

Article

Phenotypic and Functional Analysis of LCMV gp33-41-Specific CD8 T Cells Elicited by Multiple Peptide Immunization in Mice Revealed the Up-regulation of PD-1 Expression on Antigen-Specific CD8 T Cells

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The phenotype and function of antigen-specific CD8 T cells are closely associated with the efficacy of a therapeutic vaccination. Here we showed that multiple immunizations with LCMV gp33-41 peptide (KAV) in Freund's adjuvant could induce KAV-specific CD8 T cells with low expression of CD127 and CD62L molecules. The inhibitory receptor PD-1 was also expressed on a substantial part of KAV-specific CD8 T cells, and its expression level on KAV-specific CD8 T cells in spleen and lymph nodes was much higher when compared to those in peripheral blood. Furthermore, KAV-specific CD8 T cells could specifically kill KAV-pulsed target cells *in vivo* but the efficiency was low. These data suggest that prime-boost vaccination schedule with peptide in Freund's adjuvant can elicit antigen-specific CD8 T cells of effector-like phenotype with partial functional exhaustion, which may only provide short-term protection against the pathogen. *Cellular & Molecular Immunology*. 2007;4(6):431-437.

Key Words: LCMV, CD8 T cell, peptide vaccine, phenotype, PD-1

Introduction

Peptide-based therapeutic vaccination is a promising strategy for preventing or eliminating chronic infections such as hepatitis B virus infection or tumor cells (1). Clinical trials of such kind of therapeutic peptide vaccination have been carried out recently (2, 3). Although clinical response could be used to evaluate their efficacy, direct analysis of antigen-specific CD8 T cell responses, especially the quality of cytotoxic CD8 T cells, is required for better understanding of the mechanisms of cell-mediated immune responses and for substantially improving vaccination protocols (4, 5). The quality, i.e., the phenotype and function, of specific CD8 T cells is vital for a long-term efficiency (6, 7). Furthermore, these studies will provide insight into the development of

new vaccine regimens to more successfully establish protective CD8 T cell memory (6, 8). Herein, we reported the LCMV-specific CD8 T cell response elicited by multiple immunization with peptide vaccine in mice. Our data indicated that prime-boost vaccination with peptide emulsified in Freund's adjuvant could only produce low frequency of peptide-specific CD8 T cells of effector-like phenotype (CD127^{low}CD62L^{low}) with partial functional exhaustion, suggesting that this immunization strategy needs to be improved.

Materials and Methods

Reagents and animals

Peridinin chlorophyll protein (PerCP)-labeled anti-mouse CD8 mAb, phycoerythrin (PE)-labeled anti-mouse CD127 (IL-7R α) mAb, fluorescein isothiocyanate (FITC)-labeled anti-mouse CD62L mAb, and PE-conjugated anti-mouse PD-1 mAb were obtained from BD Pharmingen (San Jose, CA, USA). FITC-labeled anti-mouse CD3 mAb, PE-

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conjugated anti-mouse granzyme B mAb, allophycocyanin (APC)-conjugated anti-mouse CD8 mAb were obtained from eBioscience (San Diego, CA, USA). Succinimidyl ester of 5(6)-carboxyfluorescein diacetate (CFSE), PE-streptavidin, and APC-streptavidin were purchased from Molecular Probes (Eugene, OR, USA). Female C57BL/6 (6-8 wk old) mice were purchased from Laboratory Animal Center of Southern Medical University (Guangzhou, China). All animal experiments were performed under the local regulations.

Peptides

LCMV gp33-41 peptide KAVYNFATC (KAV) was synthesized by Invitrogen Biotechnology Co. (Shanghai, China). The peptide was purified to > 95% homogeneity by reverse-phase high-performance liquid chromatography and validated by mass spectrometry.

H-2D^b tetramer

Soluble H-2D^b LCMV KAV monomers were prepared and purified as previously described (9). In brief, prokaryotic expression vectors encoding the extracellular domain of the H-2D^b (tagged with a BirA recognition sequence) and human β 2-microglobulin were separately expressed in *Escherichia coli* and isolated from inclusion bodies. Soluble H-2D^b/KAV was obtained by *in vitro* refolding of H-2D^b heavy chain and human β 2-microglobulin in the presence of KAV peptide. Folded monomers were biotinylated and purified. H-2D^b/KAV tetramer was formed by the addition of PE-streptavidin or APC-streptavidin.

Immunization

Mice were immunized by subcutaneous injection of 100 μ g KAV peptide in complete Freund's adjuvant (CFA) into hind footpads on day 0, and boosted with 100 μ g KAV in incomplete Freund's adjuvant (IFA) on days 7 and 14. Mice injected with adjuvant only were used as negative controls.

Tetramer staining and flow cytometric analysis

For analyzing tetramer-positive CD8⁺ T cell frequency, three-color fluorescent staining was performed according to the following procedure: 50 μ l of heparinized tail vein blood or 1×10^6 lymphocytes from spleen, lymph nodes or liver, were incubated with PE-labelled tetramers (~300 ng) for 20 min at 37°C, followed by incubation with appropriate amount of FITC-CD3 and APC-CD8 antibodies for 20 min at 4°C. For intracellular and surface staining of tetramer-positive cells, APC-labeled tetramer was used in combination with PerCP-CD8 and the other two mAbs conjugated with either FITC or PE, respectively. Appropriate isotype controls were used in all experiments. For whole blood, the stained samples were then lysed with 2 ml of red blood cell lysis buffer (155 mM NH₄Cl) by incubating for 8 min at room temperature in dark. After the incubation, cells were washed twice with 2 ml of phosphate-buffered saline (PBS) and fixed in 200 μ l of 1% paraformaldehyde in PBS for flow cytometric analysis. Flow cytometric acquisition of multiple parameters was performed on a Becton Dickinson FACSCalibur (Becton Dickinson, San

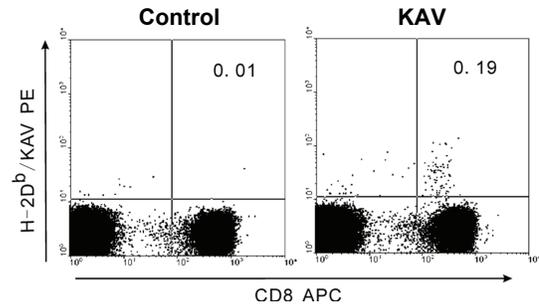


Figure 1. Low frequency of LCMV gp33-41-specific CD8 T cells in mice immunized by peptide in Freund's adjuvant. C57BL/6 mice were primed with 100 μ g KAV peptide in CFA and then boosted with the same peptide in IFA on days 7 and 14. The frequency of gp33-41-specific CD8 T cells in the blood was determined by tetramer staining. The cell populations were gated on CD3⁺ T cells. Numbers in the upper-right quadrants indicate percentages of tetramer-positive cells within CD3⁺ T cell populations.

Jose, CA, USA). At least 300,000 events were acquired for each sample. The data were analyzed using WinMDI version 2.8 (Scripps Research Institute, <http://facs.scripps.edu/software.html>).

In vivo cytotoxicity assay

C57BL/6 splenocytes, unpulsed or pulsed with 10 μ M H-2D^b-restricted KAV peptide (10 μ M) for 2 h at 37°C in a humidified incubator containing 5% CO₂, were labeled with low (0.25 μ M) and high (2.5 μ M) concentrations of CFSE, respectively, and coinjected in a 1:1 ratio (1×10^7 total cells in 100 μ l of PBS) into mice through tail vein. The efficacy of dye labeling was assessed by flow cytometry before injection. Disappearance of peptide-pulsed, fluorochrome-labeled cells was tracked using FACS analysis of fresh blood samples 5 h, 18 h, and 36 h after injection and the lymphocytes isolated from spleen, lymph nodes, and liver 36 h after injection. The level of specific cytotoxicity was calculated relative to the labeled unpulsed population using the following calculation: $100 \times [100 - (\text{percentage pulsed} / \text{percentage unpulsed})]$. WinMDI 2.8 software was used to analyze the FACS data.

Statistical analysis

Nonparametric Mann-Whitney U test for data pairs was performed using GraphPad Prism version 4.0a. All tests were two-tailed and *p* value < 0.05 was considered significant.

Results

LCMV gp33-41 peptide (KAV) immunization elicited low KAV-specific CD8 T cell responses

The mice immunized with KAV peptide were analyzed for specific CD8 T cells by H-2D^b/KAV tetramer staining. Thirty days after the last booster, only low frequency of KAV-specific CD8 T cells ($0.62 \pm 0.11\%$ gated on CD8 T

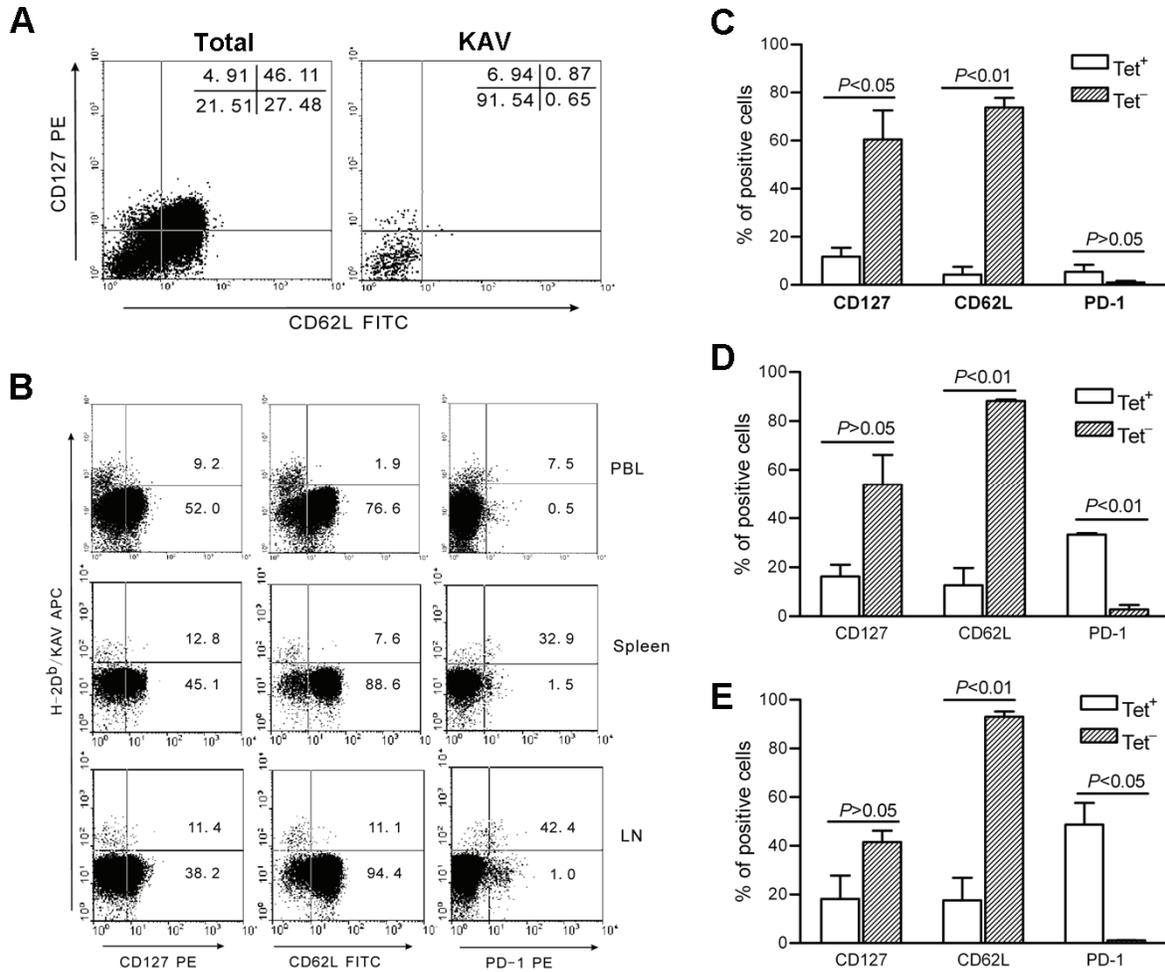


Figure 2. Phenotypic analysis of H-2D^b/KAV-specific CD8 T cells. CD127 and CD62L expression on tetramer-positive or total CD8 T cells in peripheral blood 30 days after the last booster (A). Plots are gated on CD8⁺ T cells, and values indicate the percentages of the expressions of CD127, CD62L, and PD-1 on tetramer-positive (up) and tetramer-negative (low) cells (B). The expression frequencies of CD127, CD62L, and PD-1 were compared between H-2D^b/KAV tetramer-positive and tetramer-negative cells from several tissue origins, including peripheral blood (C), spleen (D), and lymph nodes (E), of mice 30 days post the last booster (n = 3-5). PBL, peripheral blood lymphocytes; LN, lymph nodes.

cells) was detected in the peripheral blood of mice immunized with the KAV peptide, while no specific cells were stained in the blood of control mice (Figure 1). In addition, mice immunized with unrelated peptide had no specific CD8 T cells either (data not shown).

Most of the KAV-specific CD8 T cells displayed an effector-like phenotype

We next analyzed the phenotype of tetramer-positive CD8 T cells by using CD127 and CD62L antibodies. CD127 is the α subunit of IL-7 receptor (IL-7R α) which plays a critical role in the formation and survival of memory T cells, and CD62L is thought to be the marker of central memory T cells. As shown in Figure 2, a very high percentage of total CD8 T cells expressed both CD127 and CD62L, whereas only a small subset of KAV-specific CD8 T cells in the peripheral blood expressed CD127 and almost no cells expressed

CD62L. In addition, KAV-specific CD8 T cells independent of tissue origin had much lower expressions of CD62L (Figures 2C, 2D and 2E). Furthermore, KAV-specific CD8 T cells in peripheral blood, spleen, and lymph nodes had similar expression level of CD127. For CD62L, it seemed that its expression on KAV-specific cells in lymph nodes and spleen was higher but the difference was not statistically significant (Figure 3). These results indicated that most KAV-specific CD8 T cells had an effector-like phenotype (CD127⁺CD62L⁻), only a small part of them had an effector-memory phenotype (CD127⁺CD62L⁻), and very few had a central memory phenotype (CD127⁺CD62L⁺).

A subset of KAV-specific CD8 T cells expressed inhibitory receptor PD-1

We then analyzed the expression of the inhibitory receptor PD-1, which plays a role in the maintenance of self-tolerance

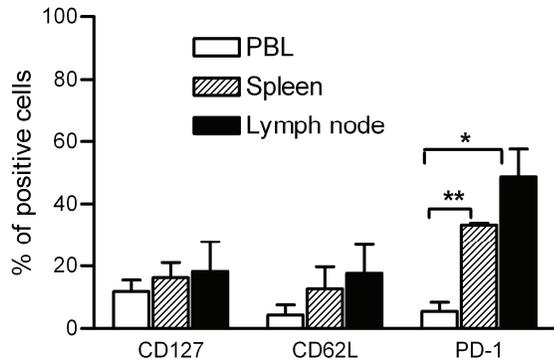


Figure 3. Comparison of the expression frequency of CD127, CD62L, and PD-1 on H-2D^b/KAV-specific CD8 T cells. Lymphocytes were isolated from several tissue origins, including peripheral blood, spleen, and lymph nodes, of mice 30 days post the last booster with KAV peptide (n = 3-5). The cells were gated on tetramer-positive CD8 T cells. * $p < 0.05$; ** $p < 0.01$.

and down-regulation of immune responses, on KAV-specific CD8 T cells. As shown in Figures 2B and 2C, PD-1 expression level on KAV-specific cells in peripheral blood was higher than that on tetramer-negative cells but the difference was not statistically significant. However, PD-1 expression was markedly upregulated on KAV-specific cells in both spleen and lymph nodes when compared to tetramer-negative CD8 T cells (Figures 2B, 2D and 2E). It is worthy noted that although more than one-third of KAV-specific CD8 T cells in spleen and lymph nodes expressed PD-1 molecules, there were significantly fewer KAV-specific cells in peripheral blood expressing these molecules (Figure 3).

KAV-specific CD8 T cells possessed cytolytic potential and in vivo cytotoxicity

To evaluate the potential cytolytic activity, granzyme-B expression was analyzed by intracellular staining. The percentage of granzyme-B^{hi} KAV-specific cells was markedly increased compared to tetramer-negative cells (Figure 4), suggesting that these cells had potential cytolytic activity. To further verify the cytolytic activity *in vivo*, we measured the *in vivo* cytotoxicity of KAV-specific CD8 T cells using *in vivo* cytotoxicity assay (10). As the time prolonged, the ratio of CFSE^{hi}/peptide-pulsed target cells and CFSE^{low}/peptide-unpulsed control cells in peripheral blood decreased gradually, whereas the ratio of these two cell populations in control group remained unchanged (Figure 5A). If the lysis level in naïve mice was defined as 0%, the target cell killing rates in peripheral blood of KAV peptide vaccinated mice were 5.26%, 13.95%, and 53.33% at 5 h, 15 h, and 36 h, respectively. When cells from other tissues were examined for CFSE^{hi} and CFSE^{low} cells 36 h after injection, the specific cytotoxicity rates in spleen and liver were 36.54% and 42.16%, respectively, similar to those in peripheral blood at the same time point. Whereas, the specific killing rates of KAV-specific cells in lymph nodes (74.76%) were higher than those in the spleen, liver, and peripheral blood (Figure 5B).

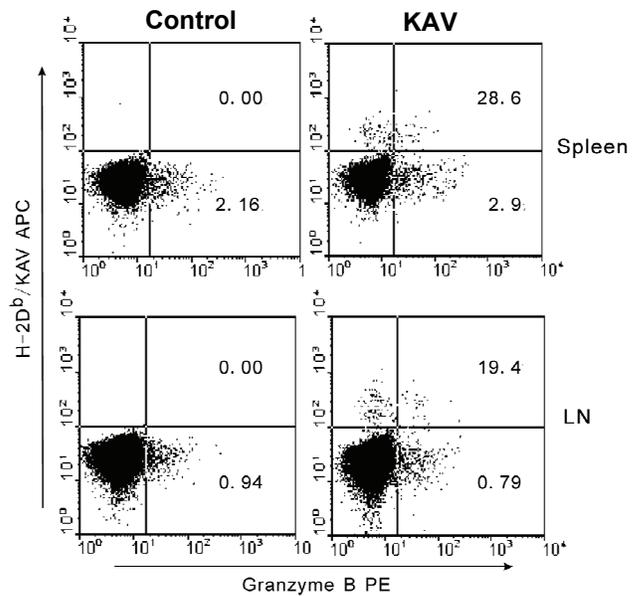


Figure 4. Expression of granzyme-B in H-2D^b/KAV-specific CD8 T cells. The lymphocytes from spleen and lymph nodes were isolated from mice 30 days after the last booster and stained intracellularly for granzyme-B. The cells were gated on CD8⁺ T cell populations. LN, lymph nodes. The values indicate the percentages of expression of granzyme B on tetramer-positive (up) and tetramer-negative (low) cells.

Discussion

Understanding of the quality of memory CD8 T cells specific for any given epitopes is important for the development of vaccines and vaccination schedules designed to promote protective cellular immunity (1, 5). Recent studies indicate that the phenotype and function of antigen-specific memory CD8 T cells is critical for providing effective protection against a pathogen or for eliminating tumor cells (4, 5, 7). In the current study, we showed that prime-boost vaccination with LCMV gp33-41 peptide (KAV) emulsified in Freund's adjuvant could induce low frequency of KAV-specific CD8 T cells, largely with CD127⁺CD62L⁻ phenotype and having inhibitory receptor PD-1 expression. The KAV-specific CD8 T cells could specifically kill KAV peptide-pulsed target cells *in vivo* in relatively low efficiency. Our results suggest that multiple peptide vaccination formulated in Freund's adjuvant may induce antigen-specific CD8 T cells of effector-like phenotype and with partial functional exhaustion, which may only provide short-term protection against the pathogen reinfection or tumor recurrence.

Recent studies have demonstrated that expression of CD127 is a marker of antigen-specific CD8 T cells which will survive the contraction phase and differentiate into memory subsets with long-term survival potential (11, 12). L-selectin (CD62L) is a critical molecule on T cells for homing from blood to lymph nodes. Three major subsets of antigen-experienced CD8 T cells have been identified

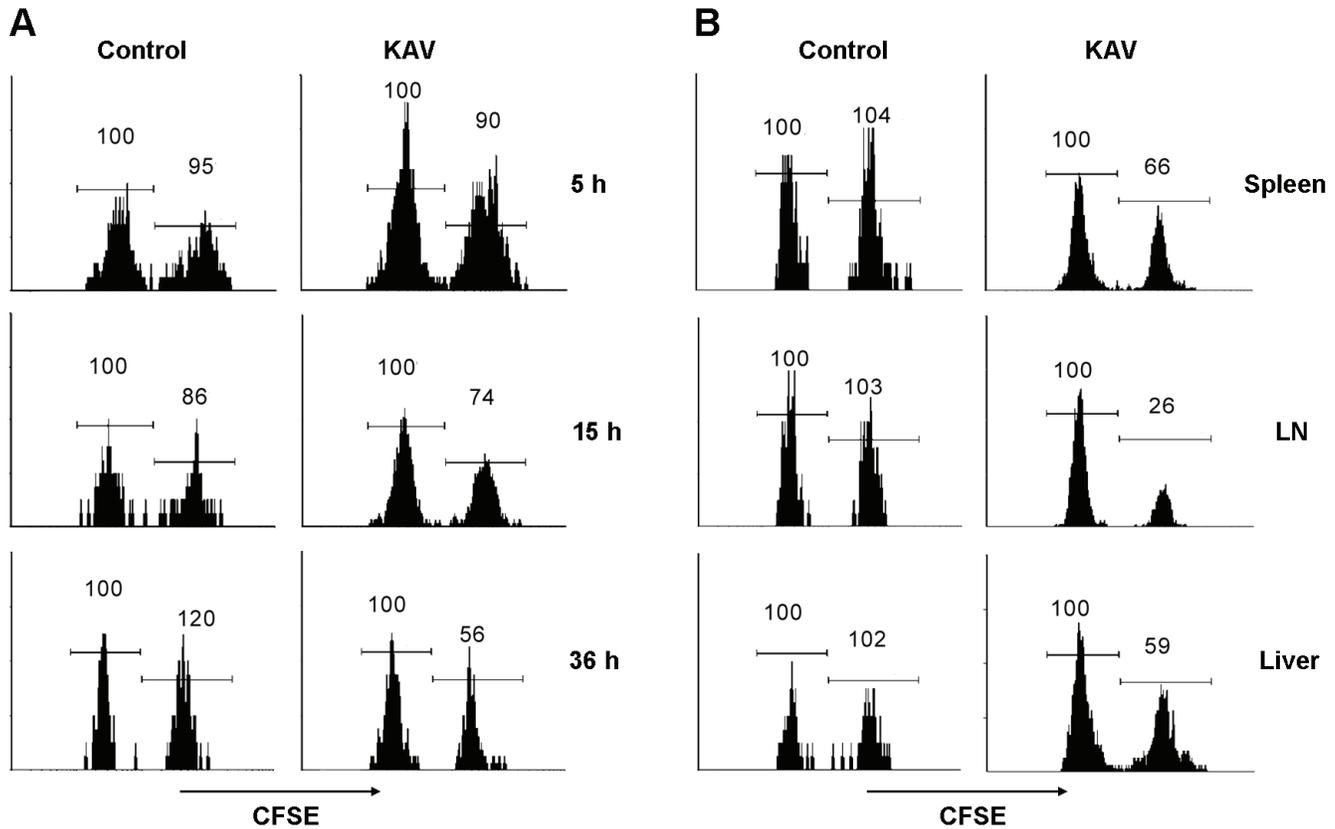


Figure 5. The *in vivo* cytotoxicity of H-2D^b/KAV-specific CD8 T cells. Splenocytes isolated from naïve C57BL/6 mice were pulsed with KAV peptide (CFSE^{hi}) or without peptide (CFSE^{low}) and co-injected into mice at a ratio of 1:1, 30 days after the last booster. (A) After appropriate time, peripheral blood was collected for analysis of CFSE fluorescence by flow cytometry. The percentages of CFSE^{low} unpulsed cells were normalized to 100%. (B) The lymphocytes in spleen, lymph nodes (LN), and liver, were isolated 36 h after injection and analyzed for CFSE fluorescence by flow cytometry.

according to their expression of CD62L and CD127 (13). These markers are associated with central memory T cells (CD62L⁺CD127⁺), effector memory T cells (CD62L⁻CD127⁺), and effector-like T cells (CD62L⁻CD127⁻) (13). Accordingly, our study showed that most of KAV-specific CD8 T cells were effector-like cells, whereas only a small part of them were effector memory cells and very few were central memory cells. It is believed that antigen-specific memory cells were generated 30 days after the last antigen stimulation (14). Then the question is why most KAV-specific cells in our study were effector-like cells 30 days after the last booster. A recent study showed that there was an inverse correlation between CD127 expression and CD8 T cell exhaustion during persistent antigen stimulation, which would lead to the downregulation of CD127 and CD62L, and T cell exhaustion (15). It is worthy noted that peptide emulsified in Freund's adjuvant could persist at least one week *in vivo* after being injected and thus mimicked chronic infection (15). The persistent peptide stimulation might drive the differentiation of antigen-specific CD8 T cells toward a late differentiation stage and functional exhaustion. In addition, proinflammatory cytokines could also influence the

relative frequency of effector versus memory cells. Therefore, limiting the inflammatory cues during CD8 T cell priming would result in more efficient memory cell generation from the effector pool (16). A more recent study indicated that inflammation directed memory precursor and short-lived effector CD8 T cell fates *via* graded expression of T-bet transcription factor (17). Taken together, these results suggested that other vaccination regimens and schedules could be employed to improve the formation of long-lived memory CD8 T cells.

In the present study, only low frequency of antigen-specific CD8 T cell was elicited. Our data are consistent with the recent report which showed that weekly vaccination in Freund's adjuvant for four doses could only elicit low specific CD8 T cell response (0.06%) (18). Thus the prime-boost vaccination at weekly interval in Freund's adjuvant seems not to be a good vaccination schedule for enhancing cell-mediated immunity. Other adjuvants such as CpG containing oligodeoxynucleotides (CpG-ODN) and vaccination schedules have already been adopted to enhance the magnitude of antigen-specific CD8 T cell response (19). In addition, it was shown that low dose of interleukin-2 could

dramatically increase peptide-vaccine-elicited CD8 T cell response (18, 20).

Apart from the low frequency and late differentiation stage of KAV-specific CD8 T cells, another surprising observation in our study was that KAV-specific CD8 T cells elicited by peptide vaccine with Freund's adjuvant had a high level of PD-1 expression. A large body of recent evidence has indicated that the expression of inhibitory receptor PD-1 is associated with CD8 T cell exhaustion during chronic viral infection and blockade of PD-1: PD-ligand pathway can revive the function of exhausted cells (21-24). Thus expression of PD-1 on KAV-specific CD8 T cells would have negative regulatory effects on their cytolytic activity. Actually, we observed that the *in vivo* cytotoxicity of antigen-specific CD8 T cells was low even though they expressed the cytolytic molecule granzyme B. Although direct evidence is lack, it is believed that PD-1 may play a negative regulatory role in killing target cells, probably by increasing the thresholds of CD8 T cell activation. In addition, expression of PD-1 on KAV-specific cells may also render them prone to apoptosis (25).

It should also be noted that PD-1 expression on KAV-specific CD8 T cells from spleen and lymph nodes was higher compared to those in blood. What is the reason? It seems that the cells in secondary lymph organs, such as spleen and lymph nodes, received more antigen stimulation than in blood. This may be true because the peptide emulsified in Freund's adjuvant can be captured by professional antigen-presenting cells which then migrate into the draining lymph nodes and spleen, in which the peptide may be presented at least for one week (15). The persistent peptide stimulation would inevitably lead to the upregulation of PD-1 on antigen-specific CD8 T cells. In the current study, we also analyzed tetramer-specific cells in the draining lymph nodes of the immunized footpads. Surprisingly, we found that the specific cytotoxicity rates in the lymph nodes were higher than the other tissues even though with high PD-1 expression level. The possible reason may be that there are more absolute numbers of KAV-specific CD8 T cells in the draining lymph nodes. However, further investigation is needed to clarify this issue.

In summary, our results indicate that peptide vaccination formulated in Freund's adjuvant can only induce low frequency of antigen-specific CD8 T cells with an effector-like phenotype. Other adjuvant such as CpG-ODN and interleukin-2 seems to be needed to augment peptide vaccine by improving the phenotype and size of vaccine-elicited CD8 T cell responses, as the efficacy of vaccines is correlated with both the quality and the quantity of vaccine-specific CD8 T cell populations.

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