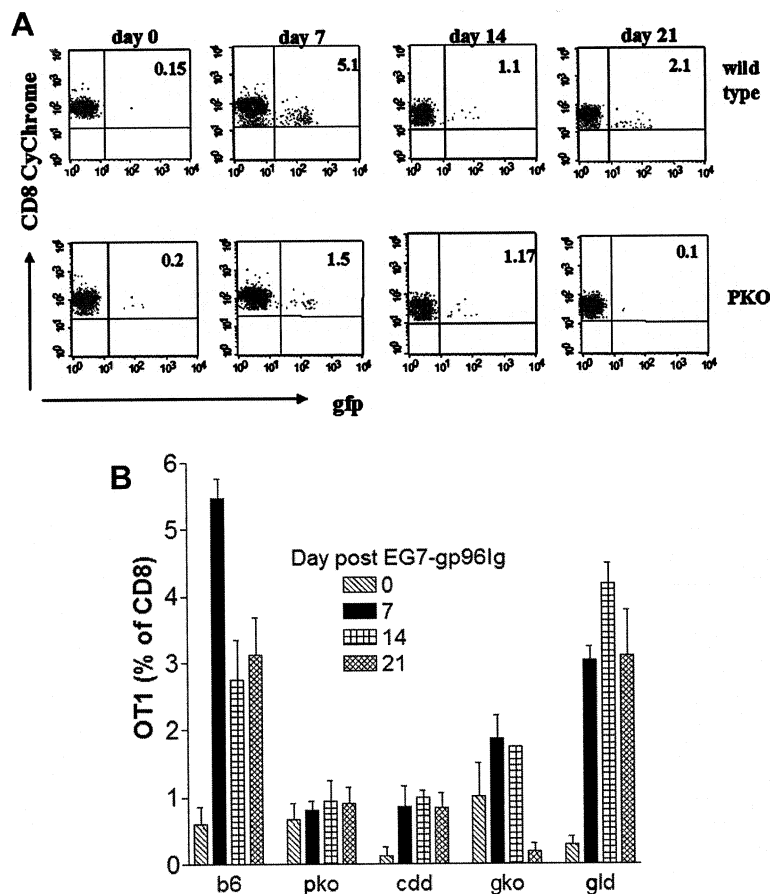
## A



## B



Figure 4. Perforin and Ifn-γ Are Required but Fas-Ligand Is Not Needed for OT-1 Expansion by gp96-Ig

(A) OT-1 do not expand in PKO mice. One million gfp-OT-1 were transferred i.v. to wt or PKO mice, and the mice were immunized i.p. on days 0 and 14 with 1 million EG7-gp96Ig. Representative FACS profiles gated on CD8 cells are shown. The numbers indicate the percentage of OT-1 cells in the CD8 gate.

(B) Wild-type and genetically deficient C57Bl/6 mice (as indicated in the graph) received one million OT-1 i.v. Two days later (day 0) and two weeks later (day 14) the mice received one million EG7-gp96Ig i.p (arrows). Mice were analyzed for tetramer/CD8 double-positive cells on the days indicated. B6 are wt C57Bl/6 mice obtained from Jackson Laboratories. Gld are homozygous Fas-ligand-defective mice, bred in our facilities under pathogen-free conditions. PKO are perforin-deficient mice also bred in our facilities. GKO are Ifn-γ-deficient mice obtained from Jackson Laboratories. Cdd mice are perforin/Fas-ligand double (cytotoxic double) -deficient mice (Spielman et al., 1998) and are maintained in our facilities. Each group was composed of four to six mice; OT-1 frequency is given as a percentage of CD8 cells ±SEM.

not shown). Specific CD8 CTL frequencies of 50% within the CD8 pool have been observed after viral challenge of adoptively transferred, TCR transgenic CD8 cells (Reich et al., 2000). The activation of endogenous NK cells is an additional indicator of the strength of the immune stimulus by gp96. As shown previously, the immune response clearly is associated with effector function, gp96Ig-secreting tumors are rejected, and specific memory is generated (Yamazaki et al., 1999), while non-gp96-secreting tumors are lethal. In ongoing studies we are assessing the effect of the presence of a wild-type, nonsecreting tumor on CD8 CTL expansion in this system. Initial data suggest that frequent vaccinations are required to sustain the CTL response.

A surprise in this study was the unexpected finding that perforin-deficient mice were unable to support CD8 CTL expansion by gp96, pointing to a role for cytotoxicity in the afferent arm of the immune response. That the cytotoxic activity of perforin is mediated by NK cells is suggested in depletion and reconstitution experiments. Perforin could not be substituted by Fas-ligand. Perforin and Ifn-γ expressed by activated wild-type OT-1 was not able to reconstitute CTL and NK expansion in PKO mice. Perforin and Ifn-γ expression in CD8 cells requires 2 to 3 days activation, while perforin and Ifn-γ are available instantly in NK cells. NK activation after EG7-gp96Ig immunization is very rapid and essential for subsequent CD8 expansion. NK cell adoptive transfer and depletion experiments support the conclusion that perforin pro-

vided by NK cells is critical for NK and CTL expansion. The question of whether normal NK cells or CD1-specific NK-T cells are involved in triggering CTL and NK expansion by gp96 is currently under study.

The inability of Fas-ligand to substitute for perforin may be explained in two ways. Fas-ligand may be unable to lyse the target cell, or Fas-ligand-mediated apoptosis, as opposed to perforin-mediated killing, does not support subsequent NK and CTL expansion. The latter explanation would suggest that lysis by perforin and granules may be qualitatively different from apoptotic death by Fas-ligand. It is still controversial, however, whether apoptotic death is less immunogenic than death by necrosis and trauma (Kotera et al., 2001; Somersan et al., 2001). PKO mice immunized with unirradiated EG7-gp96Ig, in contrast to wild-type mice, develop tumors with high incidence (data not shown). Therefore, the inability of PKO mice to mediate NK and CTL expansion correlates with increased susceptibility even to gp96-secreting tumors. Gp96-Ig vaccination, even in the absence of adoptive OT-1 transfer, allows the rejection of EG7 tumors preestablished for 3 or 5 days, supporting the effectiveness of the NK and CTL response (Yamazaki et al., 2002).

Gp96 is taken up by dendritic cells and leads to their activation (Basu et al., 2001). The ability of allogeneic, ovalbumin-transfected, and gp96-Ig secreting 3T3 cells to expand OT-1 by crosspriming clearly implicates antigen-presenting cells, most likely dendritic cells. We sug-
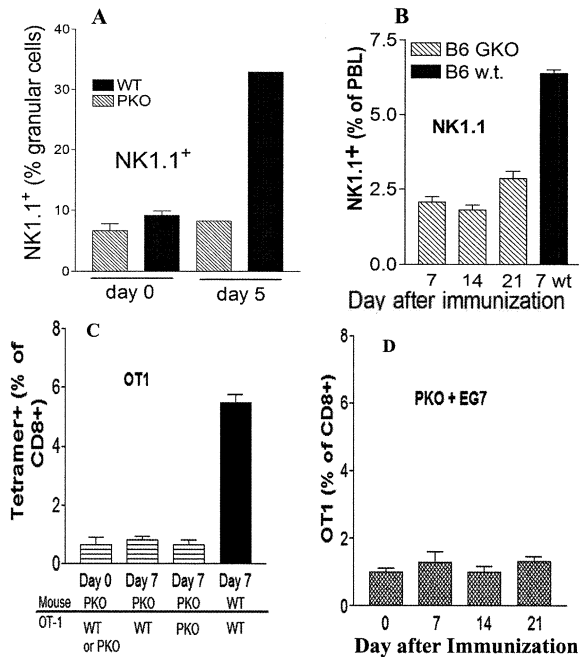
Figure 5. Perforin and Ifn-γ Deficiency Limit NK Expansion in Response to gp96-Ig

(A and B) PKO and GKO mice and wt controls received OT-1 on day −2 and were immunized with one million EG7-gp96Ig on days 0 and 14. NK1.1 frequency in PBL was analyzed on the days indicated.
(C) Perforin-deficient OT-1 do not expand in PKO mice to gp96-Ig. Perforin-deficient OT-1 cells were generated by breeding the OT-1 TCR transgene into PKO mice. Perforin-deficient mice and wt controls received one million purified normal OT-1 or purified, perforin-deficient OT-1 i.v. (as indicated) and 2 days later (day 0) one million EG7-gp96Ig. After 7 days OT-1 expansion was measured by flow cytometry. Groups of four mice were analyzed, and the mean and SEM are shown.
(D) PKO mice do not support OT-1 expansion in response to EG7. PKO mice received 1 million OT-1 on day −2 and were injected i.p. with one million EG7 on days 0 and 14 (arrows). OT-1 in PBL were identified by double staining with anti-v$_{\alpha 2}$ and anti-CD8.
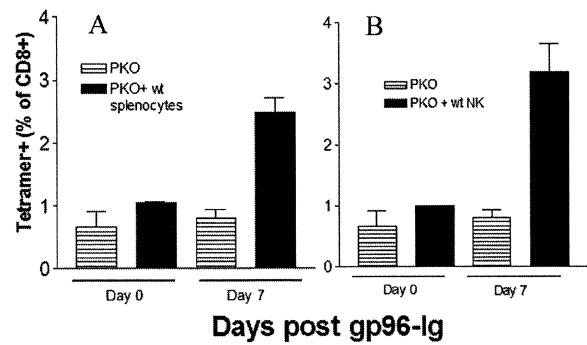


Figure 6. Reconstitution of OT-1 Expansion in Perforin-Deficient (PKO) Mice by Perforin-Competent Splenocytes or NK Cells

(A) Groups of PKO mice received five million syngeneic, wild-type splenocytes (containing 200,000 NK1.1 cells) together with one million OT-1 as indicated. Two days later (day 0) the mice received one million EG7-gp96Ig i.p. and were analyzed for OT-1 expansion by tetramer assay on days 0 and 7.
(B) Wild-type NK cells were purified to 76% purity from nonadherent spleen cells by NK1.1-positive selection on MACS columns. Five hundred thousand cells of the purified population containing 375,000 purified NK1.1 cells were adoptively transferred together with one million OT-1, and 2 days later the mice received one million EG7-gp96Ig i.p. Analysis was performed as in (A).

gest that gp96-Ig activates dendritic cells that in turn activate NK cells, signaling IFN-γ secretion by NK and triggering perforin-mediated lysis of susceptible targets. Ongoing in vitro studies using purified NK, DC, OT-1, and EG7-gp96Ig or EG7 support this conclusion. Three reports (Ferlazzo et al., 2002; Gerosa et al., 2002; Piccioli et al., 2002) published while this manuscript was written, document human NK/DC interaction in vitro. NK and DC can reciprocally activate each other, and NK cells are able to kill immature DC more efficiently than mature DC, supporting earlier reports (Carbone et al., 1999; Poggi et al., 2002; Wilson et al., 1999). Activated NK cells are also able to lyse EL4 and its derivative EG7 (Kalland et al., 1987; Matsumoto et al., 2000). Our in vivo data are consistent with the model of reciprocal activation of DC and NK and identify secreted gp96-Ig as one trigger for this positive feedback loop. Perforin and IFN-γ produced by NK according to this model are the molecular mediators sustaining the loop that is interrupted by their deficiency and terminated by the clearance of antigen. The positive feedback cycle between NK and dendritic cells requiring perforin and

IFN-γ is needed first for NK and subsequently for CTL expansion by gp96-Ig. A small proportion (up to 10%) of the CTL expanded by gp96-Ig expresses NK markers and may have antigen specificities more similar to NK in addition to MHC-restricted cytotoxicity. Expression of NK markers on CTL may regulate their interaction with DC including protection from lysis.

Lymphocytic choriomeningitis virus infection results in normal CTL expansion even in PKO mice (Badovinac and Harty, 2000; Kagi et al., 1994; Matloubian et al., 1999; Walsh et al., 1994). Viral infection and replication generates numerous inflammatory signals that may eliminate the need for perforin in the afferent phase of the immune response. NK activity and perforin therefore appear to be particularly important for the generation of CTL to immunogens, such as gp96-peptide complexes, generated in a noninflammatory environment, such as during tumor transformation or during immune suppression.

Our findings have implications in understanding the interconnections between innate and adaptive immunity, immune regulation, immune suppression, and vaccine therapy. Immune suppression by tumors and by HIV infection is frequently preceded by loss of NK activity. Diminished NK activity, similar to perforin deficiency, eliminates the ability of NK cells to lyse target cells and respond to gp96 and may be responsible for the lack of NK activation and the subsequent failure of CD8 expansion to tumor or viral antigens. In support of this hypothesis we have observed previously that perforin-deficient T cells transplanted with allogeneic bone marrow grafts cause delayed onset of graft versus host disease and delayed mortality. The inability of these grafts to mediate perforin-dependent killing of allogeneic targets may limit the amplification of the initial response (Baker et al., 1996). By the same reasoning, cellular vaccines may need to activate both NK and cognate CTL responses for effectiveness and maximal

stimulation of CD8 CTL responses and memory. Secretion of heat shock proteins by engineered tumor cells opens one way for the production of effective tumor cell vaccines that provide both adjuvanticity for NK activation and antigenicity for CTL generation.

## Experimental Procedures

### Cell Lines
EG7 were obtained from Dr. M. Bevan (U. of Washington, Seattle) and transfected with the bovine papilloma-derived vector pCMG-His containing gp96-Ig or gp96-myc as described (Yamazaki et al., 1999). Controls were transfected with vector alone. Lewis lung carcinoma (LLC) and NIH-3T3 cells, obtained from the American Tissue Culture Collection, were transfected with ovalbumin in pAC-Neo-ova (generously provided by Dr. M. Bevan (Hogquist et al., 1994) and with pCMG-His-gp96-Ig or with vector alone as control.

### Mice
C57Bl/6 and B6 IFN-$\gamma$ knockout mice were obtained from Jackson Laboratories. Gfp mice were obtained by permission of the producers (Ikawa et al., 1998); they were bred with OT-1 mice (obtained from Dr. M. Bevan) to generate gfp-OT-1-expressing green fluorescent protein. B6 perforin knockout mice (Kagi et al., 1994) and gld mice were maintained under pathogen-free conditions in our own facilities.

### Antibodies
Directly conjugated monoclonal antibodies, including PE- and Cy-Chrome-conjugated anti-mouse CD8a, FITC-conjugated anti-mouse NK1.1, PE-conjugated anti-mouse V2$\alpha$, FITC-conjugated anti-mouse V$\beta$5.1,5.2, FITC-conjugated anti-mouse CD11c, and PE-conjugated anti-mouse DX5 were purchased from PharMingen (San Diego, CA). Isotype controls, rat IgG2a (PE), mouse IgG2a (FITC), mouse IgG$_1$ (FITC), and hamster IgG$_1$ $\lambda$ (FITC) were purchased from PharMingen (San Diego, CA). Prior to staining, spleen cells and peripheral blood lymphocytes were treated with purified anti-mouse CD16/CD32 (Fc-$\gamma$III/II receptor, Pharmingen). K$^b$-tetramer associated with OVA peptide (SIINFEKL) and APC conjugated was obtain from the NIH-supported Tetramer Core Facility. Tetramer was titrated and used at a final dilution of 1:50.

### Flow Cytometry—Analysis of TCR Transgenic T Cells
The magnitude of an antigen-specific immune response was determined by enumerating CD8 T cells by staining with antibodies to a particular variable (V) region $\alpha$ (V$\alpha$2) or V$\beta$ (V$\beta$5.1,5.2) chains or with K$^b$-tetramer (loaded with SIINFEKL) using three-color flow cytometry. In some experiments as indicated in the text, gfp-OT-1 were detected in the FITC channel by FACS analysis without staining. $2 \times 10^5$ peripheral blood lymphocytes were stained with APC-labeled K$^b$-tetramer associated with SIINFEKL for 1 hr at 4°C. Cells were washed twice in FACS buffer (PBS containing 0.5% BSA, 0.1% NaN$_3$, and 2 mM EDTA). Antibodies to CD8 (PE) and NK1.1 (FITC) were then added for an additional 30 min. Events were acquired on a FACS Vantage flow cytometer (10,000–20,000 events were collected in a CD8 electronic gate), and the data were analyzed using CellQuestPro Software (Becton Dickinson).

### Flow Cytometry—Analysis of NK, NK-T, and Dendritic Cells
For detection of NK, NK-T, and dendritic cells in C57Bl/6, PKO, and GKO, peripheral blood lymphocytes were incubated with K$^b$-tetramer (SIINFEKL) as described followed by incubation with surface antibodies: FITC-conjugated NK1.1, PE-conjugated DX5, FITC-conjugated CD11c, PE- or CY-conjugated CD8.
    The cell size of NK cells and dendritic cells was determined by flow cytometry using forward light scatter (FSC) and side scatter (SSC). Electronic gates were used to display the FSC and SSC of NK1.1-positive or CD11c-positive cells. A difference in size and granularity compared to CD8 cells was observed. Therefore, in the analysis two electronic gates were used: one including only large granulated cells and the other including small and large granulated cells.

### Purification of CD8$^+$ OT-1 Cells
Spleen cells from OT-1 TCR transgenic mice were purified by positive column selection using MACS anti-CD8a (Ly-2) MicroBeads (Miltenyi Biotec, Auburn, CA). Splenocytes were resuspended in 90 $\mu$l of buffer (PBS containing 0.5% BSA) per 10$^7$ total cells and 10 $\mu$l of microbeads added. After 15 min incubation at 6°C–12°C, cells were washed and used for magnetic separation on LS columns. Purified cells were >95% CD8$^+$ as judged by FACS analysis.

### Purification of NK1.1$^+$ Cells
Spleen cells from C57BL/6 mice were allowed to adhere to plastic dishes for 1 hr. Nonadherent cells were collected and purified by positive column selection using mouse anti-DX5 MicroBeads (Miltenyi Biotec, Auburn, CA). Cells were resuspended in 80 $\mu$l of buffer (PBS containing 0.5% BSA) per 10$^7$ total cells and 20 $\mu$l of microbeads added. After 15 min incubation at 6°C–12°C, cells were washed and used for magnetic separation on a MS column. The resulting cells were 73%–76% NK1.1$^+$ as judged by FACS analysis.

### Adoptive Transfer of TCR Transgenic T Cells
Sex-matched purified CD8$^+$ cells (10$^6$) from naive OT-1 TCR transgenic mice or gfp-OT1 mice were injected intravenously in a volume of 0.2 ml PBS into normal nonirradiated B6 mice, PKO, GKO, or *gld/gld* mice.

### Immunizations
C57Bl/6 mice received one million of purified OT-1 i.v. on day 2. On days 0 and 14 mice were injected with one million nonirradiated EG7-gp96-Ig, EG7-gp96-myc-his6, LLC-gp96Ig-ova, NIH3T3-gp96Ig-ova, or with EG7, LLC-ova, or NIH-3T3-ova intraperitoneally in a volume of 0.4 ml PBS. OT-1 expansion was measured in blood or spleens by flow cytometry 4, 7, 14, and 21 days later. As controls, mice were immunized with the synthetic ovalbumin derived peptide SIINFEKL (50 $\mu$g) or EG7 and LPS (*E. coli*, Diffco, Detroit, MI) (50 $\mu$g) or a combination of the agents.

### NK Depletion
C57BL/6 mice were depleted of NK cells using rabbit anti asialo-GM1 antibody (anti-ASGM1) (WakoPure Chemicals Inc, Richmond, VA) (Wang et al., 1998). Mice were injected twice intraperitoneally with 40 $\mu$l of anti-ASGM1 diluted in 0.5 ml of PBS or only PBS as control on days 4 and 2 before OT-1 transfer. Depletion of NK1.1 cells was confirmed by flow cytometry and shown to be greater than 90%.

### NK Reconstitution into PKO
Sex-matched purified NK1.1$^+$ cells (5 $\times$ 10$^5$) and purified OT-1 CD8$^+$ were transferred into nonirradiated PKO mice on day 2, and 2 days later the mice received one million EG7-gp96Ig i.p.

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