

ABSTRACT

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GENERATING FUNCTIONAL CYTOTOXIC T LYMPHOCYTES THROUGH REPETITIVE PEPTIDE BOOSTING.

Kendra Nicole Smyth, Master of Science, 2012

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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 independent of the specific adjuvant applied and 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.

GENERATING FUNCTIONAL CYTOTOXIC T LYMPHOCYTES THROUGH
REPETITIVE PEPTIDE BOOSTING.

By

Kendra Nicole Smyth

Thesis submitted to the Faculty of the Graduate School of the
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Dedication

I dedicate this work to my parents, Paul Smyth and Denise Freeland. Thank you for inspiring me to follow my dreams and giving me the confidence and tools to do so.

Acknowledgments

First and foremost, I would like to thank my advisor Dr. Zhengguo Xiao for his direction and unyielding support of this project. I am honored to be a member of the Xiao Lab.

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List of Abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
B6	C57BL/6 mouse
CCR7	Chemokine receptor 7
CD	Cluster of differentiation
CD62L	L-selectin
CD127	Interleukin-7 receptor- α subunit
CpG	Cytosine-guanine motif, unmethylated
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
dsRNA	Double stranded RNA
ELISA	Enzyme linked immunosorbent assay
FACS	Flourescence activated cell sorter
FBS	Fetal bovine serum
GZB	Granzyme B
H2-K ^B	A classical MHC I haplotype present in B6 mice
IFN- γ	Interferon-gamma
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IL	Interleukin
I.p.	Intraperitoneal

I.v.	Intravenous
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
mAB	Monoclonal antibody
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response gene 88/encoded protein
NF- κ B	Nuclear transcription factor κ B
OTI	Transgenic mouse in which CD8 T cells are specific for the SIINFEKL peptide of chicken ovalbumin
PBL	Peripheral blood
PMBC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PFU	Plaque forming unit
Poly IC	Polyinosinic:polycytidylic acid
S.c.	Subcutaneous
T _{cm}	Central memory T cell
TCR	T cell receptor
T _{em}	Effector memory T cell
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor alpha
VV-OVA	Vaccinia virus expressing OVA 257-264
LM-OVA	Listeria monocytogenes strain expressing full-length secreted OVA

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Chapter 1: Introduction

Immunological memory

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 (1). There are two branches of immunological memory. The humoral branch consists of antibodies, B cells, and plasma cells. Antibodies are the effectors of humoral immunity and function to neutralize pathogens and toxins, promote phagocytosis, and activate complement (2). The cellular branch of immunological memory is mediated by T cells (2). CD4 T cells are essential for orchestrating immune responses. They can provide help to B cells for antibody production, stimulate CD8 T cells to release cytokines and gain effector functions, and recruit neutrophils to sites of infection (2, 3). CD8 T cells, or cytotoxic T lymphocytes (CTLs), are able to kill infected cells through direct lysis and the secretion of antiviral cytokines (e.g. IFN- γ and TNF- α) (2, 4), and they play a critical role in the control of bacteria- and virus-infected host cells and cancer cells (2, 3). The memory CTL population, derived from naïve CD8 T cells after full activation, is long-lived and rapidly initiates a robust response upon reencountering the same or a similar pathogen (5). Protective memory can last for many years after initial exposure to antigen, and in some cases it can last a lifetime (6, 7).

The CD8 T cell response to an acute infection

The specificity of T cell responses is due to the antigen-specific and clonally restricted T cell receptor (TCR). A member of the immunoglobulin superfamily, the receptor is a heterodimer that recognizes pathogen-derived peptides presented in the context of a class I major histocompatibility complex (MHC) molecule on the surface of an antigen presenting cell (APC) (3). The T cell repertoire is necessarily immense to enable responses to the limitless number of pathogens an organism may encounter in its lifetime. For example, in mice at least 2×10^5 different epitopes can be recognized, which means there are at least 2×10^5 unique TCR specificities (8). This diversity is created during T cell maturation in the thymus by random rearrangements of a series of gene segments encoding the TCR (2). Junctional flexibility and combinatorial association of chains further increase the TCR repertoire (2). The precursor frequency, or the number of naïve CD8 T cells specific for a given MHC-peptide combination before antigenic challenge, is on average about 200 cells out of the 4×10^7 naïve CD8 T cells in an adult mouse (8). Clearly, the number of CD8 T cells specific for a given pathogen is extremely low (.0005% of total naïve CTLs) prior to antigen encounter and no match for rapidly dividing microbes. To maximize the chances of encountering a pathogen, naïve CD8 T cells gather in the highly structured micro-environment of the lymph nodes, an area that facilitates their interaction with antigen presented by dendritic cells (DCs) (3).

Upon receiving the appropriate signals, naïve CD8 T cells begin their transition to memory. The first step in this journey is activation (Fig. 1). The widely accepted three-signal model of T cell activation postulates that the first activation signal is provided by TCR recognition of an appropriately presented peptide (9-12).

This signal is antigen-specific and ensures that only those T cells that recognize the antigen will be activated (13). The second signal, costimulation, is provided by contact between a group of molecules expressed by activated APCs and those expressed by CD8 T cells. This interaction between a costimulatory molecule on the T cell, such as CD28, 4-1BB, CD27, ICAMI or OX40, with its ligand on the APC is required in context with signal one to avoid T cell anergy or tolerance (5, 14). The third signal can be supplied by inflammatory cytokines produced by stimulation of toll-like receptors (TLRs) such as IL-12 and type I interferons (9-12, 15). The extent of expansion and the acquisition of functional capacities following activation is reliant on factors including the amount of antigen and the strength of its interaction with the TCR, as well as the local cytokine milieu (7).

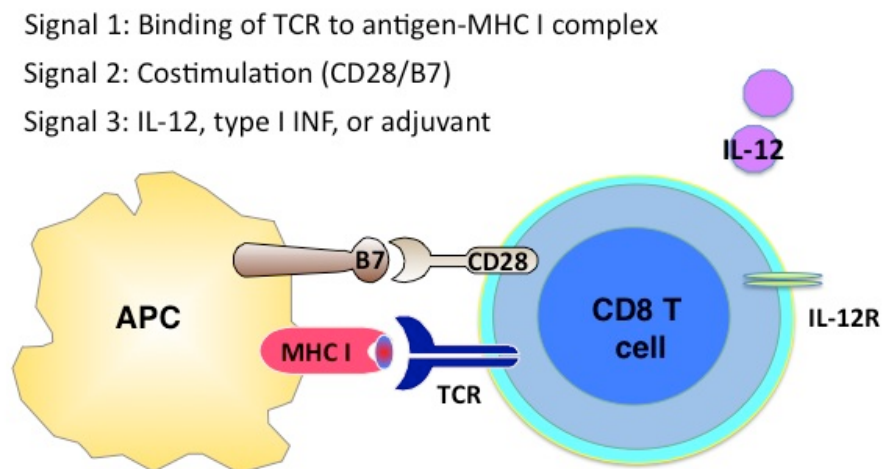


Figure 1. Three-signal model of T cell activation

In the presence of these activation signals, antigen-specific CTLs undergo massive clonal expansion, during which they increase more than 50,000-fold (8),

acquire effector function, and mediate pathogen clearance by directly killing infected cells (5, 12, 16). Clonal expansion is associated with the down regulation of the lymph-node homing molecules CD62L and CCR7 (17), which enables these effector CD8 T cells to travel through the blood to peripheral sites where they dutifully patrol for infection (17). Upon encountering the specific antigen-MHC complex on the surface of an infected cell, the integrin receptor LFA-1 on the CTL binds to ICAMs on the target-cell membrane, forming a CTL-target-cell conjugate (2). The CTL releases perforin and serine proteases called granzymes into the space between the two cells. Perforin forms pores in the target cell membrane which enable granzymes to enter and initiate apoptosis within the infected cell. Fragmentation of target cell DNA, and viral DNA within the target cell, occurs shortly after CTL contact (2). Interaction of the membrane-bound Fas ligand on CTLs with the Fas receptor on target cells is another mechanism by which CTLs are capable of inducing apoptosis in target cells. In addition to triggering these apoptotic pathways, activated CD8 T cells secrete immune-stimulating cytokines, such as IFN- γ and TNF- α , which recruit other immune cells (neutrophils, eosinophils and macrophages) to the vicinity. Macrophages up-regulate expression of MHC and costimulatory molecules, increase phagocytosis, and secrete IL-12 (3). Neutrophils and eosinophils release toxic enzymes, oxygen radicals, and additional cytokines, further contributing to the inflammatory environment (3).

Protective immunity to intracellular pathogens is largely dependent on cytolysis of infected cells and cytokine production (18). In most acute infections, the antigen-specific CTL population contracts 90-95% (8, 19, 20) 7-12 days after the

initial antigen encounter, although this can vary depending on the infection (21). Although extreme, the contraction is essential for maintaining immune system homeostasis and preventing immunopathology (21). The small percentage of surviving CD8 T cells becomes the long-lived memory population and is maintained in the absence of antigen through a process of slow but continuous homeostatic proliferation (22).

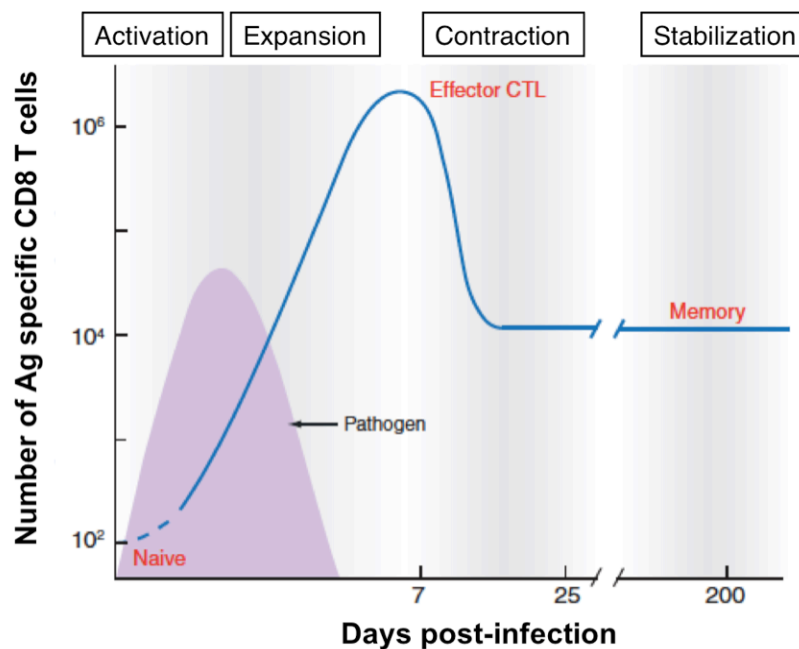


Figure 2. Kinetics of a CD8 T cell response to an acute infection
Adapted from Williams and Bevan, 2007 (5).

Memory CD8 T cells

Long-term survival and maintenance of CTLs requires IL-7 and IL-15 (7, 22, 23). These cytokines are responsible for homeostatic turnover, maintaining CTLs in a state of slow but constant proliferation (7, 20, 22). IL-7 signals are required for memory cell precursor survival (23, 24). Appropriately, expression of the alpha

subunit of the IL-7 receptor (CD127) is required functionally for memory cell development (23). Although IL-7R α is down regulated immediately following naïve cell activation, expression returns in a subset of effector cells (CD127⁺). The presence of IL-7R α on antigen-specific CTLs after the peak of expansion is predictive of their fate as long-lived memory (23, 24). Over time, the gene expression profile of antigen-specific CTLs changes as they progressively gain memory phenotype (25).

The memory population is marked by phenotypic and functional heterogeneity, making attempts to classify memory CD8 T cells based solely on phenotypic markers challenging. However, two subsets of memory CD8 T cells, effector (T_{EM}) or central (T_{CM}) memory, are traditionally categorized based on the surface expression of CD62L (L-selectin) (17). Memory CTLs that express L-selectin are classified as T_{CM} (17) and, by virtue of CD62L, these cells are able to travel directly to the highly structured microenvironment of the lymph nodes which is designed to bring them into contact with APCs presenting antigen collected from tissues and the bloodstream (17). T_{CM} cells possess high proliferative potential, produce IL-2 upon antigen recognition, which stimulates T cell proliferation and differentiation, but require a longer period of reactivation to re-acquire cytotoxic activity (4, 20). T_{EM} cells, identified as being CD62L⁻ (17), display characteristic chemokine receptors and adhesion molecules which facilitate their localization at peripheral sites of infection, such as in the lung or liver (17, 26). They are constitutively lytic and carry large amounts of pre-formed cytotoxic molecules enabling them to kill target cells without *de novo* protein synthesis (5, 17). Although these classifications of memory cell precursors and memory cell subsets are widely

accepted, caution must be taken when drawing conclusions based on evaluation of few cellular markers, as studies have shown that phenotype does not always equal function (21). Therefore, in addition to predicting the function of memory CD8 T cells based on phenotype, it is useful to characterize their function directly. In general, good memory CD8 T cells: (i) are present at substantially higher precursor frequencies compared to naïve T cells, allowing for increased surveillance at potential sites of pathogen entry; (ii) have less stringent requirements for re-activation, enabling them to promptly respond to re-infection; (iii) maintain the ability to rapidly produce IFN- γ and TNF- α and re-acquire cytotoxic activity upon re-exposure to antigen, therefore providing protection; and (iv) are capable of robust secondary clonal proliferation resulting in increased numbers of secondary memory CTLs versus primary memory CTLs (4, 7, 11, 13, 20, 21). These characteristics enable memory CTLs to respond to re-infection prior to the development of a significant inflammatory response (7, 13, 21). Appropriately, a major goal of vaccination is to generate memory CD8 T cells of sufficient quality and quantity to protect against infection.

Generating memory CD8 T cells through vaccination

Infectious diseases cause approximately 13 million deaths annually worldwide, and vaccination is the most cost-effective strategy in combating these pathogens (1, 2). While we have been successful at containing or even eradicating numerous infectious diseases through vaccination, other diseases, such as HIV and malaria, have proven more difficult to manage. Cytotoxic T lymphocytes play an important role in controlling virus infections (27, 28), and memory CTLs possess

unique functional properties, which make them an essential defense against repeat infection by the same or a similar pathogen. Yet, induction of functional memory CTLs remains a major challenge for conventional vector-based vaccination strategies (27-32), and to date no vector-based vaccines have been licensed for human use in the United States (33). Recently, however, IMOJEV – a vaccine for Japanese encephalitis utilizing the yellow fever virus vector – became available in Australia, but this vaccine works mainly through the production of neutralizing antibodies (34). 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 (11, 27, 35). 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 (30, 36-39).

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 (24, 37). 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 (24, 37). On the other hand, prime-boosting with different vectors is effective at generating memory CTLs (24, 37). 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 (30, 37, 40, 41).

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 (25, 30). KLRG1 expression, which is indicative of short-lived effectors (42), increases in secondary and tertiary memory (30), and IL-7R α , which is related to memory CTL survival (23), is down regulated in secondary memory CTLs (38). A slower transition to central memory phenotype is observed in secondary memory CTLs generated from prime-boosting with different vectors (25, 43). These studies also suggest that the memory phenotype may vary when different vectors are used in boosting (25, 30, 38, 39, 44), which further stresses 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 (40, 45). 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 (46-48). However, peptides are poorly immunogenic and, by themselves, induce immune tolerance or deletion of peripheral CTLs (49-52). 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 (53-56). It is feasible that repeated peptide vaccination with adjuvant could be used to progressively enhance this memory (46-48).

Adjuvants

One approach to increasing the efficacy of peptide immunization is co-administration of adjuvant. Adjuvants typically work by prolonging antigen persistence, enhancing costimulatory signals, increasing local inflammation, and/or triggering the nonspecific proliferation of lymphocytes (2, 3). Selection of an adjuvant depends on the species to be vaccinated, the likelihood of side effects, and the ability to promote the desired immune response (e.g. cell-mediated or humoral immunity) (57, 58).

The most widely used adjuvants in humans are alum-based (57, 58). Alum salts induce the formation of an antigen depot at the inoculation site, enabling the slow release of antigen. Unfortunately, alum-based adjuvants seldom induce cellular immune responses (57, 58). Another class of adjuvants, oil-based adjuvant emulsions, are commonly used in veterinary vaccines and two formulations have been approved in Europe for use in adjuvanted human influenza vaccines (58). Similar to alum-based adjuvants, adjuvant emulsions work through formation of an antigen depot and typically do not promote cellular immunity (57). A relatively new class of vaccine adjuvant – ligands of Toll-like receptors (TLRs) - shows promise for its ability to increase the cross talk between the innate and adaptive immune systems and enhance the specific immune response against co-inoculated antigens (59). TLRs are a family of membrane-spanning proteins that recognize pathogen-associated molecular patterns (PAMPs) of molecules derived from microbes (2). Many successful live-attenuated or killed vaccines are naturally immunogenic, as they possess motifs that trigger TLR pathways (58). TLR ligands likely contribute to CD8 T cell memory by stimulating APCs - promoting up-regulation of MHC molecules (part of signal one),

costimulatory molecules (signal two) and inflammatory cytokines (signal three) which can program CTLs to memory (5, 9, 60). Therefore, vaccines targeting TLRs may induce cellular immune responses to pathogen-derived antigens and perhaps eliminate the need for complex and toxic adjuvant formulations.

In the 1950s, lipopolysaccharide, a component of the cell wall of gram-negative bacteria, was discovered to have adjuvant activities (57). LPS acts through TLR4, utilizing the adaptor molecule MyD88 to initiate a signaling cascade through an IL-1R-associated kinase (IRAK) ultimately activating NF- κ B (61). NF- κ B activation stimulates the production of type I interferons (IFN- α and IFN- β) and promotes IL-12 production which enhances the cellular immune response (62). Although LPS can induce high fever, toxic shock, and organ failure in humans, it does not have these harmful effects in mice and is commonly used in mouse models (59). Fortunately, less toxic derivatives of LPS, which also signal through TLR4, are available. For example, monophosphoryl lipid A (MPL) retains Lipid A, the biologically active component of LPS (62), but is 1000-fold less toxic. In the United States and Europe, MPL is licensed as an adjuvant with Cevaxix, a conjugate vaccine against human papillomavirus (HPV) (62),

In addition to the aforementioned agonists of TLR4, compounds that trigger other TLRs, such as TLR3 and TLR9, are promising adjuvants. Poly IC (polyriboinosinic:polyribocytidylic acid) is a synthetic analog of dsRNA that binds to TLR3. TLR3 is located within the endosomal compartments of dendritic cells and macrophages and is naturally triggered by double stranded RNA (dsRNA) that typically appears after infection by RNA viruses (62). TLR3 is unique in that it does

not signal through the MyD88-dependent pathway but instead utilizes the adaptor molecule TRIF. The TLR3 pathway facilitates antigen cross-presentation, allowing CD8 T cells to be primed by exogenous antigen presented by MHC I molecules (62). Like LPS, poly IC can be modified for reduced toxicity (e.g. poly UC containing mismatched uracil and guanine bases) (62). TLR9 recognizes unmethylated CpG motifs, which are relatively common in bacterial and viral DNA but are rare in mammals (63). Synthetic oligonucleotides with CpG motifs can be used to mimic bacterial DNA (62). Stimulation of TLR9, located within the endosomal compartments of numerous cells of the immune system, produces an immune response geared toward eliminating intracellular pathogens by promoting the production of IL-12, IFN- γ , and TNF- α (62).

Purpose of the study

Given the importance of memory CTLs in eliminating altered self-cells, including virus-infected and tumor cells, devising effective vaccination strategies for generating memory CTLs is a priority in the field of immunology. Memory CTLs can be induced by prime-boosting with different vectors, but the availability of appropriate vectors is limited. This becomes the restrictive factor considering the sheer number of pathogens to be vaccinated against and the frequent need for multiple boosts to elicit protective immunity. A new strategy for effective and repeatable boosting without vectors is urgently needed. Therefore, the goal of the present study is to determine if repeated peptide boosting can generate a functional memory CTL population. Specific aims include: (1) determining if repeated peptide boosting requires adjuvant for the generation of memory CTLs; (2) analyzing the

phenotype and functionality of the memory CTLs after boosting; and, if adjuvant is required, (3) assessing the abilities of different TLR-ligand adjuvants to generate memory.

Chapter 2: Materials and Methods

Mice, viruses, bacteria, and reagents

This experimental system involves transgenic OTI mice and recombinant pathogens. OTI mice (gifts from MF Mescher, University of Minnesota, MN) possessing transgenic TCRs specific for H2-K^b (MHC class I) and SIINFEKL (the amino acid position 257 to 264 epitope of the antigen OVA) (64) were crossed with Thy1-congenic B6.PL-Thy1a/Cy (Thy1.1) mice (Jackson ImmunoResearch Laboratories) and bred to homozygosity. Upon transferring the OTI cells into B6 recipients, we can identify them by flow cytometry using a fluorescently labeled antibody against the congenic marker Thy1.1. This model system makes it possible to keep track of the immune response and has been widely used in the immunology field.

Recombinant Vaccinia virus expressing the gene for OVA (VV-OVA) was used for priming and mice were infected i.p. with 5×10^6 plaque-forming units (PFUs). Boosting with 50 ug peptide (SIINFEKL) in the presence or absence of 50 ug LPS, CpG, or Poly IC was performed through i.v. tail injection in a total volume of 300 ul in DPBS. Recombinant *Listeria monocytogenes* expressing full-length secreted OVA (LM-OVA) was used at 5×10^5 CFU/mouse i.v. to test the protection of memory CTLs formed during boosting.

C57BL/6NCr 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). LPS, CpG OD1826 and

Poly IC were purchased from Invivogen (San Diego, CA). SIINFEKL peptide was purchased from New England Peptide (Gardner, MA). LM-OVA, VV-OVA, and tetramer were provided by Dr. Jameson at the University of Minnesota. Collagenase D for tissue digestion was from Roche. Tissues were processed for standard paraffin histology and stained with H&E by Histoserv, Inc., Germantown, MD. Mouse TNF α ELISA Ready-SET-Go! reagent kit by eBioscience was used to quantify TNF α in serum. 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).

Adoptive transfer

Naïve OTI cells were purified from the spleens of OTI mice. Viable cell counts were done (trypan blue), and the percentage of OTI cells in the sample was determined by flow cytometry staining with anti-Thy1.1 and CD8. 10^5 /mouse naïve OTI cells were transferred into B6 recipients by i.v. injection.

Boosting plan

One day after adoptive transfer of OTI T cells, the recipient mice were infected with VV-OVA i.v. to obtain primary or background memory. At thirty days post-infection, blood was collected via tail nick, stained, and analyzed by flow cytometry to confirm the establishment of memory CTLs. This baseline memory is the first memory after priming, or M1. Next, these memory mice were divided randomly into treatment groups for boosting. Boosting occurred every thirty-two days via tail-vein injection and the OTI cells in the blood were stained for surface

markers (KLRG1, CD127, CD62L, CD8, and Thy1.1) every five and thirty days after each boost.

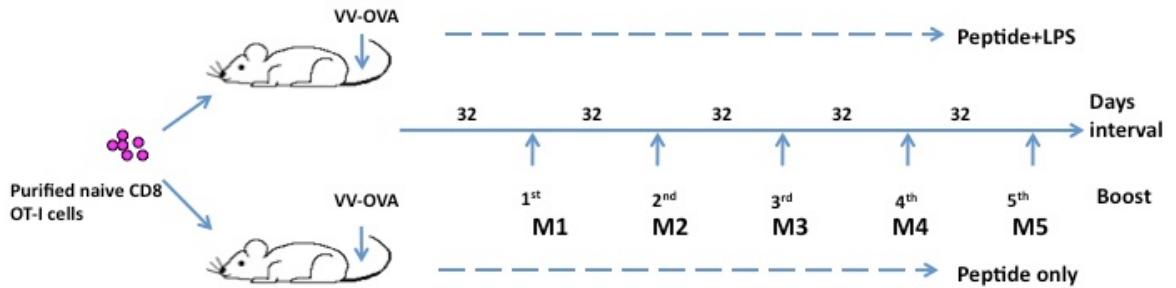


Figure 3. Boosting plan

Naïve OTI cells were purified and transferred into naïve B6 mice. The recipient mice were infected with VV-OVA the following day to obtain primary memory (M1). At day 30 post-infection, CTLs from the blood were analyzed to confirm the establishment of memory. The mice were then randomly divided into two groups – peptide only and peptide plus LPS – for boosting. We boosted these mice every 32 days and examined memory OTI cells in the blood every 30 days after each boost. M1 indicates the first memory after priming, and M2 indicates the second memory resulting from priming and one boost.

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 x 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. 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 x PBS. The final cell suspensions were passed through cell strainers before FACS analysis.

Flow cytometric analysis

Flow cytometric analysis was done using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences) to determine the percentage and total OTI cells in the samples. Background for OTI cell numbers was determined by identical staining of cells from normal C57BL/6 mice (no adoptive transfer).

Intracellular cytokine staining

Spleen cells from adoptively transferred mice were incubated at 2×10^6 cells/ml in RP-10 with 0.2 μ M OVA₂₅₇₋₂₆₄ peptide and 1 μ l of GolgiPlug (BD Biosciences) for 3.5 h at 37°C. Cells were fixed in Cytifix 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 FITC-conjugated Ab to IFN- γ for 30 min at 4°C. Cells were then washed once with Perm/Wash buffer and once with PBS containing 2% FBS, and analyzed by flow cytometry.

Statistical analysis

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

Chapter 3: Results

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

Unlike naïve CTLs, memory CTLs are sensitive to antigen stimulation (65-67) in the absence of costimulation (35, 68-76) and are not dependent on cytokines for acquiring effector function (11, 77-79). We hypothesized that repetitive intravenous peptide boosting 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) (Figs. 3 and 4A-B). Boosting with or without LPS was repeated every 30 days for a total of four times, and the memory OTI cells in the blood were examined 30 days after each boost (Figs. 3 and 4A-B). Surprisingly, 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. 4A-B). Memory CTLs increased to approximately 25% of the total CD8 T cells in the blood after the fourth boost (Fig. 4A-B) and to numbers greater than 10 million per spleen (Fig. 4C). These results indicate that repeated intravenous peptide boosting is sufficient, but requires co-administration of an adjuvant, for generating a robust memory CTL population.

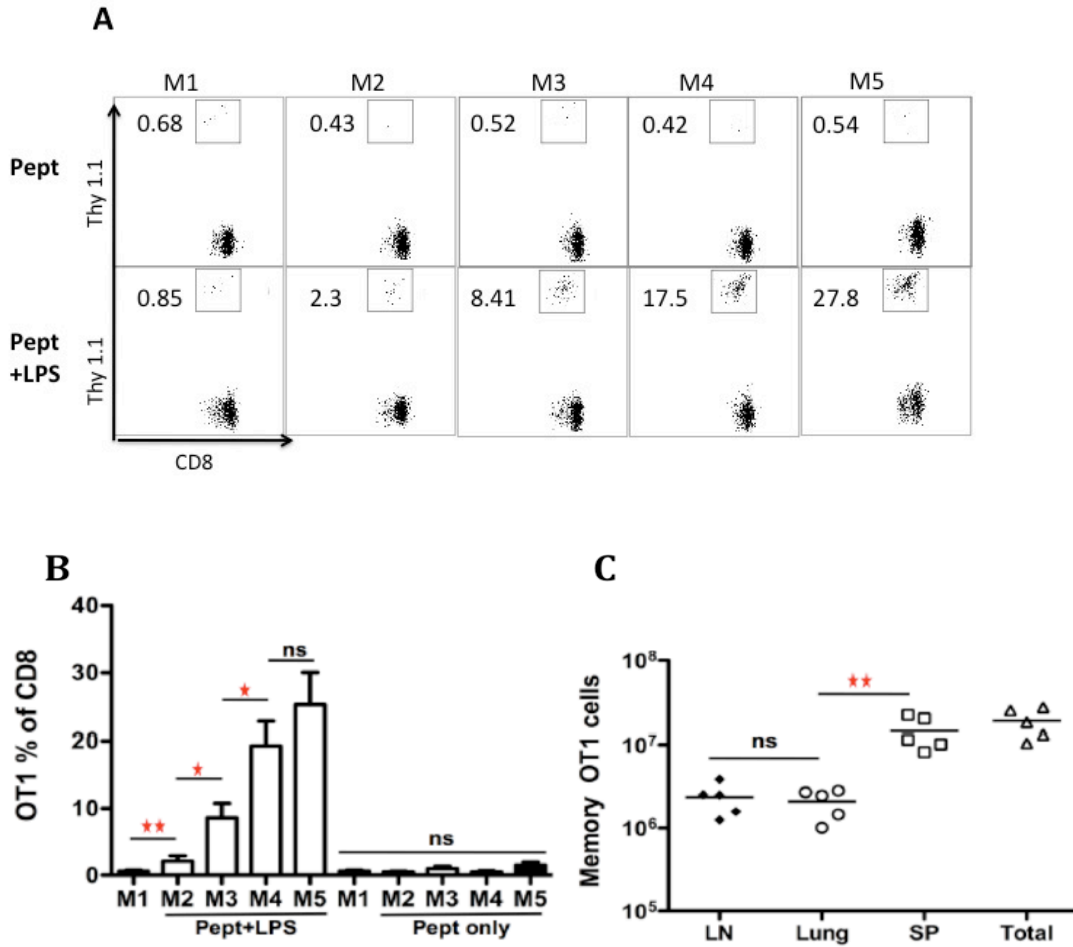


Figure 4. Repetitive boosting with peptide and adjuvant drives memory CTLs progressively to high levels.

(A) Representative dot plots illustrating the progressive increase in memory CTLs, gated on total CD8 T cells in the blood from memory mice. (B) OTI percentage of total CD8 T cells in the blood from memory mice. Pre-planned contrasts were used to test for statistical significance. (C) Distribution of memory CTLs 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.

Repeated boosting generates memory CTLs with a unique phenotype

Multiple boosts with different live vectors lead to enhanced KLRG1 expression (30) and decreased CD127 expression (38), 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. 5A), indicating that peptide boosting generates CTLs of a different phenotype compared to those from boosting with live pathogens (25, 30, 38, 44). IFN γ production is a hallmark of memory CTLs in many infections (11, 27, 30, 80). In line with this, the majority of primary memory CTLs (M1) produced IFN γ (Fig. 5B-C). However, IFN γ -producing OTI cells were significantly reduced after multiple boosts (Fig. 5B-C), from more than 60% to less than 40% (Fig. 5C). In agreement with previous reports (38, 81-83), primary memory CTLs induced by VV infection produced little to no granzyme B (GZB) (Fig. 5B and D). Yet, GZB-producing memory CTLs increased 20% after four consecutive peptide boosts (Fig. 5B and D). This phenomenon is also observed when different vectors are used for boosting (30, 38, 84).

The reduction in IFN γ production after four boosts 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. 6). 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. 6). This demonstrates that the unique effector memory CTLs resulting from repeated peptide boosting were, in fact, functional.

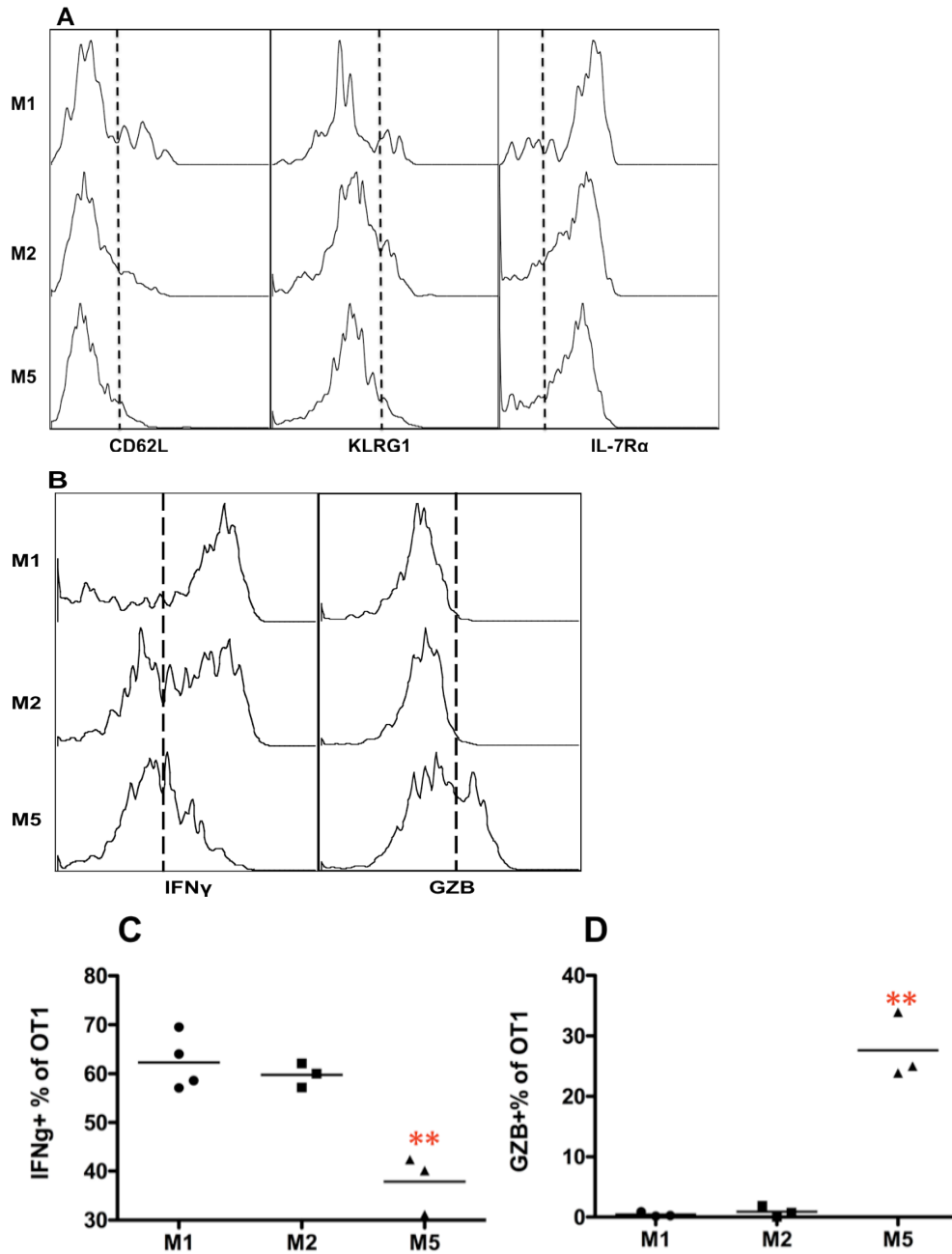


Figure 5. Memory CTLs gain a unique phenotype after multiple peptide boosts. Memory OTI cells in the spleen of memory mice after the fourth boost (M5) or first boost (M2) were compared to primary memory (M1) for surface molecule expression (A), IFN γ and granzyme B production (B-D). Similar results were obtained from three experiments. Vertical dashed lines indicate gates.

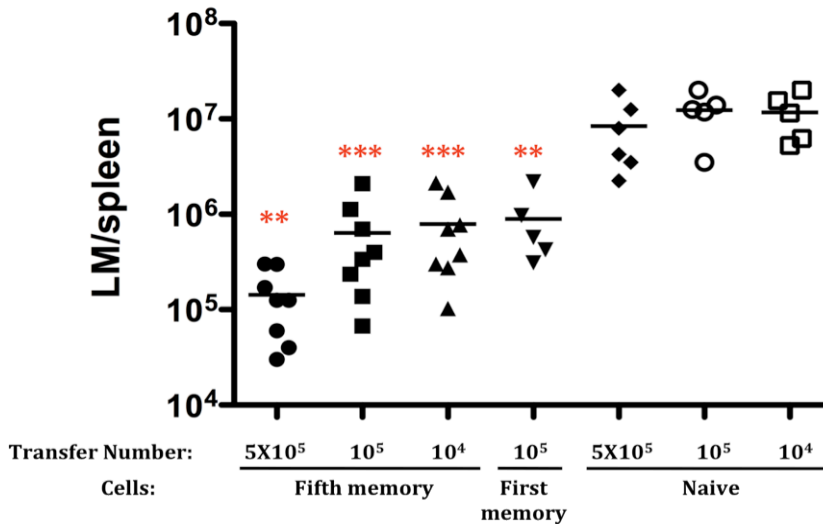


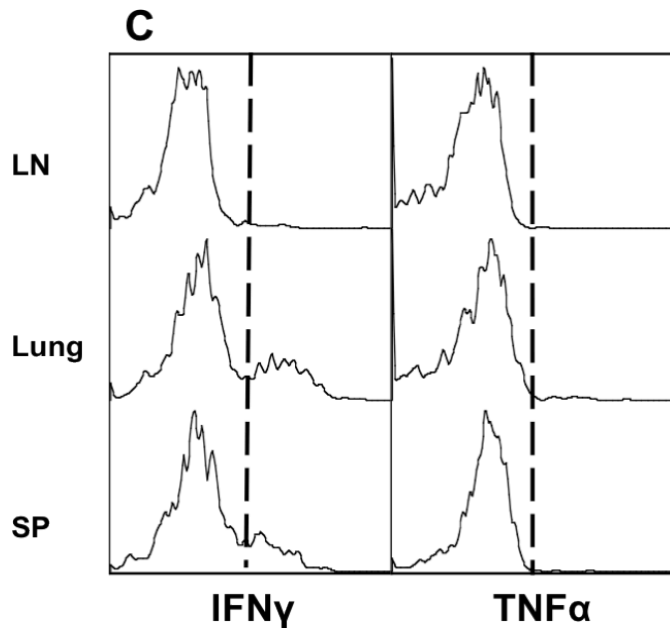
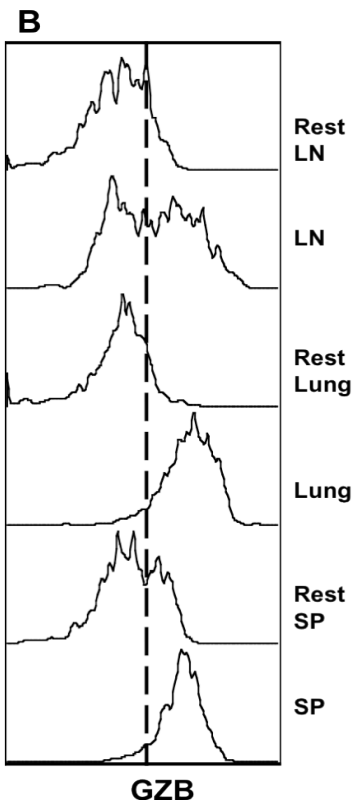
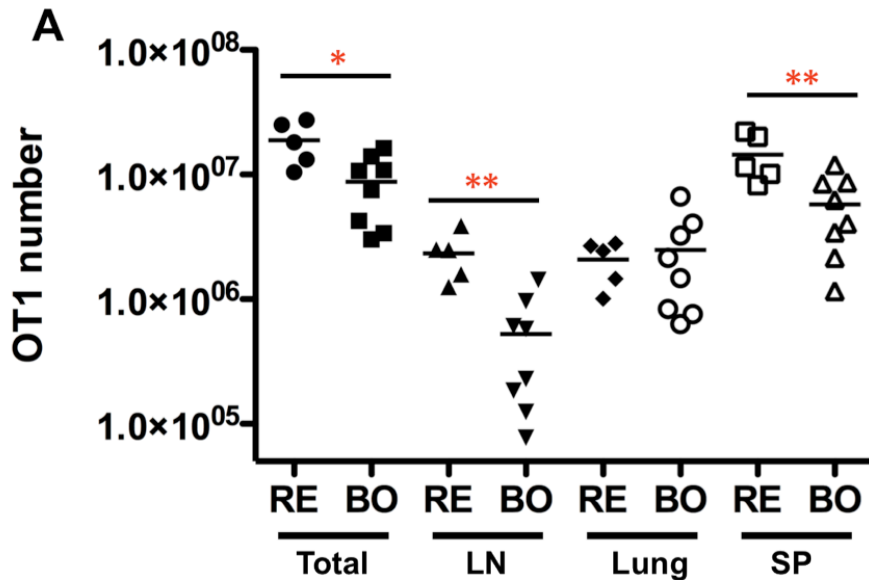
Figure 6. Memory CTLs resulting from multiple peptide boosts are functional. Splens were harvested from M5 memory mice in Fig. 3. Splenocytes containing different numbers (10^4 , 10^5 or 5×10^5 /mouse) of memory OTI cells were transferred into naive B6 mice, and the recipients were challenged with 5×10^5 LM-OVA i.v. the next day. Equal numbers of purified naïve OTI CD8 T cells were transferred as a control. Splens from VV-OVA-primed memory mice (with OTI transferred) were used as positive controls (first memory). Splens 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.

Over-induction of memory CTLs may lead to unintended immunopathology due to increased production of inflammatory cytokines, such as $\text{TNF}\alpha$ (85). Based on the large number of memory CTLs induced by repetitive peptide boosting with LPS (Fig. 4A-B), we asked if further boosting would lead to immunopathology. Indeed, mice that received five continuous boosts displayed severe clinical symptoms including decreased body temperature, humpback and lethargy and were subjected to euthanasia according to humane endpoint guidelines. Most nucleated somatic cells express MHC class I molecules on their surface and are able to present the peptide to effector CTLs upon boosting. Thus, we speculate that the lethal effects of repetitive peptide boosting may be due to the rapidly acquired cytotoxicity of reactivated memory CTLs, enabling them to destroy any cell capable of presenting the peptide. Indeed, granzyme B production was elevated 3- or 10-fold (based on the mean fluorescent intensity) in the spleens and lungs of M5 mice (Fig. 7B), but $\text{IFN}\gamma$ production remained low (Fig. 7C) and there was no noticeable $\text{TNF}\alpha$ production (Fig. 7C). However, following the fifth boost, the number of OTI cells dropped to approximately half the amount present in the resting memory stage prior to the boost (Fig. 7A). This occurred to a greater extent in lymphoid tissues than in the lung (Fig. 7A) and could be indicative of increased peripheral localization of memory CTLs upon reactivation.

$\text{TNF}\alpha$ was present in the serum only four hours after the first and fourth boosts with peptide plus adjuvant, and significantly less $\text{TNF}\alpha$ was found in the mice receiving peptide only (Fig. 7D). Since CTLs did not produce $\text{TNF}\alpha$ following five boosts, other immune cells, such as macrophages, are likely responsible for the $\text{TNF}\alpha$

present in the serum. This is evidence that repetitive boosting with peptide and adjuvant may work through cytokine induction.

It is intriguing that mice receiving up to four boosts were healthy and indistinguishable from normal mice. To examine if systemic inflammation was induced after the fourth boost of peptide with and without adjuvant, tissue samples from mice were harvested two days post-boost. Tissues were trimmed, stained using standard H&E procedures, and assessed for infiltration of lymphocytes. The results indicated that four-time boosting with peptide and adjuvant induced localized minor inflammation (infiltration) in the lung, but not in the heart, kidney and spleen (Fig. 7E). This is consistent with our finding that the number of CTLs in the lung remain relatively stable before and after boosting compared to the decreasing numbers of CTLs in lymphoid tissues following boosting (Fig. 7A). Interestingly, peptide without adjuvant also induced localized inflammation (Fig. 7E), so these effects cannot be attributed to adjuvant. In general, these data suggest that repetitive peptide boosting causes progressive tissue-specific inflammation, which is relatively minor through the fourth boost.



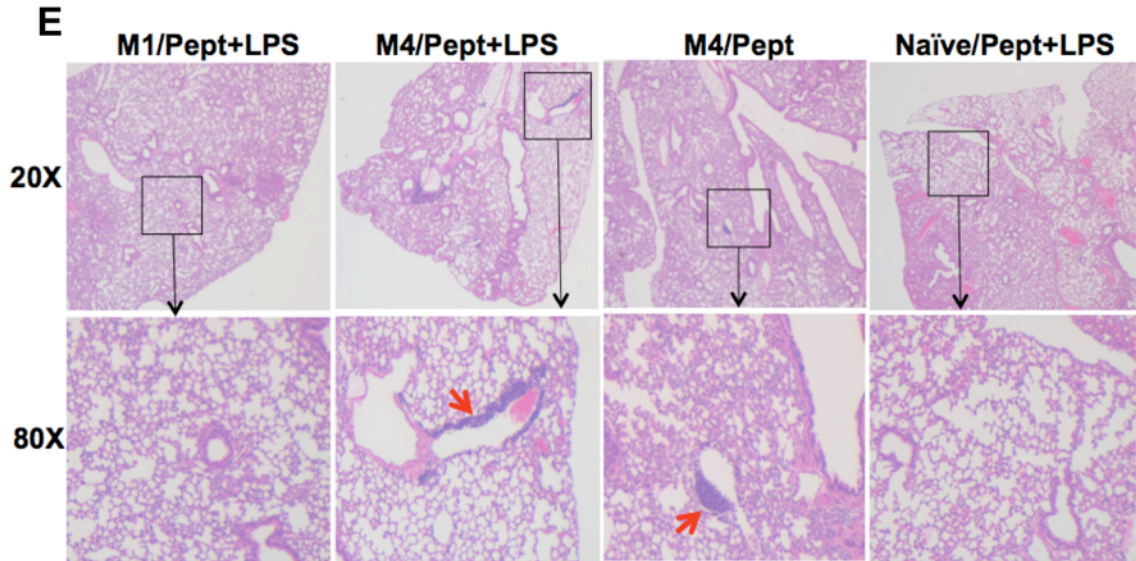
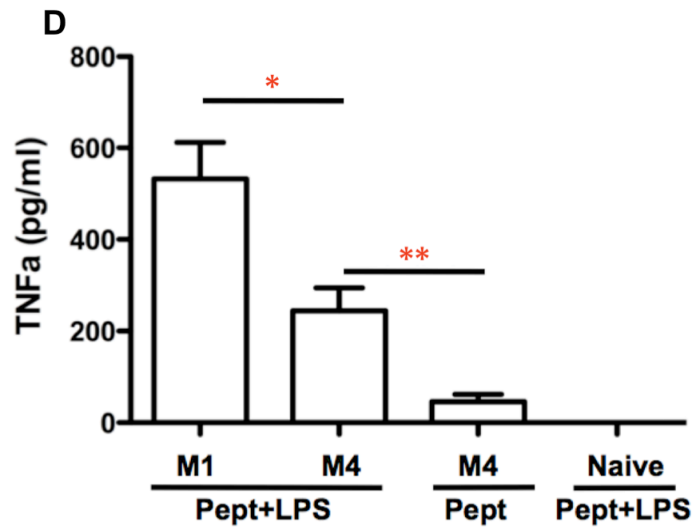


Figure 7. CTLs induced by repetitive peptide boosting can cause immunopathology.

Mice having previously received 10^5 OTI transfer were first primed with VV-OVA, and then boosted five times with peptide and LPS at intervals of 30 days as indicated in Fig. 3. Two days after the fifth boost, mice were sacrificed due to clinical complications. (A) Total OTI cells in each tissue before and after the fifth boost. RE: resting memory before the fifth boost (the same data as in Figure 4C); BO: two days after the fifth boost. (B) Granzyme B production. Rest LN: resting memory OTI in LN before the fifth boost. LN: OTI in LN two days after the fifth boost. The same description is for the lung and spleen (SP). (C) Production of $IFN\gamma$ and $TNF\alpha$ of OTI cells in different tissues two days after the fifth boost. (D-E) Recipient B6 of M1 (primed by VV-OVA), M4 (primed followed by 3 boosts with LPS+peptide), and recipients having received naïve OTI cells, were boosted with LPS+peptide or peptide alone. (D) Quantification of $TNF\alpha$ in sera harvested 4 hours after boosting. (E) Comparison of histology of the lung (magnification at 20X and 80X) two days after boosting. Tissues were processed for standard paraffin histology and stained with H&E by Histoserv, Inc., Germantown, MD. Red arrow identifies cell infiltration.

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 $\text{TNF}\alpha$ (85) (Fig. 7D). 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. 8A). Moreover, these memory CTLs retained a typical functional effector memory phenotype of $\text{CD62L}^{\text{lo}}/\text{KLRG1}^{\text{lo}}/\text{CD127}^{\text{hi}}$ (Fig. 8B). However, even though CD127 expression was generally high at all time points, its expression continued to increase over the 6-month period (Fig. 8B). This underscores the importance of IL-7 for long-term survival (35).

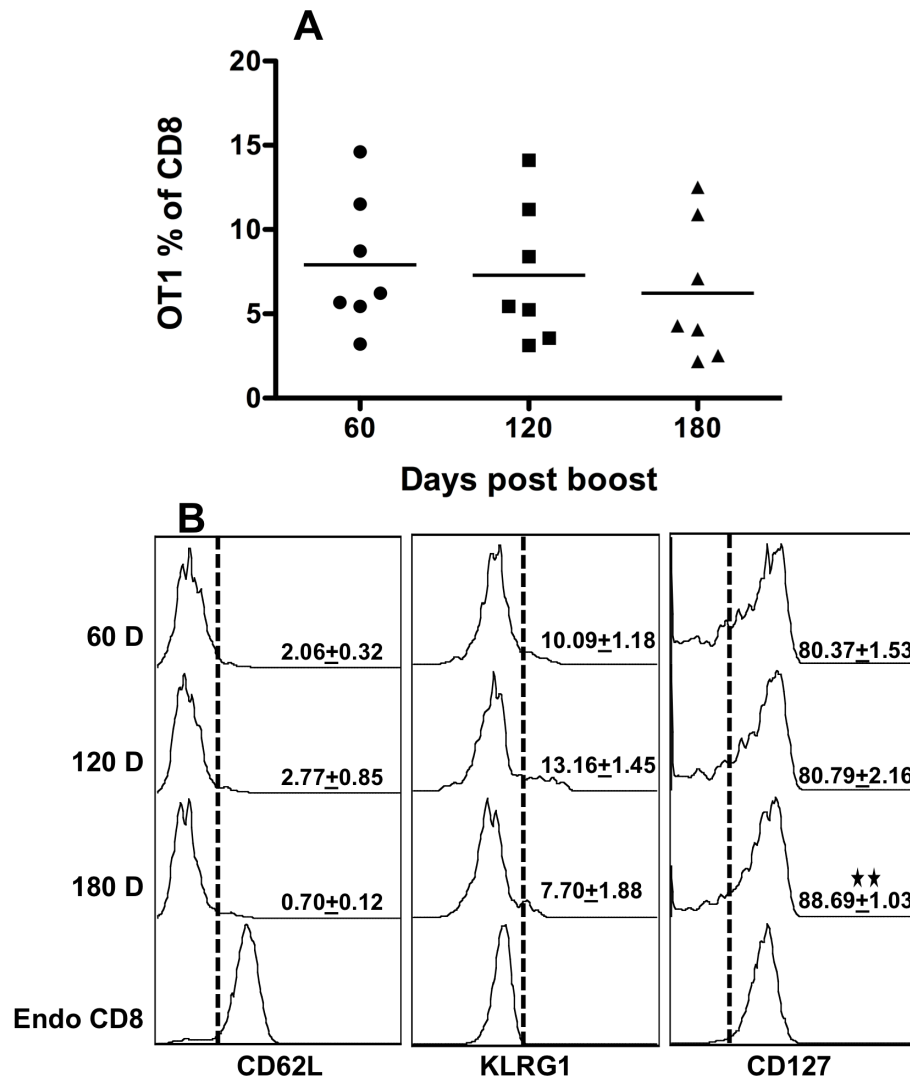
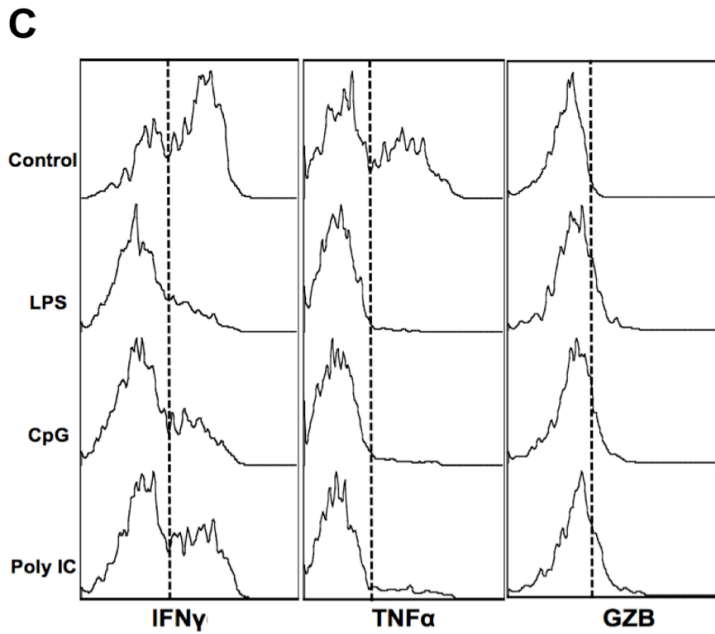
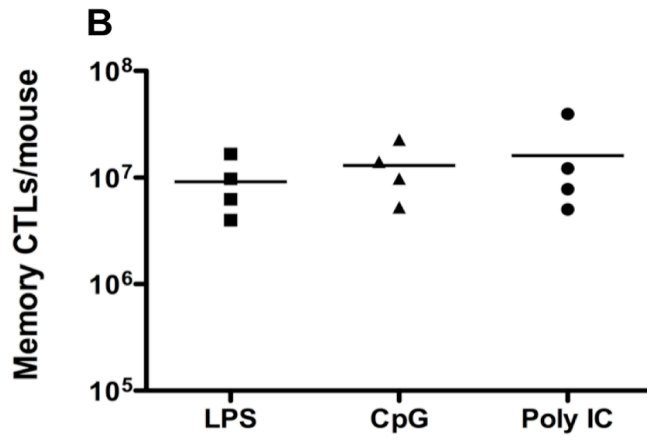
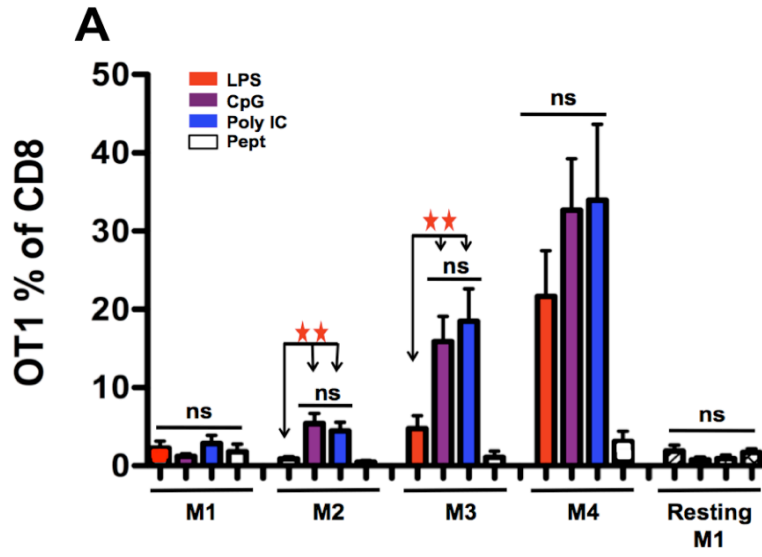


Figure 8. CTLs induced by repetitive peptide boosting are long lasting and stable in effector memory phenotype.

Memory OTI cells from M5 mice (Figure 4) 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) OTI percentage of total CD8 in the blood. (B) Representative surface molecule expression of the same samples as in A, with statistics as mean +SEM. Endo CD8 are endogenous CD8 T cells.

Repetitive peptide boosting is effective with different adjuvants

Although LPS is a well-characterized TLR-4 ligand and commonly used adjuvant in animal experiments, it is not approved for use in human trials due to its toxic effects. Several other molecular adjuvants targeting different TLRs, especially CpG and Poly IC, have proven effective in vaccination (61, 86-88). To test if these adjuvants had similar efficacy, purified naïve OTI cells were transferred into B6 mice. The experimental design was the same as in Fig. 3, except that treatments included CpG and Poly IC, in addition to LPS. Analysis of CTLs in the blood showed that LPS plus peptide progressively enhanced memory CTLs (Fig. 9A), similar to Fig. 4B. However, both CpG and Poly IC (plus peptide) were more potent in increasing memory CTLs after the first (M2) and second (M3) boosts, indicating that they may be superior to LPS as adjuvants. The third boost, on the other hand, led to an increase in memory CTLs in all three adjuvant groups (Fig. 9A). Lack of a significant difference between treatments could be explained by variation among individual animals (Fig. 9A). A few samples reached even above 60% of total CD8 (data not shown), which prompted us to stop further boosting in the same animals due to the potentially lethal effects of further antigen stimulation noted in Fig. 7 D-E. As expected, the resting memory CTLs from VV-OVA priming did not change over time, and peptide boosting alone had no effect (Fig. 9A).



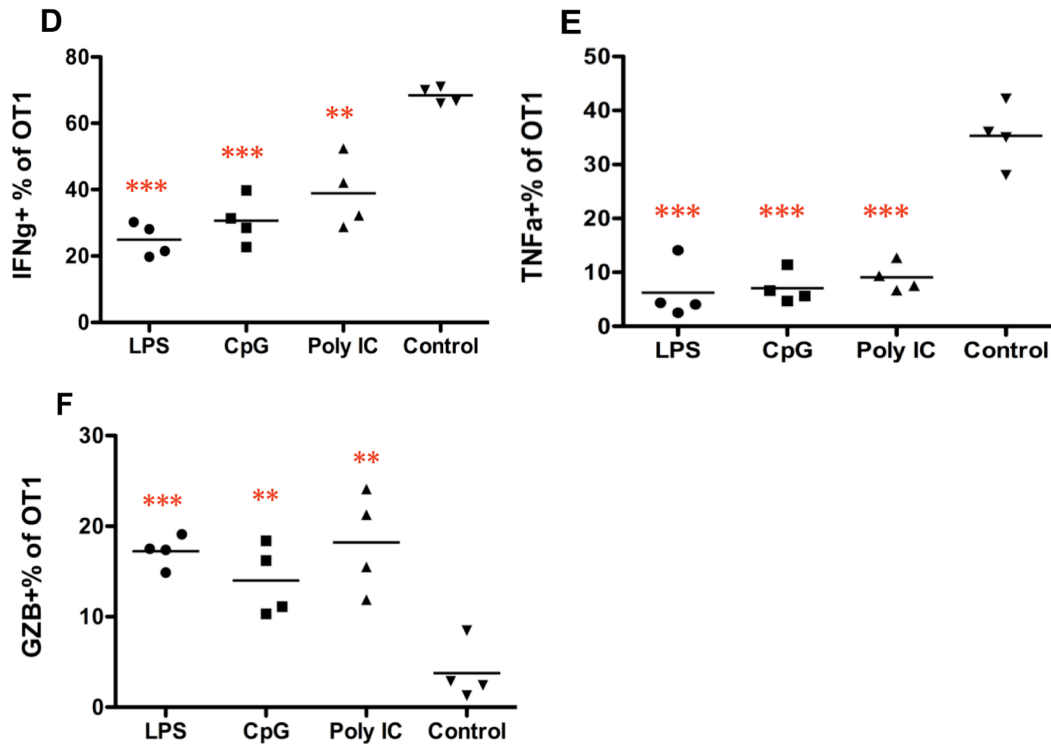


Figure 9. Repetitive peptide boosting with different adjuvants generates similar functional effector memory CTLs.

Naive OTI CD8 T cells were transferred into B6 mice 10^5 /mouse, which were primed with VV-OVA the next day. Boosts were performed three times with different adjuvants at 30-day intervals, and blood samples were drawn every 30 days after each priming or boosting. Thirty days after the third boost, memory mice were sacrificed to harvest the following tissues: peripheral lymph nodes, spleen, lung and bone marrow. (A) Memory OTI percentage of total CD8 in blood. (B) Total memory OTI cells from tissues. (C) Representative histograms of IFN γ , TNF α and GZB expression. Dashed lines indicate the gates, and statistics are shown in D-F. Each group was compared to control M1 in D-F.

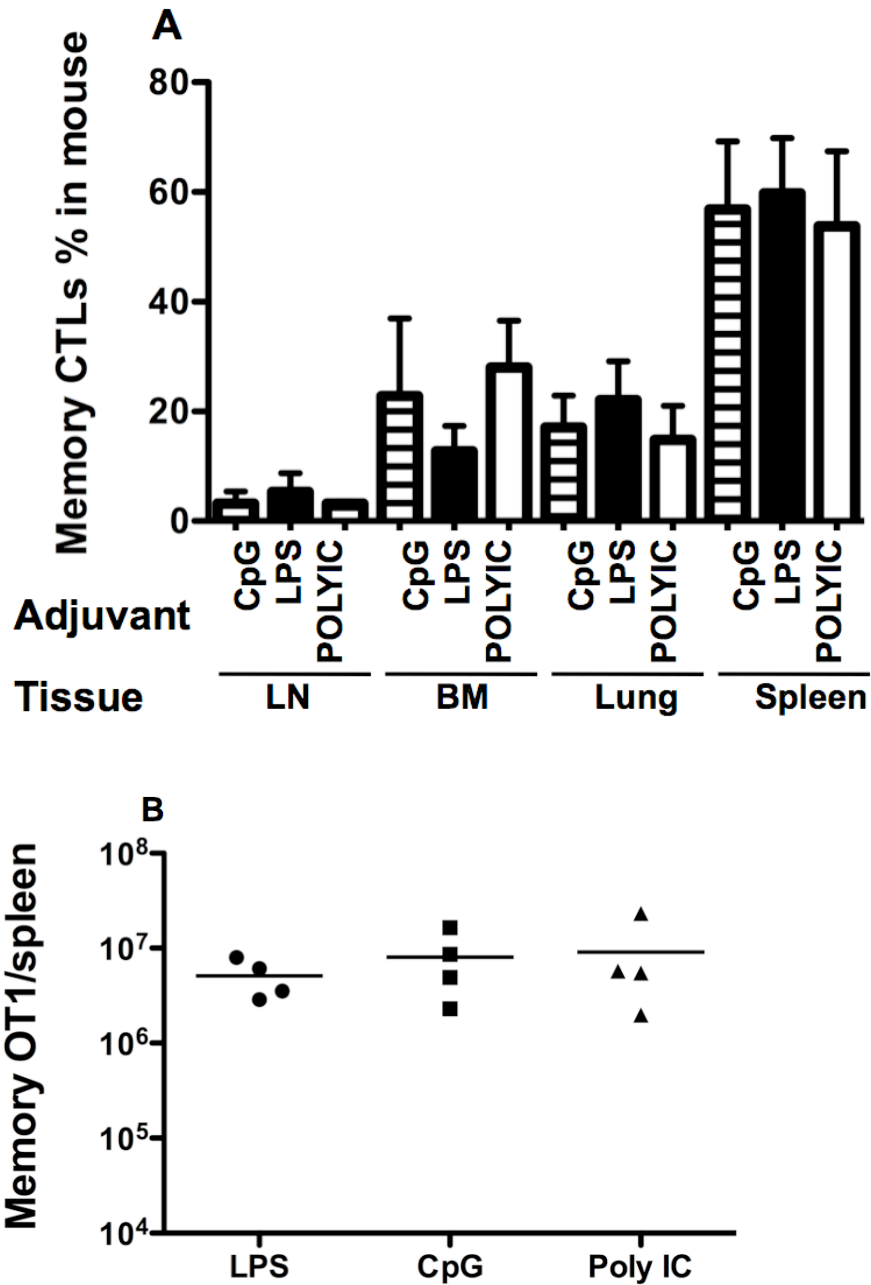


Figure 10. Memory CTLs reside mostly in the spleen after repetitive peptide boosting. Samples were from Fig. 9. (A) Memory OTI cell distribution (%) in different tissues. (B) Memory OTI cells in spleen. There are four samples for each group. Each bar represents the mean plus SEM.

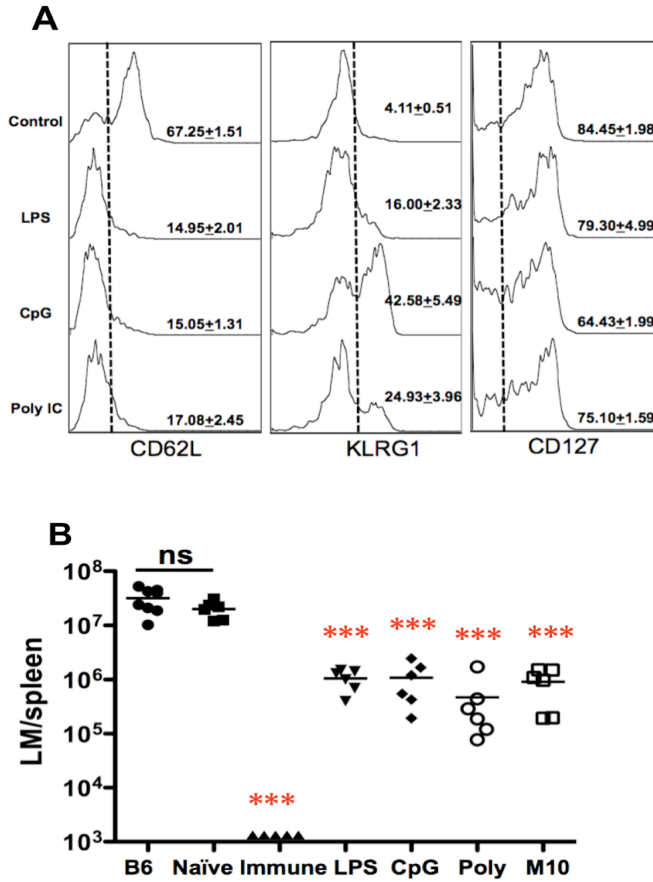


Figure 11. Phenotype of memory CTLs after repetitive peptide boosting using different adjuvants.

(A) Spleen samples were from Fig. 9. Representative histograms of expression of CD62L, KLRG1 and CD127, with statistics as mean +SEM. Every group had 4 animals. (B) Splenocytes containing 10⁵ memory or naïve OTI cells were transferred into naïve B6 mice, which were challenged and examined as described in Fig. 6. Immune memory mice (with OTI) were VV-OVA primed resting M1 (Fig. 9A). M10 memory mice: splenocytes containing 10⁵ memory OTI cells from M5 (Fig. 4B) were transferred into naïve B6 mice, which experienced five more rounds of peptide plus LPS boosting at 30-day intervals. Each group was compared to naïve CTL transferred group.

To compare the distribution and function of resultant memory CTLs from different adjuvants, we harvested peripheral lymph nodes, spleen, lung and bone marrow (two sets of femur plus tibia and fibula) 30 days after the third boost. For each adjuvant group, total memory CTLs reached numbers of approximately 10 million per animal (Fig. 9B), with the spleen harboring over 50% of all memory cells (Fig. 10). Similar to LPS, peptide boosting in the presence of CpG or Poly IC resulted in a decreased number of IFN γ - and TNF α -producing memory CTLs, but an increased number of GZB-producing cells compared to the CTLs after priming (control) (Fig. 9C-F). This indicates that the development of memory CTLs with reduced IFN γ production but enhanced GZB production is a general trend that results from repetitive peptide boosting independent of the adjuvant applied. Additionally, the memory CTLs from the three adjuvant groups had a similar effector memory phenotype (CD62L^{lo}KLRG1^{lo}CD127^{hi}), but the CpG group had higher KLRG1 expression and lower CD127 expression (Fig. 11A).

As previously mentioned, KLRG1 expression is indicative of short-lived effectors (89) and may be indicative of impaired function. To assess the protective abilities of these memory CTLs, we transferred an equal number of memory CTLs from each adjuvant group, and from a group of memory CTLs that had been boosted nine times with LPS plus peptide after VV-OVA priming (M10) (memory CTLs from M5 were transferred at 10⁵ per mouse into new hosts for five additional boosts) into naïve B6 mice and challenged with LM-OVA. Compared to the naïve group, the three adjuvant groups displayed similar protection against LM-OVA challenge (Fig. 11B). Surprisingly, memory CTLs (M10) from mice boosted nine times provided similar

protection compared to those from mice boosted three times (Fig. 11B), indicating that the function of memory CTLs resulting from multiple rounds of challenge was not compromised. Therefore, different adjuvants can similarly induce functional effector memory CTLs when used in repetitive peptide boosting.

Memory CTLs induced by repetitive peptide boosts are responsive to pathogen challenge

Although M10 memory CTLs provided similar protection compared to M4 (Fig. 11B), it was still possible that they were senescent due to experiencing six more rounds of massive expansion. Ceased proliferation in response to antigen stimulation is the major feature of this phenomenon (25, 30, 90-95). To examine the possibility of immune senescence, we compared the proliferative response of memory CTLs from Figs. 9 and 11 that had experienced three or nine rounds of peptide boosting with different adjuvants. 10^5 memory CTLs from each group were transferred into new B6 recipients, and naïve OTI and immune memory mice with OTI transfer (primed once with VV-OVA - M1) were included as controls. Recipients were challenged with LM-OVA the day after transfer. Spleens were harvested six days after challenge. Repetitively boosted memory CTLs achieved substantial expansion (Fig. 12A-B), although the number was significantly higher in the naïve OTI group compared to all except the immune group (Fig. 12A). Memory CTLs boosted three times with peptide plus LPS displayed similar expansion as those boosted nine times, suggesting that further rounds of antigenic stimulation may not decrease proliferative responsiveness until much later.

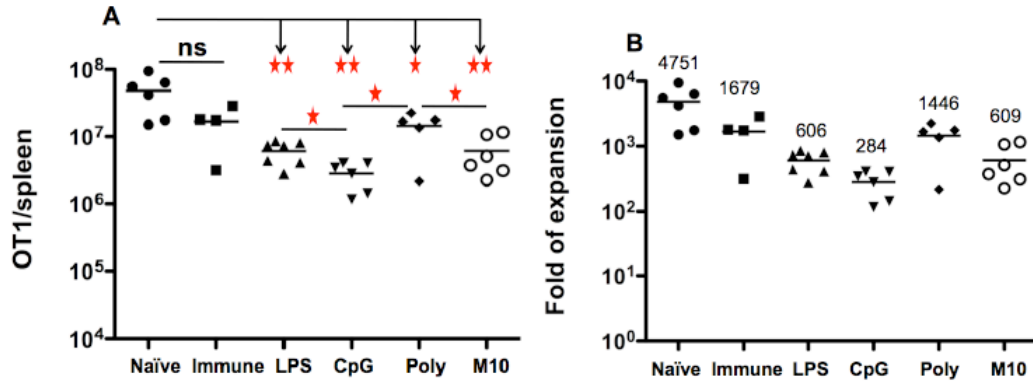


Figure 12. Memory CTLs from repetitive boosting are effective in responding to pathogen challenge.

Three-time peptide boosted memory CTLs (M4) generated by different adjuvants in Fig. 9 were transferred into new B6 recipients at 10^5 per mouse, and were subsequently challenged with LM-OVA at 10^4 CFU/mouse. M10 from Fig. 11B was transferred in the same way as M4. Six days after challenge, spleens were harvested for the analysis of OTI expansion and phenotype. (A) Comparison of OTI cells in spleens. (B) Fold change was calculated by comparing the number of OTI in the spleen with the starting number. Number above each bar represents the mean of fold changes. Overnight parking rate for transferred cells was 10% based on previous publications (8, 96), so the starting number was calculated as 10^4 .

Endogenous memory CTLs respond to repetitive peptide boosts

So far, all of the data presented have been based on transgenic OTI transfer. To test if endogenous memory CTLs could be induced by multiple intravenous peptide boosts, we adopted a similar approach as in Fig. 3, but without OTI 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⁺) present in the lymph nodes, bone marrow, lungs, and spleen was approximately 10⁷, similar to that of memory OTI cells in Fig. 9B (Fig. 13A). This suggests that repetitive peptide boosting efficiently induces endogenous memory CTLs. Similar to OTI memory CTLs (Figs. 9C-D, F and 11A), the resultant memory CTLs had lower IFN γ - (less than 40%) and increased GZB- (15%) producing cells (Fig. 13B-D) and were of effector memory phenotype (CD62L^{lo}/KLRG1^{lo}/CD127^{hi}) (Fig. 13E-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. However, this low number of endogenous M1 reached a level similar to OTI after three boosts.

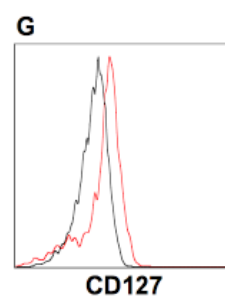
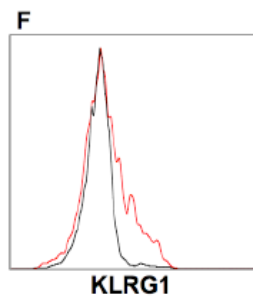
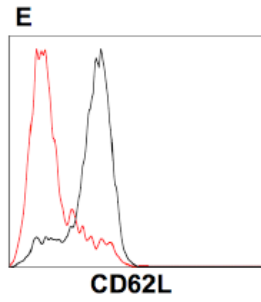
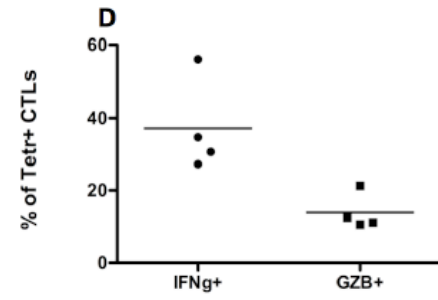
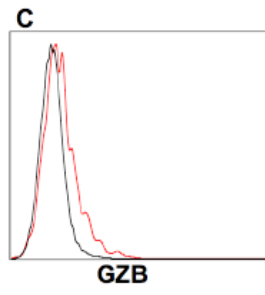
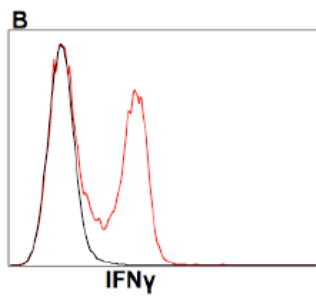
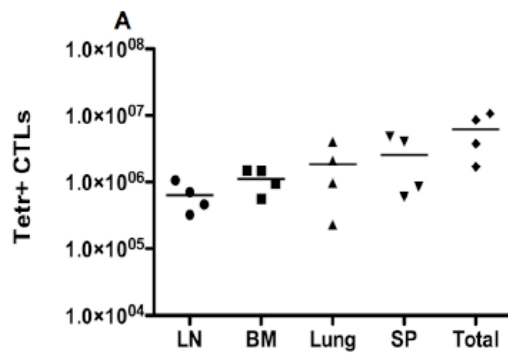


Figure 13. Repetitive peptide boosting with adjuvant induces endogenous memory CTLs.

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) Plots of IFN γ and GZB positive cells out of tetramer and CD8 double positive population in A. (E-G) Representative histograms of CD62L, KLRG1 and CD127 expression. (B-G) Data were all from memory Tetr⁺/CD8⁺ in spleen. Red histogram: Tetr⁺/CD8⁺. Black histogram: Tetr⁻/CD8⁺ (control). Bars represent mean.

Chapter 4: Discussion

Cytotoxic T lymphocytes (CTLs) are critical for the control of many intracellular infectious agents (27, 28), and memory CTLs possess unique functional properties, which make them an essential defense against repeat infection by the same or a similar pathogen. Yet, induction of functional memory CTLs remains a major challenge for conventional vector-based vaccination strategies (27-32). The goal of the present study was to develop a vector-free boosting strategy for increasing the quantity and quality of memory CD8 T cells. Specifically, we hoped to (1) determine if repeated peptide boosting requires adjuvant for the generation of memory CTLs; (2) analyze the phenotype and functionality of the memory CTLs after boosting; and (3) assess the abilities of different TLR-ligand adjuvants to generate memory.

Our experimental system utilized transgenic OTI T cells and recombinant pathogens. We adoptively transferred congenically labeled naïve OTI CD8 T cells, possessing TCRs specific for SIINFEKL (the amino acid position 257 to 264 epitope of the antigen OVA), into naïve B6 hosts. Mice were then primed with VV-OVA to generate the primary immune response and boosted multiple times with peptide (SIINFEKL) with or without adjuvant (Fig. 3). This system is advantageous as it restricts the CD8 T cell specificities to a single transgenic population, and enables us to study the effect of different boosting treatments on the same antigen-specific population over time.

In the present study, 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

(33), there are potential adverse systemic effects related to this mode of delivery (33, 97, 98), which may be derived from antigen or adjuvant (99). Although systemic immunization could cause massive activation of immune cells, such as macrophages and antigen-specific T and B cells, we did not observe any discomfort in mice boosted up to four times intravenously. In addition, the reduction in cytokine production (IFN γ and TNF α) by a large number of activated memory CTLs after repetitive boosts may be beneficial to the host for avoiding the immunopathological risks of a cytokine storm. 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.

We hypothesized that repeated peptide boosting would be effective in reactivating memory CTLs. However, our data indicate that peptide boosting without adjuvant is not sufficient for reactivating the memory CTL population. On the other hand, when adjuvant is included the number of memory cells steadily and significantly increases following each boost (Fig. 4A-B) and develops into a robust memory CTL population. 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 (107-109). In the context of an inflammatory environment, the role of 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 (110, 111), the role of costimulation in memory responses is unclear (68, 112, 113). Some studies claim that costimulation is essential (14, 115), while others suggest that high levels of costimulation are detrimental to memory cell function (114). For example, deficiency of costimulatory molecules CD27, 4-1BB and OX40, while detrimental for the establishment of primary memory CTLs, has no direct effects on the reactivation of memory CTLs (118). A high level of costimulation in vaccination may even ablate memory CTL function (114). Thus, despite some controversies (115, 119), it seems that, although required for the generation of functional primary memory, costimulation may not be necessary for memory CTL responses. This may be related to a higher TCR affinity to antigen following repeated exposure (116, 117). We believe that intravenous peptide boosting with adjuvant is working in the absence of costimulation through the rapid induction of inflammatory cytokines and extensive antigen presentation.

Adjuvants targeting TLRs can induce adaptive immune responses to pathogen-derived antigens and perhaps eliminate the need for complex and toxic adjuvant formulations. Determining TLR adjuvants that can enhance memory CTL generation was an aim of this study and is of practical interest in vaccine development. Peptide boosting with LPS, CpG or Poly IC resulted in CTLs of effector memory phenotype (Fig. 11A) with reduced IFN γ production and enhanced GZB production (Fig. 9C-D, F). Although differences in expansion were observed among the CTLs from boosting with different adjuvants (Fig. 12B), these CTLs displayed similar protection against LM-OVA challenge regardless of the adjuvant

applied (Fig. 11B). That CpG and Poly IC generated more robust immune responses after the first and second boosts compared to LPS suggests that a protective level of CTLs might be achieved with fewer boosts when these adjuvants are utilized (Fig. 9A). Future research into the molecular mechanisms by which each adjuvant influences the cytokine milieu of the immune response and directly or indirectly alters the biologically relevant functions of memory CTLs may reveal new options for enhancing the immunogenicity of vaccines.

The phenotype of the memory CTLs from repetitive peptide boosting was CD62L^{lo}/KLRG1^{lo}/CD127^{hi}. Based on expression of these surface molecules, which remained stable long-term (Fig. 8), these CTLs are classified as effector memory, possessing cytotoxic activity and the ability to localize at peripheral sites of infection (17, 26). Surprisingly, these CTLs exhibited reduced IFN γ production. IFN γ production is a hallmark of most memory CTLs, and is related to the function of both naïve and memory CTLs (11, 27, 28, 35, 120). In this study, IFN γ production was gradually reduced and remained low after multiple boosts (Fig. 5B-C), whereas TNF α production was minimal in both resting memory and reactivated memory (Fig. 7B-C). Despite reduced cytokine production, the CTLs remained functional. Indeed, proper functioning of CTLs involves many components other than IFN γ and TNF α (18). The significant increase in GZB production by multiply-boosted memory CTLs appears to have compensated for the decrease in cytokine production (Figs. 5D, 9F).

Repeated stimulation induces replicative senescence in human CD8 T cells *in vitro*, which is characterized by a cease in division, resistance to apoptosis, and production of TNF α (91, 92, 94, 95, 121). Immune senescence, which has been

attributed to the Hayflick limit, is also apparent in a mouse model employing adoptive transfer (30). In the present study, a large number of memory CTLs generated by sequential peptide boosts in the same animals (Figs. 4B, 9A) was able to expand continuously, albeit at a lower level than the naïve CTLs (Fig. 12) and produce GZB (Figs. 5D, 9F). Thus, these CTLs are not undergoing replicative senescence as observed in human CD8 T cells *in vitro* (91, 92, 94, 95, 121). Interestingly, memory CTLs boosted nine times after virus priming (M10) had a level of expansion comparable to M4 CTLs (Fig. 12A-B), indicating that repeated peptide boosting does not lead to senescence and laying to rest concerns regarding the functional limit of memory CD8 T cells. In conclusion, the data presented in this thesis have important implications for vaccine development as they demonstrate that intravenous peptide boosting with adjuvant following priming can induce long-term functional memory CD8 T cells.

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