Lentivector Prime and Vaccinia Virus Vector Boost Generate High-Quality CD8 Memory T Cells and Prevent Autochthonous Mouse Melanoma

Haiyan Xiao, Yibing Peng, Yuan Hong, Yanjun Liu, Z. Sheng Guo, David L. Bartlett, Ning Fu and Yukai He

J Immunol 2011; 187:1788-1796; Prepublished online 11 July 2011;
doi: 10.4049/jimmunol.1101138
http://www.jimmunol.org/content/187/4/1788
Lentivector Prime and Vaccinia Virus Vector Boost Generate High-Quality CD8 Memory T Cells and Prevent Autochthonous Mouse Melanoma

Haiyan Xiao,*† Yibing Peng,* Yuan Hong,* Yanjun Liu,*† Z. Sheng Guo,‡ David L. Bartlett,‡ Ning Fu,‡ and Yukai He*†

Most cancer vaccines, to date, fail to control established tumors. However, their application in preventing tumors is another question that is understudied. In the current study, we investigated the CD8 memory T cell responses of lentivector (lv) immunization and its potential to prevent melanoma using both transplantable B16 tumor and autochthonous melanoma models. We found that lv-expressing xenogenic human gp100 could induce potent CD8 responses that cross-react with mouse gp100. Importantly, the lv-primed CD8 response consisted of a high number of memory precursors and could be further increased by recombinant vaccinia virus vector (vv) boost, resulting in enhanced CD8 memory response. These long-lasting CD8 memory T cells played a critical role in immune surveillance and could rapidly respond and expand after sensing B16 tumor cells to prevent tumor establishment. Although CD8 response plays a dominant role after lv immunization, both CD4 and CD8 T cells are responsible for the immune prevention. In addition, we surprisingly found that CD4 help was not only critical for generating primary CD8 responses, but also important for secondary CD8 responses of vv boost. CD4 depletion prior to lv prime or prior to vv boost substantially reduced the magnitude of secondary CD8 effector and memory responses, and severely compromised the effect of cancer immune prevention. More importantly, the CD8 memory response from lv-vv prime-boost immunization could effectively prevent autochthonous melanoma in tumor-prone transgenic mice, providing a strong evidence that lv-vv prime-boost strategy is an effective approach for cancer immune prevention. The Journal of Immunology, 2011, 187: 1788–1796.

Vaccines are the most successful medicines for preventing infectious diseases. Based on the same premise, cancer vaccines have been extensively pursued to stimulate antitumor immunity. However, due to practical and ethical constraints, cancer vaccines, in most cases, have been tested as a therapeutic approach to treat established (and mostly advanced stages of) cancers, which is completely deviated from what vaccine does the best and is intended to do (i.e., to prevent diseases). Therefore, it is no surprise that most cancer vaccines fail to control established cancers (1). It is becoming evident that cancer vaccines alone may not work well for treating tumors due to the immune suppression established in the tumor lesions (1–3). However, their efficacy of preventing tumors is totally another question that needs to be addressed. It has been reasoned that cancer vaccines may work effectively in prophylactic setting because the tumor-induced immune suppression is not established (4, 5). However, cancer immune prevention has been understudied even though it was proposed a decade ago (5, 6) possibly due to the difficulty of identifying high-risk population, the uncertainty of cancer development, and the risk of developing autoimmune diseases. But, with the advancement of genomic analysis and gene profiling, more accurate prediction of cancer development (or relapse after conventional therapy) and better evaluation of risk/benefit ratio will become possible, which can lead to acceptance of cancer immune prevention in high-risk population (4, 7, 8).

The researches of tumor immunotherapy in the last two decades yield many different kinds of cancer vaccines, including peptide (9–), dendritic cell (DC) (10–), and gene (11, 12)-based vaccines that can stimulate potent tumor-specific immune responses. Although immunized mice were prevented from tumor cell challenge, the protection was mostly examined when the immune responses were still at the peak (13, 14), which has little relevance to the long-term effect of cancer immune prevention. Furthermore, prevention of autochthonous tumor in cancer-prone transgenic (Tg) mice was rarely conducted in a few studies. One report found that DNA immunization against oncoprotein rat Her2/neu could prevent breast cancer in the HER2/NEU Tg mice (15). However, repeated immunization every 3–4 wk was required to maintain the prevention effect. Another recent study showed that peptide vaccination against tumor Ag mucin 1 could prevent progression of colitis to colon cancer in the IL-10−/− mucin 1 Tg mice (8). But the prevention effect was again examined only 10 wk after immunization. The long-term prevention effect of cancer vaccines is unknown. In addition, the immune correlates of cancer immune prevention are controversial. For example, whereas Abs were found important in preventing autochthonous breast cancer in HER2/NEU Tg mice (15), cytotoxic T cells with (16) or without the help of Abs (13) were shown to prevent transplantable breast

*Immunology/Immunotherapy Program, Georgia Health Sciences University Cancer Center, Georgia Health Sciences University, Augusta, GA 30912; †Department of Immunology, Southern Medical University, Guangzhou 510515, China; ‡Division of Surgical Oncology, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15232; and Department of Medicine, Medical College of Georgia, Georgia Health Sciences University, Augusta, GA 30912

Received for publication April 19, 2011. Accepted for publication June 14, 2011.

This work was supported by National Institutes of Health Grant R01 CA16444 and the Distinguished Investigator fund from the Georgia Research Alliance (to Y. He).

Address correspondence and reprint requests to Dr. Yukai He or Dr. Ning Fu, Immunology/Immunotherapy Program, Georgia Health Sciences University Cancer Center, Georgia Health Sciences University, CN-4150, 1410 Laney Walker Road, Augusta, GA 30912 (Y.H.) or Department of Immunology, Southern Medical University, Guangzhou 510515, China (N.F.). E-mail addresses: yhe@georgiahealth.edu (Y.H.) and zfzu@fimmu.com (N.F.)

Abbreviations used in this article: DC, dendritic cell; Grm1, metabotropic glutamate receptor 1; hgp100, human gp100; ICS, intracellular staining; lv, lentivector; mgp100, mouse gp100; Tg, transgenic; vv, vaccinia virus vector.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11 $16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1101138

The Journal of Immunology
cancer in mice. Thus, there is a need for designing better cancer vaccines and for understanding the mechanisms underpinning the immune prevention.

We and others found that recombinant lentivector (lv) could induce potent CD8 immune responses against self/tumor Ags (17, 18) because of their effective transduction of skin DCs in vivo (19–21). Higher percentage of CD8 T cells elicited by lv possesses memory phenotype (22), suggesting that the lv-primed CD8 T cells will effectively respond to boosting immunization. Heterologous prime-boost immunization strategy has been extensively studied for stimulating potent and long-lasting memory responses to prevent infectious diseases (23–25). It was reported that lv-vaccinia virus vector (vv) prime-boost could markedly increase melanoma Ag NY-ESO–specific effector CD8 responses (26). However, it is not clear whether this strategy will generate enhanced memory CD8 T cells and prevent clinically relevant autochthonous cancers. It is also not known whether the prime-boost-induced memory responses will be qualitatively superior to that from one immunization and better prevent mice from tumor challenge. Therefore, in this study, using self melanoma Ag gp100, we studied the CD8 memory responses of lv-vv prime-boost immunization and the immune prevention of autochthonous melanoma. We found that much more potent CD8 memory responses could be elicited by lv-vv prime-boost. Critically, we demonstrated that only the CD8 memory T cells from lv-vv prime-boosted mice were able to rapidly respond to tumor cell challenge to prevent tumor establishment. Another important novel finding is that the generation of high-level effector and memory CD8 T cells requires the CD4 help during both lv prime and vv boost stage. More importantly, we demonstrated that the lv-vv prime-boost strategy could effectively prevent autochthonous melanoma growth in melanoma-prone metastatic gluta- mate receptor 1 (Gmr1) Tg mice for ≥12 mo.

Materials and Methods

Cell lines and mice

Cell lines CV-1, TK-143, 293T, and B16F10 were acquired from American Type Culture Collection and maintained in complete DMEM. C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD). Human gp100 (hgp100)-specific TCR Tg Tg (pmel-1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The melanoma-prone Gmr1 Tg mice were provided by S. Chen of Rutgers University (27). All the mice were housed under specific pathogen-free conditions in Laboratory Animal Services of Georgia Health Sciences University. Animal care protocols were approved by the Institutional Animal Care and Use Committee of Georgia Health Sciences University.

Construction of viral vectors and immunization

Plasmid hgp100 DNA was provided by W. Storkus, University of Pittsburgh Cancer Institute and Department of Dermatology, University of Pittsburgh. To construct lv-expressing xenogenic hgp100, gene fragment containing the N-terminal 340 aa of hgp100 was obtained using high-fidelity PCR and cloned into the lv plasmid. The recombinant lv was designated as hgp100-lv. Hgp100-lv was prepared and titered, as previously described (20). For immunization, 1.5 × 10^7 transduction units of hgp100-lv were injected into the footpad.

To construct recombinant vv expressing the hgp100, a shuttle plasmid vector pG10 was used (Fig. 1A). The hgp100 340-aa fragment was cloned behind the p7.5 early gene promoter to obtain pg10-hgp100. CV-1 cells in 6-well plates were infected with a wild-type vaccinia virus of WR strain at multiplicity of infection of 0.1, and then transfected with pg10-hgp100 by SuperFect reagent (Qiagen). The recombinant vv was selected in human TK-143 cells with addition of BrdU in the medium. After three rounds of plaque purification, the purity of the virus was verified by PCR assays for presence of the transgene and deletion of the viral thymidine kinase gene, and then more than 99% of the virus was titered in the infected cells. The virus, designated as hgp100-vv, was amplified in HeLa cells and purified by a standard procedure (28). For immunization, 1.5 × 10^7 PFU of hgp100-vv was injected i.p. or via footpad.

In vivo proliferation of pmel-1 by hgp100-lv or hgp100- vv immunization

To examine the induction of Ag-specific T cell proliferation by recombinant lv and vv in vivo, mice were immunized with either hgp100-lv or hgp100-vv and then injected with 1 × 10^5 pmel-1 cells labeled with CFSE dye, as previously described (19). Three days later, vaccine-draining lymph node was collected to determine the proliferation of pmel-1 cells by analyzing the progressive dilution of CFSE dye.

Flow cytometry analysis of immune responses

To detect gp100-specific CD8 T cells, single cells from peripheral blood or spleen were stained with hgp100 25–33 D^β tetramer (provided by the National Institutes of Health tetramer core facility at Emory University) together with anti-CD127, anti-CD8, and anti-CD62L (Biolegend, San Diego, CA). In addition, to measure the cytokine production, single-cell suspensions of peripheral blood or spleen were stimulated for 3 h with 1 μg/ml hgp100 peptide (GeneScript), and then intracellular staining (ICS) for IFN-γ was performed, as described (20). Cells were collected using a FACScanto system (BD Biosciences, San Jose, CA). Data were analyzed using FCS Express V3 software (De Novo Software, Los Angeles, CA).

Immune prevention of cancer development

To study the cancer immune prevention effect, B16F10 (3 × 10^5) cells were inoculated s.c. into the shaved flank of the immunized C57BL/6 mice. Tumor growth was monitored. To investigate the prevention effect in autochthonous melanoma model, lv prime with or without vv boost was conducted on 1.5- or 3-mo-old melanoma-prone Gmr1 Tg mice. Melanoma growth on the tail and ear was monitored.

Depletion of T cell subsets in vivo

To study the immune correlates of cancer prevention, immunized mice were depleted of CD4, CD8, or both by in vivo injection of 500 μg functional grade of mAbs (OK1.5 for CD4 depletion, 2.43 for CD8 depletion; Bio X Cell, West Lebanon, NH) before tumor challenge. The effect of T cell subset depletion was monitored by examining the CD4 and CD8 T cells in the peripheral blood.

Statistical analysis

Data were analyzed using Student unpaired t test, ANOVA, or log rank test of the Prism software (GraphPad Prism, La Jolla, CA).

Results

Lv-expressing xenogenic hgp100 stimulates potent CD8 responses that cross-react with mouse gp100 epitope

To characterize the lv-induced immune responses and to study the potential of lv immunization for immune prevention, we constructed recombinant lv-expressing melanoma Ag human gp100 (hgp100) because it was previously demonstrated that xenogenic hgp100 DNA could better protect mice from B16 tumor cell challenge than mouse gp100 (mgp100) (29). As a comparison, recombinant vv was also constructed (Fig. 1A). We found that, compared with hgp100- vv, hgp100- lv was more potent at inducing in vivo proliferation of hgp100 25–33-specific CD8 T cells, pmel-1 (Fig. 1B). In addition, by measuring the IFN-γ^+ T cells, we found that activation of endogenous CD8 T cells by lv was substantially higher than recombinant vv (Fig. 1C), which was in agreement with our previous findings that lv was superior to other viral vectors (19). Approximately 7% of CD8 T cells in the peripheral blood from hgp100-lv–immunized mice produced IFN-γ in responding to a 3-h ex vivo hgp100 25–33 peptide stimulation, whereas the hgp100 CD8 response was undetectable in hgp100- vv–immunized mice. Critically, the hgp100-lv–activated CD8 T cells could effectively cross-recognize the mgp100 epitope (mgp100 25–33) and produce IFN-γ (Fig. 1D). These data indicate that lv-expressing xenogenic hgp100 elicits potent CD8 T cell responses that can cross-react with mgp100 peptide. We also showed that although recombinant vv is a proven safe vaccine vector and has been widely used for immunization, it is not.
pmel-1 T cells were analyzed for proliferation by gating on Thy1.1 + CD8 adoptively transferred with CFSE-labeled pmel-1 T cells. Three days later, reduced to proliferate by hgp100-lv or hgp100-vv footpad immunization. Approximately 30–40% of CD8 T cells in the peripheral blood were hgp100-tetramer positive 2 wk after immunization. Five weeks after immunization, the proportion of hgp100-tetramer-positive cells remained at 10% of total CD8 T cells. More importantly, ~20% of tetramer-positive CD8 T cells were also CD127+ at the peak of immune responses (day 15) (Fig. 2B). Most of the hgp100-tetramer-positive cells were CD62L+. The CD127+CD62L− phenotype of activated CD8 T cells is consistent with the markers of effector memory T cells (31), suggesting lv-activated T cells contain a high level of precursors of effector memory CD8 T cells in the peripheral at early stage of immune responses. The ratio of CD127+ cells increased with time (Fig. 2C), in agreement with the notion that memory T cells are gradually accumulated as the effector cells waned (32).

**FIGURE 1.** Hgp100-lv immunization stimulates potent CD8 responses that can cross-react with mpgp100 epitope. A. The schematic diagram of hgp100-lv and hgp100-vv was shown. Hgp100-N indicated the N-terminal 340 aa of hgp100. B. Pmel-1 cells in the popliteal lymph node were induced to proliferate by hgp100-lv or hgp100-vv footpad immunization. C57BL/6 mice were immunized with hgp100-lv or hgp100-vv and then adoptively transferred with CFSE-labeled pmlt-1 T cells. Three days later, pmlt-1 T cells were analyzed for proliferation by gating on Thy1.1+ CD8 T cells. A representative of three mice in each group was shown. C. Two weeks after immunization, ICS of IFN-γ of the peripheral blood cells was performed following 3-h ex vivo stimulation with hgp100 peptide. No IFN-γ+ CD8 T cells were detected in control mice (data not shown). The number in the parentheses of upper right quadrant represents the percentage of IFN-γ+ T cells among total CD8 cells. A summary of three mice was shown on the right. D. Cross-reactivity of hgp100-lv–induced CD8 responses to mpgp100 epitope in peripheral blood was demonstrated by ICS of IFN-γ after ex vivo stimulation with indicated peptides. Summary data of three mice were also shown. The experiment was repeated twice with similar data. Statistical analysis was done with unpaired t test.

Effective for stimulating immune responses against the transgene-encoded Ag.

The gp100-specific CD8 T cells elicited by hgp100-lv consist of effector memory precursor cells with the phenotype of CD127highCD62Llow.

The ultimate purpose of vaccine administration is to generate long-lasting memory immune cells in the immunized host. To further characterize the CD8 immune responses elicited by hgp100-lv immunization, we determined the CD127 (IL-7Rα) expression on the hgp100-tetramer-positive cells because it was previously shown that CD127 was selectively expressed on the precursors of memory CD8 T cells (30, 31). Using tetramer staining, we found that whereas the ratio of hgp100-specific CD8 T cells was low following hgp100-vv immunization, hgp100-lv immunization dramatically increased the hgp100-tetramer-positive CD8 T cells (Fig. 2A), consistent with the data of cytokine staining (Fig. 1C). Five days after boost, the CD8 responses approximately reached the peak with ~15% of CD8 T cells in the blood were IFN-γ+. Reversing the order of prime-boost to vv prime and lv boost only induced the same magnitude of immune response as that of the lv-elicited primary responses. The CD8 responses induced by lv-vv prime-boost were long lasting. More than 2 mo (70 d) after boost, the ratio of IFN-γ+ CD8 T cells in the peripheral blood was still at the level of 5% (Fig. 3A). And at this stage, ~20% of CD8 T cells in the blood were hgp100-tetramer-positive cells (Fig. 3B). Importantly, >90% of the hgp100-specific CD8 T cells in the PBL were CD127+, suggesting most of hgp100-specific CD8 T cells become memory cells (lower panel, Fig. 3B). Although the hgp100-tetramer-positive cells were markedly higher in the prime-boosted mice compared with lv immunization alone, the ratio of CD127+ remained the same (lower panel, Fig. 3B). Thus, vv boost does not preferentially increase memory cells, but simply increases the overall Ag-specific responses that give rise to a higher number of memory CD8 T cells.

Lv-vv prime-boost markedly increases the magnitude of effector and memory CD8 responses

The presence of CD127+ CD8 memory precursor T cells suggests that lv-primed immune responses have the potential to be substantially increased with boosting immunization. To examine this hypothesis, lv-primed mice were then boosted with hgp100-vv. The kinetics of immune responses was determined by repeatedly examining the IFN-γ+ CD8 T cells in the peripheral blood. Consistent with the data in Figs. 1 and 2, hgp100-vv alone did not induce measurable immune responses. However, hgp100-vv boost immunization rapidly and markedly increased the magnitude of CD8 responses in the hgp100-lv–primed mice (Fig. 3A). Five days after boost, the CD8 responses approximately reached the peak with ~15% of CD8 T cells in the blood were IFN-γ+. Reversing the order of prime-boost to vv prime and lv boost only induced the same magnitude of immune response as that of the lv-elicited primary responses. The CD8 responses induced by lv-vv prime-boost were long lasting. More than 2 mo (70 d) after boost, the ratio of IFN-γ+ CD8 T cells in the peripheral blood was still at the level of 5% (Fig. 3A). And at this stage, ~20% of CD8 T cells in the blood were hgp100-tetramer-positive cells (Fig. 3B). Importantly, >90% of the hgp100-specific CD8 T cells in the PBL were CD127+, suggesting most of hgp100-specific CD8 T cells become memory cells (lower panel, Fig. 3B). Although the hgp100-tetramer-positive cells were markedly higher in the prime-boosted mice compared with lv immunization alone, the ratio of CD127+ remained the same (lower panel, Fig. 3B). Thus, vv boost does not preferentially increase memory cells, but simply increases the overall Ag-specific responses that give rise to a higher number of memory CD8 T cells.

The CD8 memory T cells generated by lv-vv prime-boost have superior quality

To prevent tumor cells from becoming established tumor, it is critical that the vaccine-induced tumor-specific memory T cells can immediately sense the emergence of tumor cells and be rapidly reactivated and expanded to kill the malignant or premalignant cells in the incipient stage. It is known that tumor cells, such as the low immunogenic B16 tumor cells, are rarely detected by the immune system because of the low level or absence of MHC molecules. Inoculation as low as 1000 B16F10 cells is sufficient to form tumor in C57BL/6 mice (33). To investigate whether the memory CD8 T cells activated by lv-vv prime-boost can protect mice from tumor cell challenge, we first examined whether the memory CD8 T cells can sense the inoculation of B16F10 tumor cells and be reactivated. Approximately 2.5 mo after vv boost and prior to...
tumor cell challenge, the lv-vv prime-boosted mice maintained significantly higher IFN-\(\gamma\) CD8 T cells compared with other mice (Fig. 4A). These data suggest that memory CD8 T cells can rapidly respond to ex vivo Ag stimulation to execute effector function. But, the most significant finding was that the memory CD8 T cells in the lv-vv prime-boosted mice could sense the B16 tumor cell challenge and immediately expanded to a significantly higher level (Fig. 4B). The hgp100 25–33-specific CD8 T cells in lv-vv prime-boosted mice were increased by 2- to 3-fold after B16 tumor cell challenge. In contrast, there was no measurable increase of CD8 T cells from the lv-primed, vv-primed, or the vv-lv prime-boosted mice in responses to the inoculation of B16 tumor cells (Fig. 4C). Our data strongly suggest that the hgp100-specific memory CD8 T cells generated from lv-vv prime-boost are better prepared and ready to be reactivated when cognate Ag is sensed.

The higher magnitude of memory CD8 T cells and their rapid responsiveness to tumor cell challenge in the lv-vv prime-boosted mice were translated into better protection. Eighty percent of the mice from lv-vv prime-boosted group were protected (Fig. 4D). Only a small fraction of vv-lv prime-boosted mice was protected from B16 tumor challenge. In contrast, although lv- or vv-alone immunization could delay tumor growth, all mice eventually succumbed to tumor growth.

Both CD8 and CD4 effector T cells are required for cancer immune prevention

To study the role of CD8 and CD4 effector T cells in cancer immune prevention, CD8, CD4, or both were depleted 2 wk after lv immunization at the peak of immune responses. Depletion of CD8

---

**FIGURE 2.** Lv-stimulated hgp100-specific CD8 T cells consist of high percentage of CD127\(^+\) memory precursors. Two weeks after immunization, peripheral blood cells from immunized C57BL/6 mice were stained with hgp100/D\(\beta\) tetramer and CD127 or CD62L. A, In agreement with data of Fig. 1, hgp100-lv induced more potent CD8 response than hgp100-vv. A representative of five mice was presented. The kinetics of tetramer-positive cells was also shown on the right. B, The tetramer-positive CD8 T cells were gated and analyzed for CD127 expression. Approximately 20–30% of hgp100-specific CD8 T cells were CD127\(^+\), and most of hgp100/D\(\beta\) tetramer-positive CD8 T cells were CD62L\(^+\). C, The percentage of memory CD127\(^+\) hgp100-specific CD8 T cells increased with time. Five mice were in each group, and the experiment was repeated twice with similar observation.

---

**FIGURE 3.** Lv-primed CD8 responses can be markedly increased by vv boost, generating long-lasting memory CD8 responses. A, The kinetics of CD8 response after lv or vv immunization was monitored by ICS of IFN-\(\gamma\). Forty-five days later, some of the lv-primed mice were boosted with vv or vice versa. Five mice were in each group. B, Seventy days after boost, the hgp100-specific CD8 T cells and their memory marker of CD127 were measured. Only the CD8 T cells were shown. Summaries of the tetramer and CD127 staining (five mice) were presented on the right. The experiment was repeated twice with similar observation. Unpaired \(t\) test or ANOVA was used for statistical analysis.
or both CD4 and CD8 effector T cells completely deprived of the immune protection of mice (Fig. 5). In contrast, mice with CD4 depletion retained some of the immune protection effect. These data suggest that both CD4 and CD8 effector cells are required for immune prevention even though CD8 T cells may be the dominant effector cells.

CD4 help is required for both primary and secondary CD8 immune response

In addition to the direct role at effector phase to prevent tumor cell challenge (Fig. 5), CD4 cell has been widely recognized for helping CD8 responses (34). To study the role of CD4 help in the induction of CD8 response by lv-vv prime-boost strategy, we immunized the mice that were pretreated with GK1.5 or isotype control Ab, as depicted in Fig. 6A. Following GK1.5 administration, CD4 T cells were completely deleted 2 d later and were undetectable for 3 wk in the peripheral blood (data not shown). We found that in the absence of CD4 T cells, the primary CD8 response by lv immunization is markedly reduced (Fig. 6B), suggesting that the induction of primary CD8 response by lv immunization is CD4 dependent.

To examine the CD4 help on the secondary CD8 immune responses, CD4 T cells were depleted either prior to lv prime or prior to vv boost. We found that the secondary CD8 responses boosted by vv were severely damaged, especially when CD4 depletion happened prior to lv prime (Fig. 6C). This conclusion was supported by the kinetics of the primary and secondary CD8 responses (Fig. 6D). In addition, in the absence of CD4, the secondary CD8 response was sharply lower 5 wk after vv boost, suggesting that the magnitude of CD8 memory T cells was also dependent on CD4 help (Fig. 6C). Not surprisingly, CD4 depletion, irrespective of time, severely compromised the cancer immune prevention effect. All the mice with CD4 depletion succumbed to tumor growth (Fig. 6E). These data suggest that CD4 help is not only important for primary CD8 responses, but also critical for generating potent secondary effector and memory CD8 responses to protect mice from tumor cell challenge.

To further examine the effect of CD4 help on the magnitude of secondary CD8 responses and on the formation of memory CD8 T cells, we also analyzed the hgp100-25–33 tetramer-specific CD8 T cells and the memory phenotype of CD127 after vv boost immunization with or without prior CD4 depletion. The data showed that, in agreement with the result of cytokine staining presented in Fig. 6C, the magnitude of secondary CD8 responses was markedly reduced in the absence of CD4 help, especially when the CD4

---

**Figure 4.** Memory CD8 T cells from lv-vv prime-boosted mice immediately respond to tumor cell challenge. A–C, Two and a half months after boost, CD8 memory responses in the lv-, vv-, lv-vv-, or vv-lv-immunized C57BL/6 mice were examined by ICS of IFN-γ following 3-h ex vivo hgp100 peptide re-stimulation in the peripheral blood of mice pre-B16 (A) and post-B16 (B) tumor cell challenge. One representative of five mice was shown. A summary of CD8 memory responses before and after tumor challenge was shown (mean ± SEM) in C. The experiment was repeated twice with similar data. Unpaired t test was used for analysis. D, The mice were monitored for tumor growth for 2 mo after B16 tumor challenge. Prevention of B16 tumor challenge was significantly more effective in the lv-vv-immunized mice. Log rank test was used for statistical analysis.

**Figure 5.** Both CD4 and CD8 effector T cells are required for immune protection of mice from tumor cell challenge. Two weeks after lv immunization, CD4, CD8, or both were depleted with mAbs in vivo. Two days later, mice were challenged with B16F10 tumor cells. Control group indicated mice without immunization, whereas hgp100-lv represented the hgp100-lv-immunized mice without depletion. Cohort data from two experiments (10 mice in each group) were presented. Log rank test was used for analysis.
depletion happened prior to hgp100-lv prime (Fig. 7A). These data suggest that CD4 help at both prime and boost stage is required to generate effective secondary CD8 responses. However, although the magnitude of secondary CD8 responses was markedly reduced without CD4 help, the ratio of CD127 + memory CD8 T cells was not markedly changed when the CD4 depletion occurred before prime (Fig. 7B). In contrast, CD4 depletion prior to vv boost also significantly reduced the ratio of CD127 + memory CD8 T cells (Fig. 7B). It is not clear why CD4 depletion prior to vv boost could markedly reduce the secondary CD8 response. In summary, CD4 help at both prime and boost stages is required for the magnitude of primary and secondary CD8 effector and memory responses.

Lv-vv prime-boost induces potent CD8 responses in the melanoma-prone Grm1 Tg mice and prevents autochthonous melanoma growth

The ultimate goal of immune prevention is to prevent spontaneous tumor growth in high-risk population. To examine whether the lv-vv prime-boost immunization strategy can prevent tumor growth in autochthonous tumor model, we used the Grm1 Tg mice. These mice contain transgene Grm1 under the control of tyrosinase-related protein 2 promoter, which allows transformation of melanocytes. Pigmentation begins to emerge at 3 mo old, and eventually all mice will develop melanoma even though malignant metastasis is rare (27). Another advantage of using the Tg mice is that melanoma appearance can be easily visible on the tail and ear. The Grm1 Tg mice were immunized with hgp100-lv at age of 1.5 or 3 mo old with or without boost with hgp100-vv 1 mo later. Lv immunization stimulated potent CD8 responses in the immunized mice. Vv boost increased the secondary and memory CD8 T cell responses in Grm1 Tg mice (Fig. 8A). However, compared with the CD8 responses in C57BL/6 mice, the primary CD8 responses elicited by hgp100-lv in Grm1 Tg mice are higher. In contrast, the boosting effect by vv in Grm1 Tg mice is not as impressive as those in C57BL/6 mice. At present, the mechanisms underpinning these differences are not clear. Nevertheless, ~90% of the mice immunized by lv-vv prime-boost at early age could be fully protected from melanoma for ≥12 mo. Lv immunization alone

---

**FIGURE 6.** CD4 help is required for generating both primary and secondary CD8 responses. A, The experimental strategy was shown. Three experimental groups of C57BL/6 mice were included, as follows: Non-depl (no CD4 depletion), Pre prime (CD4 depletion prior to hgp100-lv prime), and Pre boost (CD4 depletion prior to hgp100-vv boost). Isotype Ab was rat IgG2b. Immune responses were monitored by collecting peripheral blood and ICS staining of IFN-γ at the time points indicated in D. B, CD4 help was required for primary CD8 response. Mice were immunized with hgp100-lv with or without prior CD4 depletion. CD8 response was examined 2 wk later by ICS of IFN-γ after 3-h ex vivo stimulation with hgp100 peptide. Summary of data from two experiments (eight mice) was shown on the right. C, Five days after vv boost, the secondary CD8 response was examined by ICS of IFN-γ after 3-h ex vivo stimulation with hgp100 peptide. A summary of four mice was shown on the right. D, CD4 help at both prime and at boost stage was needed for generating secondary CD8 responses. K, The kinetics of CD8 responses also demonstrated that CD4 depletion prior to lv prime or vv boost could markedly reduce the secondary CD8 response. E, Forty-five days after vv boosting immunization, mice were challenged with B16 tumor cells. CD4 depletion abolished the cancer immune prevention effect of lv-vv prime-boost. Two experiments were repeated with similar data. Unpaired t test and log rank test were used for analysis.
can protect up to 50% of the mice from melanoma growth (Fig. 8B). However, if preventive immunization started at 3 mo old when skin pigmentation was developing, the protection rate declined (Fig. 8C), a phenomenon more obvious in the lv-alone group. The reduced prevention effect in elder mice was also observed previously (35, 36), and is consistent with the notion that immune prevention effect of cancer vaccines declines when tumors begin to grow. Whereas the lv-vv prime-boosted mice were protected from melanoma growth, a mild hair depigmentation (patched vitiligo) was visible in the tumor-free mice. But the overall condition of the immunized mice was very healthy. No weight loss and no wasting signs were observed. These data suggest that effective tumor prevention can be induced by lv-vv prime-boost immunization strategy in cancer-prone mice without stimulating severe autoimmune diseases.

Discussion
In the current study, we found that lv-expressing xenogenic hgp100 melanoma Ag could induce potent CD8 T cell responses that can cross-react with mpg100 Ag. A significant portion of the gp100-specific CD8 T cells expressed the memory marker of CD127 at the peak of immune responses, suggesting that they were CD8 memory precursor cells. The memory CD8 T cells were markedly increased by vv boost. Importantly, CD8 memory T cells from the lv-vv prime-boosted mice could be rapidly reactivated and expanded after sensing tumor cells, which was required for prevention of B16 tumor cell challenge and for prevention of autochthonous melanoma in melanoma-prone Tg mice. The cancer prevention effect is mediated by both CD4 and CD8 T cells. The memory CD8 T cells were markedly increased by vv boost. Two weeks after vv boost, the hgp100-specific CD8 T cells were analyzed by tetramer (A) and CD127 (B) staining. CD4 depletion markedly reduced the secondary hgp100-D9 tetramer-positive CD8 T cells in the blood. In addition, CD4 depletion prior to hgp100-vv boost also reduced the ratio of CD127+ T cells. The summary of data from four mice was shown. Statistical analysis was done with unpaired t test.
system, the CD8 memory T cells generated in the presence of CD4 help from the primary immunization were not responding well without CD4 help during the secondary stimulation, suggesting the secondary CD8 response by boost immunization was also CD4 dependent. The reasons for such divergence on CD4 dependence may include the nature of Ag, the inflammation status during priming, and the DC subsets involved.

In our opinion, the stringent and appropriate criteria to measure the quality and usefulness of memory CD8 T cells are to determine their responsiveness to tumor cell challenge. On this aspect, we found that only the CD8 memory T cells from the lv-vv prime-boosted mice were able to sense and respond to the B16 tumor cell challenge (Fig. 4B, 4C), resulting in effective cancer prevention in both transplantable B16 tumor and autochthonous melanoma models. It remains to be studied why only the memory CD8 T cells from lv-vv prime-boosted mice are able to recognize and respond to B16 tumor cell challenge. It was reported that the MHC I and II molecules on B16 tumor cells are not detectable in vitro and remain low in vivo (33), which allows B16 tumor cells to easily evade the surveillance of immune system. It is possible that memory CD8 T cells generated from multiple stimulations (such as the prime-boost) are qualitatively different from that generated by one immunization. The CD8 memory T cells from lv-vv prime-boosted mice may be more prepared and ready to respond, which allows them to recognize the B16 tumor cells and expand even when the MHC molecules are low (45). An alternative explanation is that the absolute number of gp100-specific CD8 T cells in the lv-vv prime-boosted mice is higher and contributes to more IFN-γ production upon encountering the tumor cells, which then increases the MHC expression to a higher level, making the B16 tumor cells become visible by the memory T cells. A third explanation is that the high number of effector memory CD8 T cells can immediately execute their effector function, including tumor lyses, which release sufficient tumor Ag to be cross-presented by APCs to further expand the memory T cells and to generate Ag spreading to prevent tumor growth. All these explanations suggest that for an effective cancer immune prevention, high-level and qualitatively fit memory CD8 T cells may be required for them to recognize, respond, and kill the tumor cells to prevent tumor establishment.

The prospective clinical applications of cancer immune prevention depend on accurate evaluation of risk/benefit ratio and the safety of both the immunization vector and the vaccine-induced immune responses. With technology development such as gene profiling and genomic analysis, the risk of developing certain malignancy can be better measured and the risk-benefit can be more accurately estimated. Under such conditions, high-risk population will be more likely to accept the procedure of cancer immune prevention if the risks associated with immune prevention can be minimized. Recombinant lv has been demonstrated to be less likely to induce tumorigenesis compared with conventional oncoretroviral vector in mice (46). The data from recent clinical trials using lv-modified cells (including stem cells) demonstrated no severe side effects (47–49). In addition, lv immunization with the purpose of targeting nonstem cells (such as skin DCs and epidermal cells) should be safer. Thus, based on safety profile of lv and vv and the potency of lv-induced Ag-specific immune responses against desired tumor Ag, we reason that lv-vv prime-
boost immunization strategy has a great potential for cancer im-
munoprevention in humans.

Acknowledgments

We thank the National Institutes of Health tetramer core facility at Emory
University for synthesizing the hp1g1023,3D4 tetramer and Dr. Suzie
Chen of Rutgers University for kindly providing the melanoma-prone
Grm1 Tg mice. We also greatly appreciate the stimulating discussion on
this project with Drs. Andrew Mellor and David Munn, Immunotherapy
Center, Georgia Health Sciences University.

Disclosures

The authors have no financial conflicts of interest.

References

A. Mellor, N. Fu, and Y. He. 2010. Blockade of programmed death-1 pathway
rescues the effector function of tumor-infiltrating T cells and enhances the an-
Cancer Res. 70: 4049–4059.
of cancer: is the time ripe? Cancer Res. 60: 2571–2584.
against MUC1 antigen expressed in inflammatory bowel disease and cancer
lessens colonic inflammation and prevents progression to colitis-
EpCam-2 DNA protects mice from mammary tumor growth without anti-EpCam-2
Allogeneic cytotoxic T cell genetic immunotherapy for hepatocellular carcinoma.
Cancer Res. 59: 3064–3067.
15. Rovero, S., A. Amici, E. Di Carlo, R. Bei, P. Nanni, E. Quaglino, P. Porcedda,
era2/HER-2/neu tumor rejection results in a long-term complete remission.
D. Weintraub, and E. M. Jaffee. 2001. The collaboration of both humoral and
cellular HER-2/neu-targeted immune responses is required for the complete
Lentivector immunization stimulates potent CD8 T cell responses against mel-
anoma self-antigen tyrosinase-related protein 1 and generates antitumor immu-
18. Esslinger, C., L. Chapatte, D. Finke, I. Micomet, P. Guillaum, F. Lévy, and
H. R. MacDonald. 2003. In vivo administration of a lentiviral vaccine targets DCs
cells induce potent CD8(+) T cell immunity in recombinant lentivirus-mediated
vector-transduced dendritic cells elicits strong and long-lasting T cell responses and
21. Furmanov, K., E. Elnekave, D. Lehmam, B. E. Clausen, D. D. Kotton, and
A. H. Howey. 2010. The role of skin-derived dendritic cells in CD8(+) T cell
22. Chapatte, L., S. Colombetti, J. C. Cerottini, and F. Lévy. 2006. Efficient in-
duction of tumor antigen-specific CD8+ memory T cells by recombinant lenti-
R. Cao, et al. 2009. Improved efficacy of DNA vaccination against prostate
cancer by boosting with recombinant protein vaccine and by introduction of a