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Repetitive peptide boosting progressively enhances functional memory CTLs

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Abstract

Cytotoxic T lymphocytes (CTLs) play a critical role in controlling intracellular pathogens and cancer cells, and induction of memory CTLs holds promise for developing effective vaccines against critical virus infections. However, generating memory CTLs remains a major challenge for conventional vector-based, prime-boost vaccinations. Thus, it is imperative that we explore nonconventional alternatives, such as boosting without vectors. We show here that repetitive intravenous boosting with peptide and adjuvant generates memory CD8 T cells of sufficient quality and quantity to protect against infection in mice. The resulting memory CTLs possess a unique and long-lasting effector memory phenotype, characterized by decreased interferon- γ but increased granzyme B production. These results are observed in both transgenic and endogenous models. Overall, our findings have important implications for future vaccine development, as they suggest that intravenous peptide boosting with adjuvant following priming can induce long-term functional memory CTLs.

Keywords

T cells; cytotoxicity; peptide; memory; adjuvant; boosting

Introduction

Immunological memory is the cardinal feature of adaptive immunity. This intrinsic characteristic of the immune system grants long-lasting and effective protection from reinfection and is the foundation for vaccination, which is the most effective tool for combating or even eradicating infectious disease. CD8 T cells, or cytotoxic T lymphocytes (CTLs) play an important role in controlling virus infections [1], and memory CTLs possess unique functional properties which make them an essential defense against repeat infection by the same or a similar pathogen. Appropriately, a major goal of vaccination is to generate memory CD8 T cells of sufficient quality and quantity to protect against infection. Yet, induction of functional memory CTLs remains a major challenge for conventional vector-based vaccination strategies [1,2,3], and to date no vector-based vaccines have been licensed

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for human use in the United States [4]. Priming with a live vector is superior to priming with a killed or subunit vaccine, in that a live vector mimics natural infection by stimulating both the innate and acquired immune systems to achieve the optimal orchestrated immune response [5,6]. However, even after priming with a live vector, a CTL response is normally not induced to a protective level, and subsequent boosting is required to generate a sufficient level of functional CTLs [7,8,9].

Repeated vaccination, or boosting, using the same viral or bacterial vector is one way to establish strong humoral, but not cellular, immunity to specific pathogens [7]. In this case, the pre-existing immunity to the vector accelerates its clearance after secondary exposure, limiting the immune response by impairing antigen presentation and the production of inflammatory cytokines [7]. On the other hand, prime-boosting with different vectors is effective at generating memory CTLs [7]. This strategy involves priming the immune system to an antigen expressed by one vector followed by boosting with a second vector containing the same antigen. This circumvents the issue of inducing strong immunity against the vectors themselves and focuses the immune system on the common antigen [7,9,10]. However, prime-boosting with different vectors generates memory CTLs with potentially impaired functions. For example, immune senescence has been observed in memory CTLs after multiple boosts [9,11]. KLRG1 expression, which is indicative of short-lived effectors [12], increases in secondary and tertiary memory [9], and IL-7R α , which is related to memory CTL survival [13], is down regulated in secondary memory CTLs [8]. These studies also suggest that the memory phenotype may vary when different vectors are used in boosting [8,9,14,15] and stress the need to characterize function directly. Despite these challenges, the biggest obstacle to future applications of the vector-based prime-boost strategy is limited availability of appropriate vectors, especially when multiple boosts are needed to elicit protective immunity [10,16]. Although vectors are required for optimal priming in vaccination, a new strategy for effective and repeatable boosting without vectors is urgently needed.

One promising strategy is the use of peptide vaccines instead of vector-based vaccines to generate memory CTLs. A peptide vaccine allows for focused induction of peptide-specific CTLs. However, peptides are poorly immunogenic and, by themselves, induce immune tolerance or deletion of peripheral CTLs [17,18]. When adjuvant is co-administered with peptide in a single subcutaneous immunization, low levels of memory CTLs are generated and the resulting immunity is relatively weak [19,20,21,22]. It is feasible that repeated peptide vaccination with adjuvant could be used to progressively enhance this memory. Therefore, the goal of this study was to determine if vaccination with peptide and adjuvant is an effective repeatable boosting method – generating memory CTLs of sufficient quantity and quality to protect against infection.

Materials and Methods

Mice reagents and immunization

OT-I mice, gifts from MF Mescher, University of Minnesota, MN, have a transgenic TCR specific for H-2K^b and OVA_{257–264} [23]. Mice were maintained under specific pathogen-free conditions at the University of Maryland, and these studies have been reviewed and approved by the Institutional Animal Care and Use Committee (protocol ID R-09-22). C57BL/6 mice were purchased from the National Cancer Institute. All conjugated fluorescent Abs were purchased from BD Biosciences (San Diego, CA), eBioscience (San Diego, CA) or Biolegend (San Diego, CA). Lipopolysaccharide (LPS) was purchased from Invivogen (San Diego, CA), and used at 50 ug per mouse. SIINFELK peptide was purchased from New England peptide (Gardner, MA), and was used at 50 ug per mouse. Boosting was

performed through i.v. tail injection in a total volume of 300 μ l in DPBS. Tetramer was a gift from Dr. Jameson in University of Minnesota.

Viruses and bacteria

Recombinant VV-GFP-JAW-OVA (VV-OVA) expressing the OVA_{257–264} epitope and recombinant LM expressing full-length secreted OVA (LM-OVA) were gifts from SC Jameson, University of Minnesota, MN, the same as we described before [23,24]. Mice were infected i.p. with VV-OVA at 5×10^6 PFUs and LM-OVA at 10^4 CFU/mouse i.v. for primary infection, and 5×10^5 CFU/mouse i.v. for test of protection.

Naive T cell purification

Inguinal, axillary, brachial, cervical, and mesenteric lymph nodes (LN) were harvested from wild-type (WT) OT-I mice, pooled, and disrupted to obtain a single-cell suspension. CD8⁺CD44^{low} cells were enriched by negative selection using MACS magnetic cell sorting (Miltenyi Biotec, Auburn CA) as we described before [23,24].

Adoptive transfer and flow cytometric analysis

Purified naive OT1 cells were adoptively transferred into normal C57BL/6NCr mice by i.v. tail injection at the numbers indicated for each experiment. One day after transfer, mice were infected. Single-cell suspensions were prepared, viable cell counts were done (trypan blue). Since OT-1 T cells possess the congenic marker Thy1.1, we can identify them in a B6 host by flow cytometry staining with anti-Thy1.1 and CD8, as described before [23,24].

Memory CTLs are formed at the end of the contraction phase[1], and the memory population remains stable after a few weeks following acute virus infection or immunization [25]. As is common practice in immunology[26], we use one month as a cutoff point to examine memory CTLs as we reported previously[23,27,28]. Analysis was performed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences) to determine the percentage of total OT-I cells in the samples.

Tissue harvest and digestion

Mice were euthanized by CO₂ and peripheral lymph nodes and spleens were directly picked up and homogenized using 15 ml glass grinders. Lungs were perfused using $1 \times$ PBS at about 30 ml per mouse, cut into small pieces (1mm³), homogenized with a 10 ml pipette and resuspended in 4 ml Collagenase D (Roche, Indianapolis, IN). For complete digestion, lung tissues were kept in a water bath (37°C) for 25 minutes. Digestion was stopped by the addition of 0.1 M EDTA, and digested tissues were homogenized using glass grinders. Bone marrow was harvested by flushing cut bones with $1 \times$ PBS.

Intracellular cytokine staining after in vitro rechallenge

Isolated cells from adoptively transferred mice were incubated at 2×10^6 cells/ml in RP-10 with 0.2 μ M OVA_{257–264} peptide and 1 μ l of GolgiPlug (BD Biosciences) for 3.5 h at 37°C. Cells were then fixed in Cytofix buffer (BD Biosciences) for 15 min at 4°C, permeabilized in saponin-containing Perm/Wash buffer (BD Biosciences) for 15 min at 4°C, and stained with conjugated Abs for 30 min at 4°C. Cells were washed once with Perm/Wash buffer and once with PBS containing 2% FBS and analyzed by flow cytometry.

Statistics

Unpaired two-tailed Student's t-tests were used to determine significant differences between treatments using Prism (GraphPad Software).

Results

Repeated peptide boosting with adjuvant progressively increases the number of memory CTL

We hypothesized that repetitive intravenous peptide boosting with adjuvant would be effective in generating memory CTLs. To test this hypothesis, purified OTI cells were transferred into B6 recipients and primed with VV-OVA the next day to induce low but detectable background memory. At day 30 post-infection, circulating CTLs in the blood were examined to confirm the presence of memory (designated as M1) (Fig. 1A). Boosting with or without LPS was repeated every 32 days for a total of four times, and the memory OTI cells in the blood were examined 30 days after each boost (Fig 1A). As expected, peptide alone did not induce any significant increase of memory CTLs. However, repeated boosting with peptide plus LPS steadily and significantly enhanced the number of memory OTI cells following the first three boosts (Fig. 1C). Memory CTLs increased to approximately 25% of the total CD8 T cells in the blood after the fourth boost (Fig. 1B). *In M5 mice, lungs had the lowest percentage of CD8 T cells out of live cells, whereas the secondary lymphoid tissues, such as lymph nodes and spleens, had higher percentages of CD8 (Fig.1D). Memory OT1 percentage of total CD8 T cells was the highest in the spleen (Fig. 1E). Also, numbers of isolated cells in spleen were about 3 folds of those in lymph nodes and lungs (data not shown).* Of note, endogenous peptide-specific CTLs were not detectable using tetramer[28] in all of the memory mice regardless of the number of times boosted (data not shown). These results indicate that repeated intravenous peptide boosting is sufficient, but requires co-administration of an adjuvant, for generating a robust memory CTL population.

Repeated boosting generates functional memory CTLs with a unique phenotype of down-regulated IFN γ and up-regulated granzyme B production

Multiple boosts with different live vectors lead to enhanced KLRG1 expression [9] and decreased CD127 expression [8], suggesting impaired function of the resultant memory CTLs. To determine if repeated peptide boosting generated CTLs of similar phenotype, memory CTLs from spleens of mice having received various rounds of intravenous peptide boosting were examined based on their phenotype and production of functional molecules. Memory CTLs obtained following primary infection (M1), first boost (M2) and fourth boost (M5) showed a similar effector memory phenotype of CD62L^{lo}/KLRG1^{lo}/IL-7R^{hi} (Fig. 2A), indicating that peptide boosting generates CTLs of a different phenotype compared to those from boosting with live pathogens [8,9,14]. However, in agreement with previous reports [8,29], primary memory CTLs induced by VV infection produced minimum or no granzyme B (GZB) (Fig. 2B and D). After boosting with different vectors, GZB-producing memory CTLs have been shown to increase [8,9,30]. Similarly, GZB-producing memory CTLs were elevated by 20% after four consecutive peptide boosts (Fig. 2B and D).

IFN γ production is a hallmark of memory CTLs in many infections [1,6,9,27]. In line with this, the majority of primary memory CTLs (M1) produced IFN γ (Fig. 2B–C). However, IFN γ -producing OTI cells were significantly reduced after multiple boosts (Fig. 2B–C). This reduction in IFN γ production was concerning, as it could be indicative of impaired function. To determine the protective ability of the memory CTLs on a per cell basis against LM-OVA, we transferred memory CTLs into naïve recipients. Low numbers were used for transfer to avoid the possibility of a massive lethal reactivation upon antigen stimulation. Indeed, the resultant memory CTLs from multiple rounds of peptide boosting provided similar protection against LM challenge compared to primary memory (Fig. 2E). Likewise, there seems to be a general positive correlation between protection against LM-OVA and the

number of memory CTLs transferred, especially for the groups that received 10^5 and 5×10^5 CTLs per mouse (Fig. 2E).

Memory CTLs induced by repetitive peptide boosting are long-lasting and stable in effector memory phenotype

Long-lasting and stable memory is essential for successful vaccination. We examined the long-term memory of CTLs subjected to a total of seven boosts after virus priming: memory CTLs from M5 in Fig. 1 were transferred into naïve B6 mice at 10^5 /mouse, which then received three additional boosts of peptide plus LPS at 30-day intervals. We took this approach to avoid the reactivation of a large amount of memory CTLs (in this case more than 25% of total CD8), which could be lethal due to increased production of inflammatory cytokines, such as TNF α [31]. After the last boost, memory CTLs in the blood were examined over a period of six months. The percentage of memory CTLs out of total CD8 T cells in the blood was not significantly altered over the entire 6-month period, indicative of long-lasting memory (Fig. 3A). Moreover, these memory CTLs retained a typical functional effector memory phenotype of CD62L^{lo}/KLRG1^{lo}/CD127^{hi} (Fig. 3B). However, even though CD127 expression was generally high at all time points, its expression continued to increase over the 6-month period (Fig. 3B). This underscores the importance of IL-7 for long-term survival [5].

Repetitive peptide boosts generate endogenous memory CTLs

So far, all of the data presented were based on transgenic OT1 transfer. To test if endogenous memory CTLs could be induced by multiple intravenous peptide boosts, we adopted a similar approach as in Fig. 1A, but without OT1 transfer. Multiple tissues were harvested from memory mice boosted three times with LPS plus peptide after VV-OVA priming. The total number of endogenous memory CTLs (tetramer⁺ CD8⁺) in the lymph nodes, bone marrow, lungs, and spleen was approximately 10^7 (Fig. 4A). This suggests that repetitive peptide boosting efficiently induces endogenous memory CTLs. Similar to OT1 memory CTLs (Fig. 2A–D), the resultant memory CTLs had lower IFN γ - (less than 40%) and increased GZB- (15%) producing cells (Fig. 4B–D) and were of effector memory phenotype (CD62L^{lo}/KLRG1^{lo}/CD127^{hi}) (Fig. 4E–G). While it would be ideal to compare endogenous M1 to endogenous M4, the frequency of endogenous M1 was extremely low and undetectable from background noise, *but the effects of boosting on endogenous CTLs was similar to transgenic OT1s* (Fig. 4H). This low number of endogenous M1 reached a level similar to OT1 after three boosts (Fig. 4H), suggesting that intravenous peptide boosting may be even more effective in the induction of endogenous memory CTLs than in OT1 transferred animals.

Discussion

Our results demonstrate that repetitive intravenous boosting with peptide and adjuvant in live vector-primed animals progressively enhances functional memory CTLs. Peptide and adjuvant were administered intravenously for rapid distribution to tissues and prompt interaction with immune cells. Although intravenous vaccination has been used successfully in some human cancer vaccines [4], there are potential adverse systemic effects related to this mode of delivery [4,32], including massive activation of immune cells. However, we did not observe any discomfort in mice boosted up to four times intravenously. Memory CTLs that were boosted repeatedly had considerably reduced production of IFN γ (Fig. 2C); thus the potential for immunopathology was decreased. Although caution must be taken when boosting a large number of antigen-experienced CTLs, it appears that intravenous delivery of peptide and adjuvant is an appropriate method for progressively enhancing memory CTLs in this animal model.

The memory CTLs from multiple rounds of boosting acquire a unique phenotype—CD62L^{lo}CD127^{hi}KLRG1^{lo} effector memory, with reduced IFN γ but increased granzyme B production. Despite the decrease in IFN γ , the CTLs remain functional and able to protect against pathogen challenge. The significant increase in GZB production by multiply-boosted memory CTLs appears to have compensated for the decrease in cytokine production (Fig. 2D). Furthermore, the resultant memory from this boosting strategy is long-lasting and phenotypically stable. Therefore, intravenous peptide boosting may be a useful approach for inducing a large quantity of functional memory CTLs in vaccination.

The requirement for adjuvant in this peptide boosting strategy suggests that inflammatory cytokines induced by adjuvants are needed for the reactivation of memory. The intravenous delivery of adjuvant makes it rapidly available to macrophages and DCs in tissues, which may produce IL-12 or IFN γ soon after stimulation [33]. In the context of an inflammatory environment, the role of macrophages and DCs in memory reactivation could be the simultaneous provision of cytokines and antigens rather than direct costimulation. Although costimulation is indispensable for the activation of naïve CTLs and induction of functional primary memory [34], its role in memory responses is unclear [35,36]. We believe that this boosting strategy is working in the absence of costimulation through the rapid induction of inflammatory cytokines and extensive antigen presentation by any cell capable of presenting peptide. However, future studies that directly test the role of costimulation in memory reactivation are needed.

Determining TLR adjuvants that can enhance memory CTL generation is of practical interest in vaccine development. In this study, we confirmed that LPS, which signals through TLR4, is an effective adjuvant for generating memory CTLs (Fig. 1B–C). Although LPS can induce high fever, toxic shock, and organ failure in humans, [37] less toxic derivatives of LPS, which also signal through TLR4, are available. For instance, monophosphoryl lipid A (MPL) retains Lipid A, the biologically active component of LPS, but is 1000-fold less toxic [38]. Future studies will seek to determine if other TLR adjuvants can similarly enhance functional memory CTLs. In sum, these results have important implications for vaccine development as they reveal a vector-free boosting method for inducing long-term functional memory CD8 T cells.

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Highlights

- Intravenous peptide boosting with adjuvant can induce large numbers of functional memory CTLs.
- Repetitively boosted CTLs are long-lasting and stable in effector memory phenotype.
- CTLs possess a unique phenotype of down-regulated IFN γ and up-regulated granzyme B production.
- These results are observed in both transgenic and endogenous models.

Figure 1. Repetitive boosting with peptide and adjuvant drives memory CTLs progressively to high levels

(A) Scheme for experimental design. (B) Representative dot plots of M1 and M5. (C) OT1 percentage of total CD8 T cells in the blood from memory mice. (D–E) CD8 T cell percentage of live cells and OT1 percentage of total CD8 T cells in the tissues of M5 memory mice. Data represent mean $\pm$ SEM of six to ten animals. Asterisks indicate statistical significance. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ns: not significant. These will be followed in the rest of paper. Similar results were obtained from three experiments.

Figure 2. Functional memory CTLs gain a unique phenotype after multiple peptide boosts
 Memory OT1 cells in the spleen of memory mice after the fourth boosting (M5) or first boosting (M2) were compared to primary memory (M1) for surface molecule expression (A), IFN γ and granzyme B production (B–D). Similar results were obtained from three experiments. Endo: endogenous CD8 T cells. (E) Spleens were harvested from M5 memory mice in Fig.1. Splenocytes containing different numbers (10^4 , 10^5 or 5×10^5 /mouse) of memory OT1 cells were transferred into naïve B6 mice, and the recipients were challenged with 5×10^5 LM-OVA i.v. the next day. Equal numbers of purified naïve OT1 CD8 T cells were transferred as a control. Spleens from VV-OVA-primed memory mice (with OT1 transferred) were used as positive controls (first memory). Spleens were harvested for LM-OVA counting three days after LM-OVA challenge. Comparisons were made between the same number transfer of memory CTLs and naïve CTLs.

Figure 3. Memory CTLs from multiple peptide boosts are long lasting and of stable effector memory

Memory OT1 cells from M5 mice (Fig. 1) were transferred into B6 mice at 10^5 /mouse, followed by three additional injections with peptide plus LPS at 30-day intervals. Blood samples were taken from these memory mice at days 60, 120 and 180 after the last boost. (A) OT1 percentage of total CD8 in the blood. (B) Representative surface molecule expression of the same samples as in A. Dashed lines indicate the gate, and the *number on each histogram represents the mean of gated areas plus SEM of 7 individual samples in (A).*

Figure 4. Repetitive peptide boosting with adjuvant induces endogenous memory CTLs
 Naïve B6 mice were primed with VV-OVA, and boosted with peptide and LPS a total of three times at 30-day intervals. Tissues were harvested 30 days after the third boost (M4). Peptide-specific CD8 T cells were defined by K^b /OVA tetramer and CD8 staining. Data were all based on tetramer and CD8 double-positive cells. (A) Distribution of tetramer positive CTLs in different tissues. (B–C) Representative histograms of IFN γ and GZB production. (D) Statistics of IFN γ and GZB positive cells out of tetramer and CD8 double positive population in B–C. (E–G) Representative histograms of CD62L, KLRG1 and CD127 expression. (B–G) Data were all from memory Tetr $^+$ /CD8 $^+$ in spleen. Non-shaded histogram: Tetr $^+$ /CD8 $^+$. Shaded histogram: Tetr $^-$ /CD8 $^+$ (control). Bars represent mean. (H) K^b /OVA tetramer positive CD8 cells were quantified in blood for M1 through M4 as a percentage of total CD8 T cells.