2005

Cytotoxic T lymphocyte response to adenovirus gene therapy.

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CYTOTOXIC T LYMPHOCYTE RESPONSE TO ADENOVIRUS GENE THERAPY

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2005

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Program Pathology

Name of Candidate Jian Chen

Committee Chair John D. Mountz

Title Cytotoxic T Lymphocyte Response to Adenovirus Gene Therapy

The virus-specific cytotoxic T lymphocyte (CTL) response is a major obstacle to effective delivery of adenovirus (Ad) gene therapy. However, due to a poor understanding of the role and the mechanisms underlying the CTL activation and effector functions, there is a lack of efficient strategies to suppress the Ad-specific CTL response. To enable analysis of the specific CTL response to Ad vectors, we employed computer-driven algorithms to predict mouse class I MHC binding peptides from Ad capsid protein and validated the fiber peptide (*391 VGNKNNDKL*) as a H2-D^b restricted epitope by an IFNγ ELISPOT assay and an *in vivo* CTL assay. To determine the mechanisms regulating the primary Ad specific CTL response, an Ad5 specific tetramer that contains the epitope peptide, E1Bp (*192 VNIRNCCYI*), in the context of H2-D^b was developed. Using this specific tool in combination with an *in vivo* CTL assay, we were able to quantitatively characterize the primary CTL response after Ad infection. First, we established that a strong CTL response occurred 8 days after infection. The tetramer positive cells expressed high levels of granzyme B and IFNγ and the numbers of tetramer positive cells strongly correlated with the *in vivo* lysis of target cells, indicating that the tetramer positive cells have high cytolytic activity. Surprisingly, viral load in the liver and liver enzyme elevation peaked at 4 - 6 days, before the specific CTL response was detectable.

We next examined the requirement for CD28-B7 interactions in the primary CD8 T cell activation and the effector functions of activated CD8 T cells after Ad infection. We also assessed the role of effector molecules, including perforin and FasL, in mediating target cell lysis. During the primary response, there was a substantial defect in both the generation and effector function of Ad-specific CTLs in CD28 knockout mice. Perforin deficiency resulted in a 28% decrease in the cytolysis mediated by Ad-specific CTLs. Defects in FasL resulted in a decrease in the generation of Ad-specific CTLs, but the effector function was not impaired except when perforin was also deficient, indicating a synergistic role of these two pathways. These findings together will help to develop efficient adenovirus gene therapy protocols by specifically monitoring and suppressing the adverse cytotoxic effects caused by the host immune response.

DEDICATION

To my parents, my sister, my brother and my wife, Ping Hu, who have supported me unconditionally.

ACKNOWLEDGMENTS

I sincerely thank my tireless mentor, Dr. John D. Mountz, for his extraordinary insight into the complex workings of our field. I appreciate him all the more for being approachable, open to ideas contrary to his own, and tenacious in pursuit of mechanisms to test hypotheses definitively.

I likewise wish to extend my sincerest thanks to the following persons: my committee members Peter D. Burrows, Paul A. Goepfert, Jay M. McDonald, and Selvarangan Ponnazhagan provided patience, support, and critical insights, not only on this work but also on my career.

Our collaborators at the University of Alabama at Birmingham, Allan J. Zajac, taught me most of the key techniques employed in the work; Sylvia McPherson helped me develop the MHC/peptide tetramer; Kohtaro Fujihashi, taught me the ELISPOT technique; and David T. Curiel provided us with adenoviral vectors.

Our collaborator in Children's Hospital of Philadelphia, Katherine A. High kindly provided plenty of AAV-hF.IX vectors as well as critical technical support.

My laboratory colleagues, in particular, Hui-Chen Hsu, provided stimulating and productive discussions. Xin Xu, Ping Ar Yang, and Qi Wu helped me selflessly on my project.

I am also grateful for Carol Humber, Jennifer Allen and Evelyn Rogers for their excellent assistance.

Finally, I would like to thank my wife, Ping Hu, who has given me enormous support during my graduate studies.

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LIST OF ABBREVIATIONS

Ad	adenovirus
APC	antigen-presenting cell
$CD28^{-/-}$	CD28 knockout
CTL	cytotoxic T lymphocyte
CTLA4	cytotoxic T lymphocyte associated protein 4
ELISPOT	enzyme-linked immunospot
FasL	Fas ligand
Ig	immunoglobulin
IL	interleukin
MHC	macrohistocompatibility complex
NK	natural killer
TCR	T cell receptor
TNF	tumor necrosis factor

INTRODUCTION

Biology of adenovirus and adenovirus vectors in gene therapy

Adenovirus (Ad) is a non-enveloped icosahedral virus with a single, linear double-stranded DNA genome of approximately 38kb in size.[1] During infection, Ad enters the cell by receptor-mediated endocytosis.[2-5] The early gene products of the E1A and E1B genes are the predominant transcription factors that transactivate other genes E2, E3, and E4. E1B proteins cooperate with E1A products to induce cell growth and block apoptosis induced by E1A proteins by inhibiting p53. During the late stage of transcription, structural genes are transcribed from the L1-L5 genes, which are mainly translated into the capsid proteins.

Many features of Ad make it one of the most popular choices as a gene delivery vector. Thus far, around 27% of clinical protocols used Ad vectors in gene therapy clinical trials (Journal of Gene Medicine, http://www.wiley.co.uk/genmed/clinical). These features include the ability to grow recombinant viruses to high titers, a relatively high capacity for transgene insertion, and efficient transduction of both quiescent and actively dividing cells, usually without incorporation of viral DNA into the host cell genome. First generation Ad vectors were E1/E3-deleted and were developed for treatment of monogenic diseases.[6] Second generation Ad vectors were produced by additional deletion of E2 or E4. Both first and second generation Ad vectors have been associated with severe cellular immune response, resulting in the destruction of transduced cells and direct cytopathic effect.[7] Therefore, third generation Ad vectors, often called gutless or helper-

dependent vectors, have been developed.[8] These vectors are devoid of viral proteins. Consequently, they display reduced immunogenicity and long-term transgene expres sion.[8-10] Alternatively, in order to exploit the cytotoxic functions of wild-type Ad, conditionally-replicative Ad vectors have also been utilized in treatment of tumors.[11-15]

Immune response to Ad gene therapy

One important limitation of Ad gene therapy is the toxicity associated with the use of Ad vectors, which is complex involving both the innate and adaptive immune responses.[16-19] The initial response to Ad vectors administered intravascularly occurs within minutes, peaks at 6 hrs, and occurs in the absence of viral gene expression. This response has been attributed to the innate response characterized by the release and/or production of several proinflammatory cytokines, including interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-8, granulocyte-macrophage colony-stimulating factor, and macrophage inhibitory protein-2.[20,21] The cells that participate in the innate immune response are nature killer (NK) cells, macrophages and dendritic cells, the so-called professional antigen presenting cells (APCs) which serve as a functional bridge between the innate and the acquired immune response.[22,23] Immature APCs are activated, resulting in the upregulation of the major histocompatibility complex (MHC) antigens as well as costimulatory and adhesion molecules, via a nuclear factor κB-dependent pathway.[24] Mature dendritic cells, loaded with antigenic peptides, migrate to draining lymph nodes, where they deliver "signal 1" to naïve T cells through the interaction of peptide-MHC complex with a T cell receptor (TCR). B7-1/B7-2 (CD80/CD86) molecules on the APCs interact with CD28 on T cells, delivering "signal 2", which is critical for cytotoxic T lymphocyte (CTL) cross-priming by activated

APCs. In addition, vector interaction with epithelial cells results in the release of C-X-C chemokines, especially interferon-inducible protein-10, which are potent chemoattractants for activated T lymphocytes and polarizes the reaction towards a Th-1 type response.[25,26]

CTL Response against Ad Vectors

The antigen-specific CTL response plays a major role in limiting transgene expression by eradiation of transduced cells.[27,28] CTLs can be activated in response to both viral antigens and transgene products.[27,29] Although the antibody response to Ad is well characterized as extensively reviewed elsewhere,[19,30] the activation, expansion, as well as the effector mechanisms of cytotoxic CD8 T cells remain unclear.

Identification of Ad CTL epitopes

The interaction between CD8 T cells and the antigen presenting cells, or target cells, requires recognition of the peptide epitopes presented by the class I MHC molecules. Several Ad proteins have been identified as the principal antigens for CTLs generated in humans.[31,32] In mice, two CTL epitopes have been identified from Ad E1A and E1B encoded proteins.[33-35] However, the deletion of the E1 region in most of the Ad vectors restricts the application of the epitopes in the study of Ad specific CTLs.

Previously, CTL epitopes were mapped using large arrays of overlapping synthetic peptides. The large number of protein sequences available for mapping has made this method prohibitively expensive and time-consuming. Recent advances in the development of computer-driven algorithms have offered a significant advantage over other methods of epitope selection since high-throughput screening can be performed using these prediction

programs, followed by confirmatory studies *in vitro* and *in vivo*. CTL epitopes discovered using these tools have been used to develop novel vaccines and therapeutics for the prevention and treatment of infectious diseases and tumors.[36-39]

The most frequently used method to test the predicted peptide is detection of interferon (IFN)-γ by activated CD8 T cells after incubation with the peptide. This is normally done by the IFNγ enzyme-linked immunoSPOT (ELISPOT) assay or intracellular staining of IFNγ. The *in vivo* CTL assay using a green fluorescent dye, 5,6-carboxysuccinimidyl-fluoresceine ester, labeled target cells is a sensitive and specific method to assess the CTL function *in vivo*,[40,41] However, the latter method has not been used in validation of predicted peptides.

The requirement for CD28 costimulation in CD8 T cell activation after primary Ad infection

The role of CD28-B7 co-stimulatory pathway in CD4 T cell activation has been analyzed using *in vivo* models. CD28 knockout (*CD28*$^{-/-}$)mice are deficient in the T cell dependent component of the antibody response and other responses that depend on CD4 T-helper activity,[42] indicating the importance of CD28 signaling in CD4 T cells. In contrast, much less is known about the role of CD28 in activation of CD8 T cells. Goldstein et al. [43] have shown that immobilized MHC class I peptide complexes can activate CD8 T cells from TCR transgenic mice and these CD8 T cells can be stimulated to proliferate and secrete IL-2 independently of the molecular interactions between CD28-B7. Similarly, Wang et al. [44] showed that TCR engagement by peptide/MHC tetramers is sufficient for the activation of naive CD8 T cells and was not inhibited in the absence of anti-CD28. Shahinian et al. [42] first showed that CD28 mutant mice exhibited low IL-2 production, low immu-

noglubilin (Ig) production, but could produce CTLs in a delayed-type hypersensitivity after infection with lymphocytic choriomeningitis virus. These results suggested that CD28 is not required for all T cell responses *in vivo*, and that alternative co-stimulatory pathways may exist.[42] In contrast, Dyson et al. [45] showed that treatment of HY-specific TCR transgenic mice with soluble anti-B7.2 antibody led to an inhibition of CD8 T cell activation. Suresh et al. [46] showed that the primary CD8 T cell response to both dominant and subdominant lymphocytic choriomeningitis virus CTL epitopes was 2 – 3 times lower in *CD28*$^{-/-}$ mice. Similar results were found in vesicular stomatitis virus infected *CD28*$^{-/-}$ mice.[47] These results indicate that *CD28*$^{-/-}$ mice may exhibit either no decrease or a two-fold decrease in the number of CTLs. It is not clear if this decrease is due to lack of CD28 expression by CD4 T cells or by CD8 T cells. It also remains unknown whether there is a decrease in the number or the effector function, or both, of CTLs in the absence of CD28-B7 interaction.

These questions become especially important as related to viral delivery of gene therapy. Realizing the potential role of CD28 signaling in T cell activation, efforts have been taken to block CD28-B7 interaction using cytotoxic T lymphocyte-associated protein 4 (CTLA4)-Ig, a construct containing the extracellular domain of CTLA-4, which has been fused to the Fc portion of IgG to promote solubility. Blocking of CD28-B7 by CTLA4-Ig has resulted in prolonged transgene expression in the context of Ad gene therapy.[48-51] In these experiments, a reduced T cell dependent antibody response was detected, but the impact on CD8 T cell activation was not determined. One factor that has limited these analyses is the lack of an MHC class I/peptide tetramers to measure the number of specific CTLs generated in response to Ad administration.

The requirement for CD4 T cell help in the primary CD8 T cell response to Ad infection

The importance of CD4 T cell help for the primary CD8 T cell responses remains controversial. After Ad administration to CD4 deficient mice, there was a prolonged transgene expression despite the infiltration of CD8 T cells, indicating that CD4 T cells may be necessary for a fully competent CTL response.[52,53] Further studies, however, have shown that the primary CTL response to infectious agents is often independent of T helper cells,[54-56] and it was hypothesized that recognition of microbial products by Toll-like receptors can directly activate APCs and thus bypasses the need for CD4 T cell help.[55] A recent study showed contrasting results that support the essential role of CD4 help in the primary CD8 T cell response.[57] The presence of CD4 T cells during the priming phase is critical for generating a fully functional CD8 memory response,[58] and direct CD40-CD40 ligand interaction between CD4 and CD8 cells might be involved in this process.[59]

Effector mechanisms of CTL response to Ad infection

In response to virus infection, activated CD8 T cells produce perforin, Fas ligand (FasL), as well as IFN-γ and TNFα to clear virus infected cells.[60,61] However, the primary mediators of CTL function after virus infection are believed to be FasL and perforin/granzymes.[62-65] Although the original findings by Nagata et al. [66] indicated the importance of FasL in hepatotoxic injury, the actual role of FasL in the CTL response in the liver is still unclear. One major concern has been the reliance on FasL knockout *gld* mice or Fas-mutant *lpr* mice, which are known to exhibit autoimmune disease, low production of IL-2,[67] and do not produce a normal CTL response.[68] Therefore, a likely mechanism for the decreased CTL response in FasL mutant *gld* mice may be due to a decrease in number

rather than a decrease in function of the CTLs. In contrast, the role of perforin is less controversial, since perforin knockout mice have normal numbers of CTLs after virus infection as determined by virus peptide/MHC tetramers, but exhibit decreased CTL activity and impaired viral clearance.[63-65]

Previous studies have shown that Ad vector-infected hepatocyte death is mediated by Fas and TNF receptor, but not perforin.[69,70] However, direct analysis of the specific CTL response to Ad in these studies has not been possible due to a lack of an antigen specific tetramer to measure the precise number of specific CTLs. Furthermore, direct analysis of CTL function after Ad infection has not been possible due to a lack of a specific CTL epitope to enable a specific and sensitive analysis of direct killing of target cells by potential CTLs.

Overview of the dissertation work

Based on our current understanding, CD8 CTL response induced by Ad gene therapy is the major cause for transient transgene expression and cytotoxicity of the infected tissues. Although much effort has been employed in developing immunomodulation strategies, these problems still limit the successful application of Ad vectors. Considering the specific nature of the CTL response, it is important to analyze Ad specific CTL response with advanced specific approaches.

The current work focused on three main aspects of Ad CTL response:

1. Identification of Ad CTL epitopes that can be used in the analysis of CTL response in the context of Ad gene therapy.

2. Presentation of a comprehensive analysis of Ad CTL response with two specific methods, MHC I/peptide tetramer staining and *in vivo* CTL assay.

3. Determination of the role of major factors that regulate Ad CTL activation and effector functions.

Key findings from these studies are:

1. Ad fiber contains an H2-D^b-restricted CTL epitope, which can be used to detect Ad specific CTL response *in vivo*.

2. The *in vivo* CTL function correlates with the number of CTLs generated after a primary Ad infection.

3. Ad specific CTLs are recruited into the infected tissues, such as liver and lung, at high frequencies.

4. The peak of CTL response occurs after the majority of virus has been cleared.

5. The CD28 signaling pathway in CD8 T cells is necessary for CD8 T cell activation and full function.

6. Perforin-mediated pathway is the major mechanism for target cell lysis induced by Ad specific CTLs and Fas-pathway plays a synergistic role with perforin.

The details that have been published or communicated are presented in the following dissertation sections and in an overall discussion of the entire project given at the end.

ADENOVIRUS FIBER CONTAINS A MOUSE H2-DB-RESTRICTED EPITOPE PEPTIDE

by

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Submitted to *Molecular Therapy*

Format adapted for dissertation

Abstract

The adenovirus (Ad) capsid is the major target of both humoral and cellular immune response to E1-deleted Ad vectors. However, analysis of the Ad capsid specific cytotoxic T lymphocyte (CTL) response has not been possible due to a lack of specific methods to detect Ad vector-induced CTLs. To identify Ad specific CTL epitopes derived from viral capsid proteins, we utilized two computer-driven algorithms, BIMAS and SYFPEITHI, to predict Ad fiber, penton, and hexon peptides that bind to the mouse class I MHC. The ELISPOT results showed that a fiber peptide (*391 VGNKNNDKL*) can stimulate Ad5-primed spleen cells to secrete IFNγ. The number of spot-forming cells that respond to this peptide is comparable to that of the known immunodomiant E1B peptide (*192 VNIRNCCYI*). As confirmed by an *in vivo* CTL assay, the fiber peptide-pulsed target cells were completely deleted in mice infected with the wild-type Ad5. E1-deleted, replication-deficient Ad vector also induced an anti-fiber CTL response, but the response was weaker than that induced by wild-type Ad5, as evidenced by the decreased target cell killing in the *in vivo* CTL assay. Using this epitope peptide, we were able to show that CD28 signaling pathway is necessary for the primary CTL response and perforin, rather than FasL, is the major effector molecule in mediating target cell lysis. The finding of the new CTL epitope from the capsid protein provides a powerful tool for assessing the CTL response induced by any Ad5-derived vectors.

Introduction

Adenovirus (Ad) has been used as a gene delivery vector in gene therapy since early 1990s.[1] Many unique features make it well suited for gene delivery, including the

ability to grow recombinant viruses to high titers, capacity for large DNA inserts, and high-efficiency of transduction of both quiescent and actively dividing cells, usually without incorporation of viral DNA into the host cell genome.[2] However, the successful application of Ad vectors has been greatly limited by the stimulation of host immune response.[3]

The initial response to Ad vectors administered intravascularly occurs within minutes, in the absence of viral gene expression and is attributed to the innate response.[4-6] The vast majority of Ad is localized to the liver.[7] During the first two days after a primary infection, more than 90% of the virus is eliminated through a nonspecific immune response. In spite of the clearance of the majority of virus, administration of high titer of Ad results in more than 95% of hepatocytes being transduced by Ad vectors.[8] In immunocompetent mice, transgene expression rapidly declines to baseline levels by 2 to 3 weeks after infection.[9,10]

It has been well-established that the antigen specific cytotoxic T lymphocyte (CTL) response induced by Ad infection is the major reason for the transient transgene expression.[11,12] However, the regulatory mechanisms underlying CTL response against Ad infection remains unclear. As a result, there are very few immunosuppressive methods developed that can specifically reduce the CTL response to Ad vector transduced cells. These limitations are partly due to a lack of specific methods to assess the CTL response.

Both the activation and cytotoxic function of CTLs require recognition of the peptide epitopes presented by the class I MHC molecules. Thus, the CTL epitope is an important component to the study of specific $CD8^+$ CTLs. Several Ad proteins have been identified as the principal antigens for CTLs generated in humans.[13,14] In mice, two CTL

epitopes have been identified from Ad E1A and E1B encoded proteins,[15-17] both of which are H2-D^b molecule restricted. In most Ad-derived vectors, the E1 region is deleted or substituted by an expression cassette,[1] which restricts the application of the E1A or E1B epitope in the study of Ad specific CTLs.

To identify class I MHC restricted CTL epitopes from the Ad structural proteins, which comprise the hexon, penton, and fiber, we employed two computer-driven algorithms, BIMAS [18] and SYPFEITHI,[19] to screen potential CTL epitopes derived from these proteins. By an IFNγ ELISPOT assay and an *in vivo* CTL assay, we identified an H2-D^b restricted CTL epitope derived from Ad fiber protein. We also justified the immunogenecity of this fiber peptide in the context of E1-deleted, replication-deficient Ad vectors.

Classification of the major factors that regulate the CTL generation and effector function would help to define the targets for specific immunomodulation, thus prolonging the transgene expression. With the newly identified CTL epitope, we assessed the requirement for CD28 signaling pathway in CTL response to Ad infection. The relative role of the two major effector molecules, perforin and FasL, in mediating target cell lysis was also determined with the fiber epitope.

Materials and Methods

Peptide prediction and synthesis

The protein sequences of Ad hexon, penton, fiber, E1A, and E1B were obtained from GenBank and analyzed for $H2^b$ binding motifs using two computer-driven algorithms, BIMAS [18]and SYPFEITHI.[19] Peptide sequences that were given high binding

scores in both prediction programs were chosen. Peptides were synthesized in the University of Alabama at Birmingham Protein Sequencing and Synthesis Core Facility and were > 98% pure as indicated by analytical high-performance liquid chromatography. Peptides were dissolved in 100% DMSO at a concentration of 10 mM and stored at -20°C until use.

Mice

Female C57BL/6 (B6; H-2^b), B6.CD28$^{-/-}$ (*CD28*$^{-/-}$; H-2^b), B6.perforin$^{-/-}$ (*prf*$^{-/-}$; H-2^b), B6.FasL$^{-/-}$ (*gld*; H-2^b), and DBA/2 (H-2^d) mice at 6-8 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Groups of at least 3-5 mice were analyzed in each experiment. All animal protocols were approved by the Institutional Animal Care and Use Committee at University of Alabama at Birmingham.

Adenovirus and infection

Wild-type Ad type 5 was obtained from the American Tissue Culture Collection (Rockville, MD). The recombinant Ad expressing *LacZ* gene with the cytomegalovirus (CMV) promoter (AdLacZ) was generated by *in vitro* recombination of two plasmids as described previously.[20] Briefly, the PCR-amplified *Escherichia coli lacZ* gene from pcDNA3His (Invitrogen, San Diego, CA) was inserted directionally into the adenoviral shuttle vector, pCA13 (Microbix, Biosystems, Toronto, Canada), resulting in pCA13LacZ, which was then cotransfected with pJM17 (Microbix) into 293 cells using the Lipofectin method (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's manual. The recombinant virus was picked after staining with 5-bromo-4-chloro-

3-indolyl-b-D-galactopyranoside (X-Gal), and subjected to three rounds of plaque purification. Both wild-type Ad5 and AdLacZ were propagated in the HEK293 cells as described previously.[21] Infected cells were harvested and lysed by 3 freeze-thaw cycles to release the virus, which was then purified through two cesium chloride gradients. The purified wild-type Ad5 or AdLacZ was then titrated by the plaque assay,[22] aliquoted, and stored at -80°C until used. Mice were administered either with wild-type Ad5 at $8x10^8$ infection units (i.u.) per mouse or with AdLacZ at 1 $x10^{11}$ i.u. per mouse through tail vein injection.

Cell preparation

Single-cell suspensions of spleen were prepared as described previously.[21] Briefly, spleens were disrupted using wire mesh screens, and erythrocytes were lysed by treatment with buffered-NH_4Cl (8.29 g of NH_4Cl, 1.0 g of $KHCO_3$, and 0.037 g of EDTA/liter). The cells were washed by phosphate buffer solution (PBS) containing 2% (w/v) fetal bovine serum (FBS) and 0.2% (w/v) NaN_3 before staining with fluorescent antibodies.

IFNγ ELISPOT

To determine the frequency of peptide epitope specific T cells in the spleen, IFNγ ELISPOT assay was performed. 96-well polyvinylidene difluoride-backed plates (Millipore, Bedford, MA) were coated overnight with 5 μg/ml anti-mouse IFN-γ mAb at 4°C, washed, and then blocked with complete medium. Cells from spleens of naïve or immunized mice were washed and adjusted to a concentration of 2 x 10^6/ml in complete medium, then added to the microtiter wells together with either 10 μg/ml predicted peptide

or the peptide control, human influenza virus M1 peptide (*58 GILGFVFTL*), to give a final volume of 200 µl containing 2 x 10^5 cells. After 3 days incubation, cells were washed three times with PBS/0.05% Tween 20 before overnight incubation at 4°C with 1 µg/ml biotinylated anti-mouse IFN-γ mAb in PBS/10% FBS. Plates were washed three times with PBS/0.05% Tween 20, before adding 40 ng of HRP-conjugated goat anti-biotin Ab (Vector Laboratories, Burlingame, CA), in 100 µl of PBS/10% FBS, to each well. Plates were incubated at room temperature for 1 hr, and washed with PBS/0.05% Tween 20 and then with PBS alone before developing spots using 3-amino-9-ethylcarbazole. Plates were read on a CTL automatic ELISPOT reader (CTL, Cleveland, OH) and analyzed using Immunospot 3.1 software (CTL). All results were repeated in triplicate.

In vivo CTL assay

For target cells, 10^7 spleen cells from naïve B6 mice were incubated with either 2 µM (high dose) 5,6-carboxysuccinimidylfluoresceine ester (CFSE; $CFSE^{Hi}$) or 0.2 µM (low dose) CFSE ($CFSE^{Lo}$) in PBS, at 25°C for 12 min. CFSE labeling was then quenched by the addition of FBS to a final concentration of 20% (v/v). Cells were washed with RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 50 µM β-mercaptoethanol and 25mM HEPES. The $CFSE^{hi}$ cells were then incubated in the presence of 1 µM synthesized peptides at 37°C for 1 hr, whereas $CFSE^{lo}$ cells were incubated in medium only. Human influenza virus M1 peptide (*58 GILGFVFTL*) was used as control to pulse a separate population of $CFSE^{hi}$ cells. After labeling and peptide pulsing, both populations of target cells were washed and mixed together in 0°C PBS such that recipient mice received 10^7 cells of each population in a sin-

gle *i.v.* injection. As a control, naïve B6 mice were adoptively transferred with an identical number of target cells. Recipient mice were sacrificed 6 hr following cell transfer, spleen cells were prepared as described above and then analyzed by flow cytometry. Alternatively, blood samples from the recipient mice were drawn from the retro-orbital sinus at 6 hr or 48 hr following cell transfer and CFSE labeled target cells were analyzed by flow cytometry. Percent specific lysis was determined using the following formulas: [23]

The ratio of recovery of nonpeptide-treated control spleen cells to peptide-sensitized spleen cells = (percentage of $CFSE^{lo}$ cells)/(percentage of $CFSE^{hi}$ cells).

The percent specific lysis (%) = 100 x (1 – (ratio of cells recovered from naive mice/ratio of cells recovered from infected mice)).

Statistical analyses

Significant differences between mean values were determined by Student's *t* test. $p < 0.05$ was considered statistically significant.

Results

Prediction of H2-D^b-restricted T-cell epitopes derived from Ad capsid proteins

To identify peptides derived from Ad capsid proteins that could potentially bind to H2-D^b, the amino acid sequences of hexon, penton, and fiber were analyzed by BIMAS and SYFPEITHI, and only nonameric sequences were selected. BIMAS is a computer program designed to predict MHC-binding peptides on the basis of an estimation of the half-time dissociation of the MHC-peptide complex.[19] SYFPEITHI is a matrix-based MHC-binding prediction program. In contrast to BIMAS, only naturally occurring MHC

ligands are used to generate the matrices in the SYFPEITHI database.[19] Only those peptides which were predicted to bind to mouse $H2^b$ molecules with the top 4 scores in both BIMAS and SYFPEITHI were chosen for further analysis. The results are summarized in Table 1. As a test of the efficiency of the prediction programs, the prediction scores for two known H2-D^b-restricted peptides from Ad E1A and E1B proteins,[15-17] respectively, were also listed (Table 1).

Table 1 Prediction of H2-D^b binding peptides

	Peptide		BIMAS		SYFPETHI	
Protein	Position	Sequence	Score	Ranking	Score	Ranking
Hexon	567	FAIKNLLLL	1235.0	1	31	1
	418	GGVINTETL	780.0	2	28	2
	730	SWPGNDRLL	660.0	3	26	3
Penton	253	SRLSNLLGI	792.0	1	27	1
Fiber	391	VGNKNNDKL	343.0	2	26	1
	465	GVLLNNSFL	600.0	1	23	4
E1B	192	VNIRNCCYI	660.0	1	26	1
E1A	234	SGPSNTPPEI	8.6	4	30	1

Analysis of immunogenecities of the predicted H2-D^b-restricted epitopes by IFN-γ ELISPOT assay

To test and validate the predicted MHC-binding peptides, we first used an IFN-γ ELISPOT assay. Spleen cells isolated from B6 mice, which had been immunized with wild-type Ad5 8 days earlier were analyzed using an IFNγ ELISPOT assay to detect CD8 T cells that specifically responded to the peptides. The strongest responses were induced by the fiber peptide (*391 VGNKNNDKL*) and the known E1B peptide (*192 VNIRNCCYI*) (Figure 1A). The other peptides induced an equivalent response as did the control peptide, M1 flu (*58 GILGFVFTL*) (Figure 1A and data not shown). The frequencies of spot-

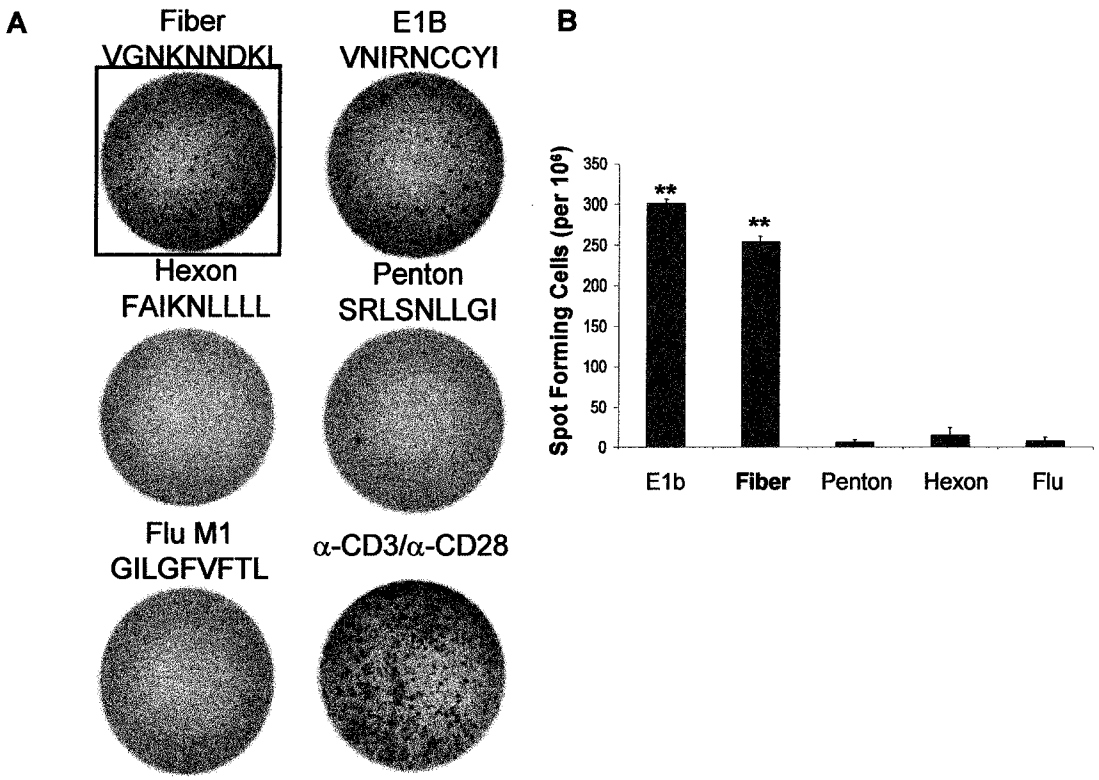

Figure 1 *Fiber peptide (391 VGNKNNDKL) stimulated Ad-primed spleen cells secrete IFNγ. B6 mice were intravenously injected with $8x10^8$ i.u. of wild-type Ad5 and analyzed 8 days later. Mononuclear cells from spleens of B6 mice were isolated and IFNγ ELISPOT assay were performed as described in Materials and Methods (A). The number of spot-forming cells (SFC) per 10^6 cells was shown (B). The results are representative of 3 independent experiments. Data shown are the mean ± s.e.m. of SFC/10^6 MNCs from 3-5 mice each group. ** $p < 0.001$ compared to flu M1 peptide.*

forming cells to individual peptide were compared (Figure 1B). There were 250±17 spot-forming cells per 10^6 spleen cells after incubation with fiber peptide (*391 VGNKNNDKL*), which is comparable to 298±9 spot-forming cells per 10^6 spleen cells in response to E1B peptide (*192 VNIRNCCYI*) (Figure 1B).

In vivo killing of predicted peptides pulsed target cells in immunized B6 mice

To further confirm that this fiber epitope can serve as a specific target for CTLs generated in Ad5 immunized mice, an *in vivo* CTL assay was performed. Spleen cells from naïve B6 mice were either pulsed with the predicted peptide and labeled with a high dose of CFSE ($CFSE^{hi}$) or unpulsed and labeled with a low dose of CFSE ($CFSE^{lo}$). The spleen cells (10^7/mouse) were then injected intravenously into B6 mice that had been infected with Ad5 8 days earlier. The spleens of the recipient mice were recovered 6 hours after injection, a single-cell suspension was prepared, and the relative recoveries of $CFSE^{lo}$ (unpulsed) cells and $CFSE^{hi}$ (peptide-pulsed) cells were compared. In comparison with unpulsed $CFSE^{lo}$ cells, 98% of the fiber peptide (*391 VGNKNNDKL*)-sensitized $CFSE^{hi}$ target cells were deleted *in vivo* during this 6 hr period (Figure 2). There was no specific lysis of hexon or penton peptides pulsed target cells (Figure 2 and data not shown). Similarly, H2-D^b specific lysis was confirmed by the observation that there was no specific lysis of fiber peptide (*391 VGNKNNDKL*) pulsed syngenic target cells recovered from D2 (H-2^d) mice.

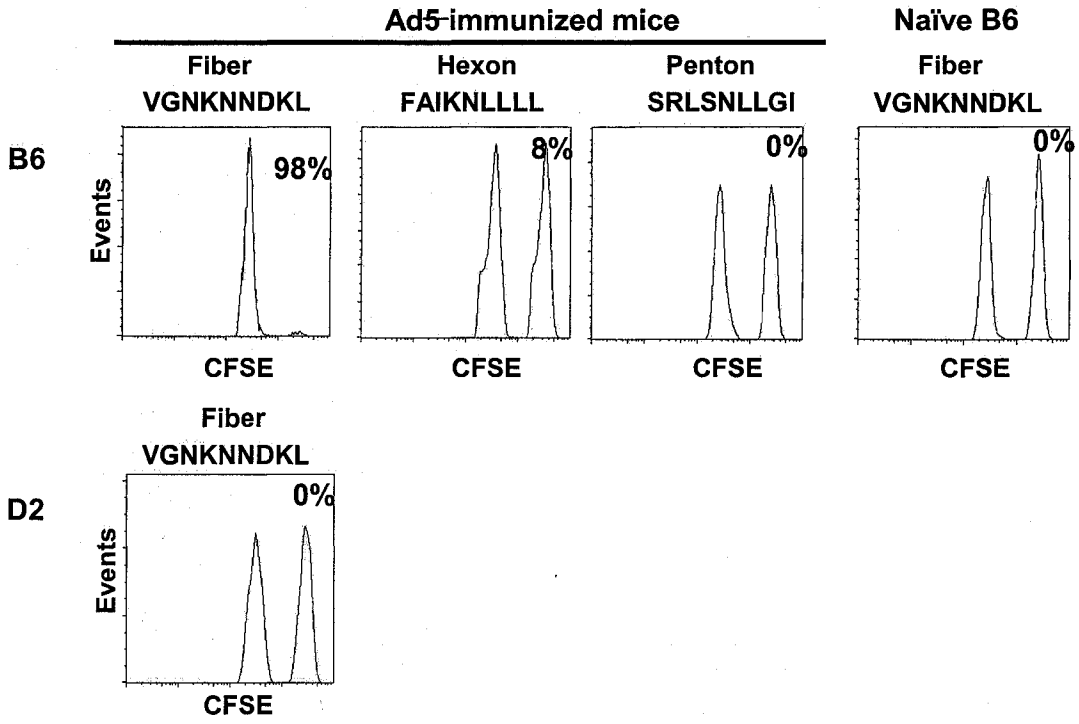

Figure 2 *Fiber peptide (391 VGNKNNDKL) pulsed target cells were specifically deleted in B6 mice after Ad infection. B6 (H-2^b) mice and D2 (H-2^d) mice were intravenously injected with 8x10^8 i.u. of wild-type Ad5 and analyzed 8 days later. Target cells from the spleen of naïve B6 mice were labeled with a high concentration of CFSE (CFSE^{hi}) and then pulsed either with E1Bp peptide or irrelevant flu peptide M1. Peptide-pulsed CFSE^{hi} cells were cotransferred intravenously into Ad-primed B6 mice with unpulsed target cells that were labeled with a low concentration of CFSE (CFSE^{lo}). Target cells from the spleen of naïve D2 mice were treated similarly and transferred into Ad-primed D2 mice. Six hours later, single cell suspensions of the spleen were analyzed by flow cytometry (100,000 events) for CFSE fluorescence. The numbers in the histograms represent the percentage of target cell killed. The results are representative of 5 independent experiments.*

T cell response to fiber peptide after infection of replication-deficient Ad vector

To reduce the immune response for Ad gene therapy, the Ad-derived vectors are deleted of the E1 gene which is necessary for viral gene expression. It remains controversial whether CTLs were formed against the incoming virus or against *de novo* synthesized Ad Proteins.[24,25] To assess the CTL response to the fiber peptide in mice immunized with replication-deficient Ad vector, we injected B6 mice with a recombinant Ad vector expressing the LacZ gene (AdLacZ) and then determined the fiber peptide specific CTL response by an *in vivo* CTL assay. Target cells pulsed with the fiber peptide (*391 VGNKNNDKL*) were adoptively transferred to AdLacZ immunized B6 mice. The mononuclear cells from the peripheral blood (PBMC) of the recipient mice were recovered 6 hr after injection and the relative recoveries of $CFSE^{lo}$ (unpulsed) cells and $CFSE^{hi}$ (peptide-pulsed) cells were compared (Figure 3; left panels). There was no specific killing of peptide pulsed cells. However, when the PBMCs from the same mice were analyzed 48 hr after target cell transfer, 92% of the fiber peptide (*391 VGNKNNDKL*) pulsed target cells were deleted (Figure 3; right panels). These results indicated that the E1-deleted Ad vector induced a weak CTL response to the viral capsid antigen compared with wild-type Ad and that the fiber peptide can be used to examine the anti-Ad specific CTL response in the context of replication-deficient vectors.

Characterization of CTL response in immunodeficient mice with fiber peptide

Due to the lack of a specific epitope to assess the CTL response to Ad gene therapy, it has not been possible to clearly dissect the immune mechanisms that contribute to the generation and effector mechanisms. In previous studies, nonspecific or indirect methods

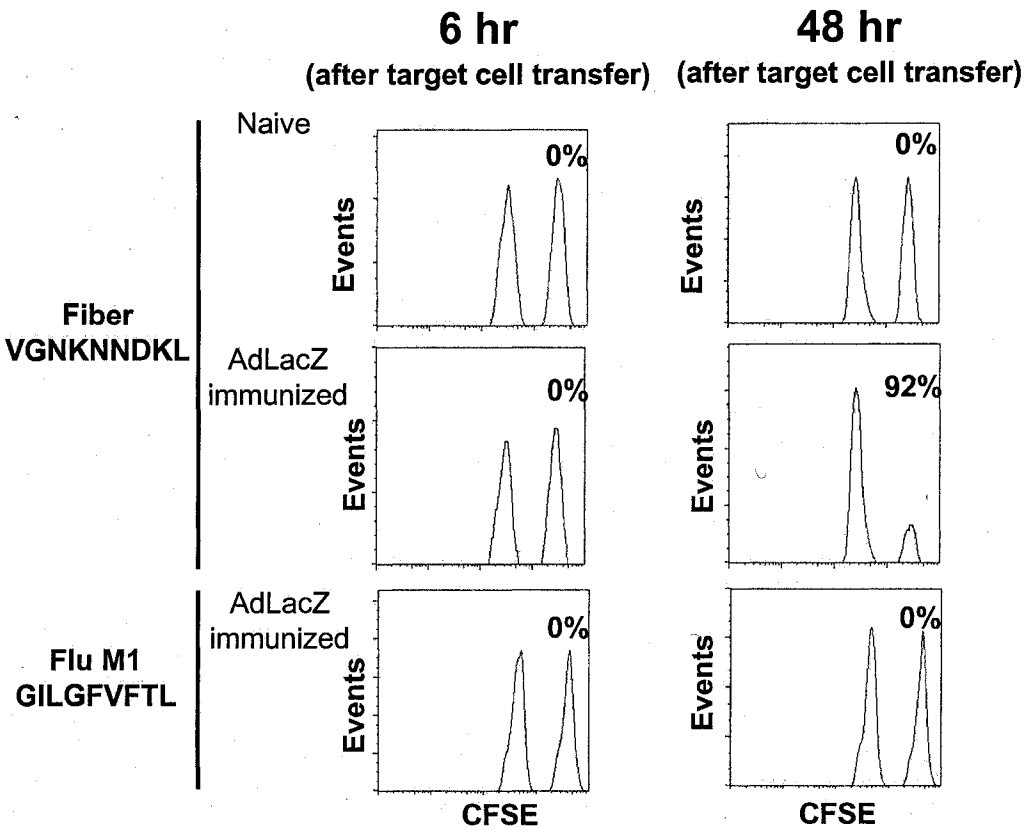

Figure 3 *E1-deleted, replication-deficient AdLacZ induced a weak fiber peptide specific CTL response. B6 mice were i.v. injected with $8x10^8$ i.u. of wild-type Ad5 or 10^{11} i.u. of AdLacZ. Eight days later, spleen cells isolated from naïve B6 mice were pulsed either with fiber peptide or irrelevant flu peptide M1 and then adoptively transferred to the immunized recipient mice. Blood samples from the recipient mice were drawn from the retro-orbital sinus at 6 hr or 48 hr following cell transfer and CFSE labeled target cells were analyzed by flow cytometry. The numbers in the histograms represent the percentage of target cell killed. The results are representative of 5 independent experiments.*

were employed in analysis of T cell response in immunodeficient gene knockout mice,[12,26,27] such as the Cr^{51} based *in vitro* CTL assay, clearance of vector or transgene, or liver enzymes. Thus, it is not possible to correlate the observed prolonged gene expression to the reduced CTL response in these immunodeficient mice. To examine the Ad-specific CTL response in immunodeficient mice, we immunized *gld* mice, *prf*$^{-/-}$ mice and CD28 *CD28*$^{-/-}$ mice with Ad5 and performed an *in vivo* CTL assay using the fiber peptide (*391 VGNKNNDKL*) pulsed target cells. There was a significant decrease in target cell killing in *CD28*$^{-/-}$ mice (14% vs 98% in wild-type B6 mice) 8 days after Ad infection. In *prf*$^{-/-}$ mice, 63% of target cells were deleted indicating a 35% decrease compared to wild-type B6 mice. There was no significant decrease in target cell killing when FasL is deficient (Figure 4). These results indicate that the CD28 signaling pathway, by delivery of costimulatory signals to CD8 T cells, is necessary for anti-Ad CTL response, but perforin, not Fas, is the major pathway in mediating target cell lysis.

Discussion

The role of the cytotoxic T lymphocyte response to Ad vectors in elimination of transduced cells, thus limiting transgene expression has been well established.[10,12] However, until now, the analysis of the CTL response could only be explored using indirect approaches, i.e., the duration of transgene expression, *in vitro* CTL assay, or hepatic toxicity assayed by serum metabolic enzymes.[27-30] This has made it difficult to investigate the Ad CTL response and the mechanisms underlying its generation and effector functions. In the present study, we identified a mouse H2-D^b-restricted CTL epitope contained in the fiber protein of human Ad5. CTLs specific for this epitope were generated in B6

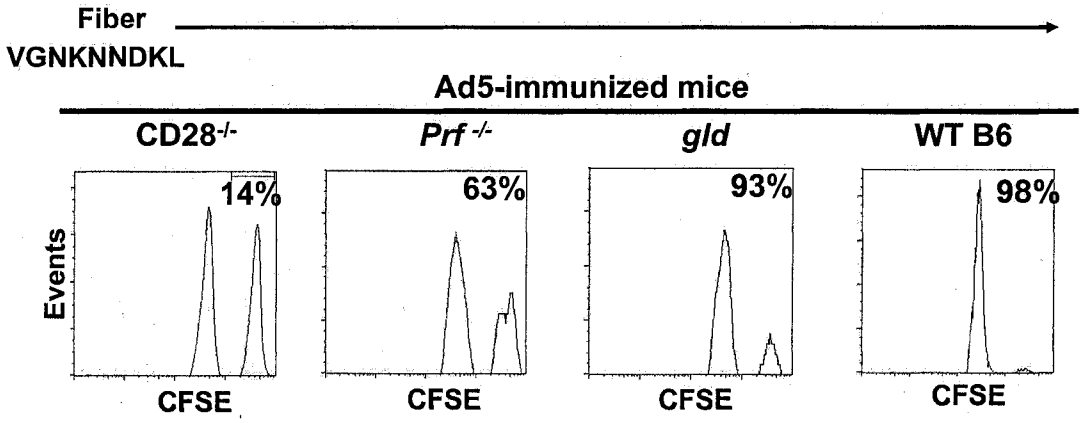

Figure 4 *Reduced target cells lysis in immunodeficient mice. CD28$^{-/-}$ mice, prf$^{-/-}$ mice and gld mice, and WT B6 mice were i.v. injected with 8x10^{8} i.u. of wild-type Ad5. Eight days later, spleen cells isolated from naïve B6 mice were pulsed with fiber peptide and then adoptively transferred to the immunized recipient mice. Six hours later, spleen cells recovered from the recipient mice were analyzed by flow cytometry for CFSE fluorescence. The numbers in the histograms represent the percentage of target cell killed. The results are representative of 3-5 independent experiments.*

mice either infected with replicative, wild-type Ad, or E1-deleted, replication-deficient Ad vectors.

Several computational methods are now available for analysis of binding of peptides to MHC.[31] In this study, we have used two programs, BIMAS and SYFPEITHI, which are based on different algorithms to predict CTL epitopes from Ad capsid protein. BIMAS has been developed using experimental data of half time of dissociation of β2 microglobulin from MHC/peptide complex since β2 micro globulin dissociates from the complex when the peptide is released.[18] In contrast, the SYFPEITHI scoring system values the amino acids in specific sequence positions that are conserved among different epitopes, and sums up these individual values to assess the capacity of the peptide to bind to the MHC molecule.[19] These MHC-binding peptide prediction programs have been successfully employed in identification of HLA-A2 restricted T cell epitopes fom HIV [14,32] and the tumor-associated protein SSX2.[33] The advantages of the present method for identification of CD8 T cell epitopes over traditional vaccinia virus expressing overlapping peptides or peptide library is that the restriction MHC class I molecule could be determined readily, which is important for subsequent tetrameric MHC class I/peptide complex development.[34]

To test and validate these predicted peptides, we employed two sensitive methods, IFN-γ ELISPOT assay and *in vivo* CTL assay. In the ELISPOT assay, the activated CD8 T cells were detected via the detection of IFN-γ secretion by these cells after incubation with the specific antigenic peptides.[35] In the *in vivo* CTL assay, the generation of CTLs was detected by the killing of target cells that artificially present specific CTL epitopes. In this assay, bystander toxicity is minimized since killing of peptide-pulsed target cells is

compared to that of unpulsed target cells. Another advantage of the *in vivo* CTL assay is that the killing efficiency can be determined by recovery of target cells from the spleen or blood. In the present experiments, fiber peptide (*391 VGNKNNDKL*) was verified by both approaches. First, the fiber peptide could specifically stimulate IFN-γ production by the spleen cells from Ad5-immunized mice. The fiber peptide specific CTLs were next testified by the killing of fiber peptide pulsed target cells in Ad5-immunized B6 mice.

The replication-deficient Ad vectors used in gene therapy lack the E1 gene, which encodes proteins necessary for the expression of other early and late genes.[36] It remains controversial whether the replication-deficient Ad vectors induce an attenuated CTL response to infected cells.[24,25] Kafri et al. showed that CTL response to adenoviral vector infected cells does not require *de novo* viral gene expression.[24] In contrast, Jooss et al showed that *de novo* synthesis of proteins is essential for presentation of epitopes.[25] The discrepancy might be explained by the use of UV inactivated E1-deleted Ad vectors and the nonspecific measurement of the infiltration of $CD4^+$ and $CD8^+$ cells. The UV treatment results in the crosslinking of the viral DNA and consequently prevents transcription, gene expression, and replication from these vectors.[37] However, it is very likely that the UV treatment only ablates CTL epitopes from transgene expression but leaves viral capsid epitopes intact. T cell infiltration is now believed to result from a nonspecific inflammatory response. In the present experiment, we compared the CTL response induced by the wild-type Ad5 and an E1-deleted, replication-deficient Ad vector with the specific *in vivo* CTL assay. As determined by the standard procedure of *in vivo* CTL assay, no specific killing occurred during the 6 hr period in replication-deficient Ad vector infected

mice. However, when the assay time was prolonged to 48 h, most of the target cells were deleted.

In order to prolong the transgene expression delivered by Ad vectors, strategies have been proposed to suppress the immune response. However, there are very few immunosuppressive approaches that can specifically reduce the CTL response against Ad vector transduced cells. CTLA-4Ig is a chimeric protein composed of the heavy chain constant region of the human immunoglobulin bound to the extracellular binding domain of CTLA-4.[38] The recombinant fusion protein is a homologue of the T cell costimulatory receptor CD28. CTLA-4Ig competes effectively for binding to the CD28 ligand, B7, on antigen-presenting cells, inhibiting costimulatory signals between APC and T cells, leading to T cell anergy and peripheral tolerance.[38] Recombinant Ad vectors expressing CTLA-4Ig have been extensively studied aiming at inhibiting T cell response to Ad gene therapy or transplants.[39-43] Although the reduction of antibody response has been shown, the effect on CTL response remains unclear.[39,44]

The relative role of the major effector molecules, perforin, FasL, and TNFα is also controversial. Yang et al. has reported that clearance of Ad vector by specific CTLs is mediated by perforin/granzyme pathway.[45] In contrast, Kafrouni et al. showed a resistance of Ad infected hepatocytes to cytotoxicity mediated by perforin-dependent mechanisms, and these investigators proposed that Fas/FasL-dependent, cell-mediated cytotoxicity is the major pathway for CTL-mediated killing of Ad infected.[27] In the present study, we have used the fiber epitope and an *in vivo* CTL assay to assess the Ad specific CTL response in gene knockout mice. We found that CD28 deficiency resulted in a significant decrease in target cell killing and *prf*$^{-/-}$ mice exhibited a moderate defect in CTL

function, whereas FasL knockout *gld* mice showed normal CTL function. These results provide the rationale for the immunosuppressive approaches aimed at blocking the CD28 signaling pathway to ablate Ad specific CTL response. Our studies also demonstrate the use of the fiber epitope in determining the mechanisms underlying CTL generation and function, and it will help to define new target for immunomodulation.

Acknowledgements

We thank Carol Humber and Jennifer Allen for excellent secretarial work. This work was supported in part by National Institutes of Health Grants RO1-AI-42900 (to J.D. Mountz) and RO1-CA-86881 (to D.T.Curiel), by a VA Career Development Award, and a Merit Review Award (to J.D.Mountz).

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PRIMARY ADENOVIRUS SPECIFIC CYTOTOXIC T LYMPHOCYTE RESPONSE OCCURS AFTER VIRAL CLEARANCE AND LIVER ENZYME ELEVATION

by

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Gene Therapy, 2005, 12: 1079-1088

Abstract

The virus-specific cytotoxic T lymphocyte (CTL) response is a major obstacle to effective delivery of adenovirus gene therapy. However, its relative role in viral clearance, transgene elimination and hepatotoxicity remains unclear. In this paper, we present an analysis of viral clearance and liver toxicity in relation to the induction of the virus-specific CD8 T cell response revealed by an MHC class I tetramer. A surprisingly high number of tetramer$^+$ CD8 T cells were found in the liver and lung and reached peak values at days 8 and 10, respectively, post infection. Nearly 100% of these tetramer$^+$ CD8 T cells expressed high levels of granzyme B and IFNγ. Remarkably, liver viral load and liver enzyme elevation peaked early, at days 2 and 4, respectively, post infection, before the specific CTL response was detectable. After generation of CTLs, there was only minimal liver damage or further decrease in virus titer. These results indicated that the primary peak response of tetramer$^+$ CTLs does not correlate with the elimination of adenovirus or liver cytotoxic response.

Introduction

Clearance of adenovirus (Ad) and subsequent elimination of transgene is a major limitation of adenovirus gene therapy.[1,2] These events occur during the first few weeks after Ad administration.[3] The immune response to Ad consists first of an innate immune response followed by a specific CD8$^+$ CTL response and production of neutralizing antibody.[4] The antigen-specific cytotoxic T lymphocyte (CTL) response is thought to have a major role in the elimination of cells transfected with the Ad vectors that express the transgene.[5,6] However, the relative contribution of the specific CTL response and innate

immune responses to the clearance of Ad and associated transgenes has been a subject of debate.

There is clear evidence that for some viruses such as vaccinia virus and lymphocytic choriomeningitis virus (LCMV), the $CD8^+$ CTL response is necessary for viral clearance following primary infection.[7,8] However, the evidence for CTL involvement for clearance of adenovirus during a primary response is less certain. In fact, several previous studies showed that the adenovirus genome is rapidly cleared within 4 – 6 days after inoculation, well before the generation of potent CTLs.[9-11] The elevation of liver enzymes occurs as a result of the acute response and peaks by day 4 after Ad administration.[12] Moreover, we have previously shown that administration of soluble TNF receptor greatly inhibits transgene clearance and the elevation of liver.[13] Thus, unlike the critical role of the CTL response after challenge with a highly replicative virus, the role of the CTL response after Ad gene therapy is unclear.

To detect the numbers and functions of adenovirus-specific CTLs that might contribute to the clearance of adenovirus and associated liver enzyme elevation, we developed an Ad5 specific tetramer that contains the epitope peptide, E1Bp (*192 VNIRNCCYI*),[14-16] in the context of class I MHC, D^b. We then quantified the primary $CD8^+$ T cell response during the two week time period following Ad administration. No Ad-specific $CD8^+$ T cells were detectable before day 5, low levels were present at day 6, and peak levels were observed at day 8 after infection. In contrast, liver viral load and serum alanine transaminase (ALT) levels peaked at day 2 and 4, respectively, and returned to baseline values at day 6. There was no further clearance of Ad virus or liver cytotoxicity during or after the peak of the CTL response. These results indicate that the Ad-specific

CTLs do not contribute significantly to the clearance of Ad or liver enzyme elevation after a primary administration of Ad.

Materials and Methods

Mice

Female C57BL/6 (B6; H-2^b), B6.SCID (SCID; H-2^b), and DBA/2 (D2; H-2^d) mice at 6-8 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Groups of at least 3-5 mice were analyzed in each experiment or at each time point. All animal protocols were approved by the Institutional Animal Care and Use Committee at University of Alabama at Birmingham.

Adenovirus and infection

Wild-type adenovirus type 5 was obtained from the American Tissue Culture Collection (Rockville, MD) and propagated in the HEK293 cells as described previously.[13] Infected cells were harvested and lysed by 3 freeze-thaw cycles to release the virus, which was then purified through two cesium chloride gradients. The purified wild-type Ad5 was then titrated by the plaque assay,[17] aliquoted, and stored at -80°C until used. Mice were administered at $8x10^8$ infection units (i.u.) per mouse through tail vein injection on day 0 and sacrificed at different time points for CTL analysis.

Cell preparation

Single-cell suspensions of spleen and lung were prepared as described previously.[13] Briefly, spleens and lungs were disrupted using wire mesh screens, and erythro-

cytes were lysed by treatment with buffered-NH_4Cl (8.29 g of NH_4Cl, 1.0 g of $KHCO_3$, and 0.037 g of EDTA/liter). The livers were strained through a 70 μm nylon cell screen (BD Falcon, Bedford, MA). Cells were collected and mononuclear cells were centrifuged at 1500 rpm for 5 min. The cell pellets were resuspended in 8 ml 40% Percoll, pipetted onto 3 ml 70% Percoll and centrifuged at 2500 rpm for 20 min at room temperature. The cells were washed by PBS containing 2% (w/v) BSA and 0.2% (w/v) NaN_3 before staining with fluorescent antibodies.

Determination of serum alanine transaminase

Blood samples from injected and control mice, drawn from the retro-orbital sinus at the indicated time points, were centrifuged for serum isolation. Serum samples were analyzed for alanine aminotransferase (ALT) levels, a marker of hepatocellular damage, using a colorimetric ALT detection kit (TECO Diagnostics, Anaheim, CA) according to the manufacturer's protocol without modifications. ALT levels were measured in triplicate by using plasma samples obtained from at least three mice.

RT-PCR

Total RNA was isolated from the liver of infected mice at different time points by a single step method using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Thereafter, RNA was transcribed to cDNA with a first strand cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Real time PCR

For detection of adenovirus type 5, the following forward primer and reverse primer specific for hexon gene, respectively, were used: 5'-GCGCTACCGCTCAATGTT-3' and 5'-AGGCACCTGGATGTGGAA-3'. The mouse GAPDH gene was detected as internal control with the forward primer, 5'-AGCCGCATCTTCTTGTGC-3', and reverse primer, 5'-TCCGTTCACACCGACCTT-3'. To detect E1B cDNA, the following primers were used: forward, 5'- AGACACAA-GAATCGCCTGCT-3' and reverse, 5'-CTGCTCCTCCGTCGGTATTA-3'. Mouse β-actin cDNA was used as internal control with the forward primer, 5'-GACGGCCAGGTCATCACTAT-3' and reverse primer, 5'-AAGGAAGGCTGGAAAAGAGC-3'. The reaction mixture contained SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with 100 ng RNase-treated DNA, 25 pmol of each primer, and in 25 μl volumes. Reactions were performed in ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster city, CA) with thermal cycling conditions as follows: 50 °C for 2 min, then a 95 °C hold for 10 min, followed by 40 cycles at 95 °C for 20 sec, and 60 °C for 1 min. For each sample, DNA copies of the Ad5 hexon gene were normalized for DNA loading using the number of DNA copies of the mouse GAPDH gene and the results were reported as the number of adenovirus genome copies per 10^6 GAPDH genome copies. Ad5 E1B cDNA copies were normalized to β-actin cDNA content and expressed as the number of E1B mRNA copies per 10^6 β-actin cDNA copies.

IFNγ ELISPOT

To determine the frequency of peptide epitope specific T cells in the spleen and liver, IFNγ ELISPOT assay was performed. 96-well polyvinylidene difluoride-backed plates (Millipore, Bedford, MA) were coated overnight with 5 μg/ml anti-mouse IFN-γ mAb at 4°C, washed, and then blocked with complete medium. Cells from spleens or livers of naïve or immunized mice were washed and adjusted to a concentration of 2 x 10^6/ml in complete medium, then added to the microtiter wells together with either 10 μg/ml Ad E1Bp or human influenza virus M1 peptide (*58 GILGFVFTL*) to give a final volume of 200 μl containing 2 x 10^5 cells. Control wells received no peptide. After 3 days of incubation, cells were washed three times with PBS/0.05% Tween 20, before overnight incubation at 4°C with 1 μg/ml biotinylated anti-mouse IFN-γ mAb in PBS/10% FCS. Plates were washed three times with PBS/0.05% Tween 20, before adding 40 ng of HRP-conjugated goat anti-biotin Ab (Vector Laboratories, Burlingame, CA), in 100 μl of PBS/10% FCS, to each well. Plates were incubated at room temperature for 1 hr, and washed with PBS/0.05% Tween 20 and then with PBS alone before developing spots using 3-amino-9-ethylcarbazole. Plates were read on a CTL automatic ELISPOT reader (CTL, Cleveland, OH) and analyzed using Immunospot 3.1 software (CTL). All results were repeated in triplicate.

Preparation of MHC class I tetramers

MHC class I tetramers were prepared, as described previously.[18] Briefly, β_2-microglobulin as well as recombinant H-2D^b H chains fused to a BirA substrate peptide was produced in *Escherichia coli* BL21 (DE3). Monomeric MHC-peptide complexes

were refolded with the Ad5 peptide epitope E1Bp (*192 VNIRNCCYI*) and subsequently enzymatically biotinylated using BirA. Tetramers were formed by the stepwise addition of allophycocyanin-conjugated streptavidin (Molecular Probes, Eugene, OR).

MHC class I tetramer staining

Mononuclear cells from spleen, lung, liver, and peripheral blood were stained with allophycocyanin (APC)-conjugated MHC class I tetramer, D^b-E1Bp, anti-CD8 FITC and anti-CD44 PE or anti-CD127 PE (BD PharMingen, San Diego, CA). Staining procedures were performed at 4°C in PBS containing 2% (w/v) BSA and 0.2% (w/v) NaN_3. After incubation with the antibodies, the samples were washed and then fixed in PBS containing 2% (w/v) paraformaldehyde. At least 100,000 events were acquired using a FACSCalibur (BD Biosciences, San Jose, CA) and analyzed using the computer program CellQuest.

Intracellular cytokine staining

Mononuclear cells from spleen or liver were either left untreated or stimulated with E1Bp peptide (10 μg/ml) for 3 days at 37°C. Before intracellular staining, cells were stained with D^b-E1Bp tetramer and anti-CD8 PE as described above. After fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Biosciences), cells were then stained with anti-granzyme B [19] (BD Pharmingen) or anti-IFNγ (eBioscience, San Diego, CA) Abs per manufacture's instructions.

In vivo CTL assay

For target cells, 10^7 spleen cells from naïve B6 mice were incubated with either 2 μM (high dose) 5,6-carboxysuccinimidylfluoresceine ester (CFSE; $CFSE^{Hi}$) or 0.2 μM (low dose) CFSE ($CFSE^{Lo}$) in PBS, at 25°C for 12 min. CFSE labeling was then quenched by the addition of fetal bovine serum (FBS) to a final concentration of 20% (v/v). Cells were washed with RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 50 μM β-mercaptoethanol and 25mM HEPES. The $CFSE^{hi}$ cells were then incubated in the presence of 1 μM E1Bp peptide at 37°C for 1 hr, whereas $CFSE^{lo}$ cells were incubated in medium only. Human influenza virus M1 peptide (*58 GILGFVFTL*) was used as control to pulse a separate population of $CFSE^{hi}$ cells. After labeling and peptide pulsing, both populations of target cells were washed and mixed together in 0°C PBS such that recipient mice received 10^7 cells of each population in a single *i.v.* injection. As a control, naïve B6 mice were adoptively transferred with an identical number of target cells. Recipient mice were sacrificed 6 hr following cell transfer. Spleen and liver mononuclear cells were prepared as described above and then analyzed by flow cytometry. Percent specific lysis was determined using the following formulas: [20]

The ratio of recovery of nonpeptide-treated control spleen cells to peptide-sensitized spleen cells = (percentage of $CFSE^{lo}$ cells)/(percentage of $CFSE^{hi}$ cells).

The percent specific lysis (%) = 100 x (1 – (ratio of cells recovered from naive mice/ratio of cells recovered from infected mice)).

Serum ELISA

Before and at days 1, 2, 4, 7 and 12, after Ad5 injection, serum samples were collected from ~50 μl of blood obtained from mice via retro-orbital bleeds, and assayed on ELISA plates coated with capture antibodies for IFNγ. All subsequent steps in the ELISA were performed according to manufacturer's instructions (eBioscience).

Statistical analyses

Significant differences between mean values were determined by Student's *t* test. $p < 0.05$ was considered statistically significant. Curve estimation for the regression of *in vivo* lysis to tetramer-positive cells was conducted using the Statistical Package for Social Sciences version 10.0 for Windows.

Results

Kinetics of the viral clearance and liver enzyme elevation after adenovirus infection

C57BL/6 (B6) mice were injected intravenously (*i.v.*) with $8x10^8$ i.u. of wild-type Ad5. At different times after Ad administration, the level of replicating virus and viral genomes were determined and liver enzyme levels were monitored. There were high levels of Ad viral genome and E1B mRNA copies detectable by day 1 after Ad5 administration with a smaller further increase by day 2 followed by a rapid decrease by days 4 and 6 (Figure1A). However, the levels of viral DNA and mRNA in the liver did not decrease to pre-treatment levels and remained at values that were at least 10 - 50-fold higher than pre-administration values, and were nearly constant from day 8 to day 15. There was a significant increase in the levels of serum alanine transaminase (ALT) at day 2, which

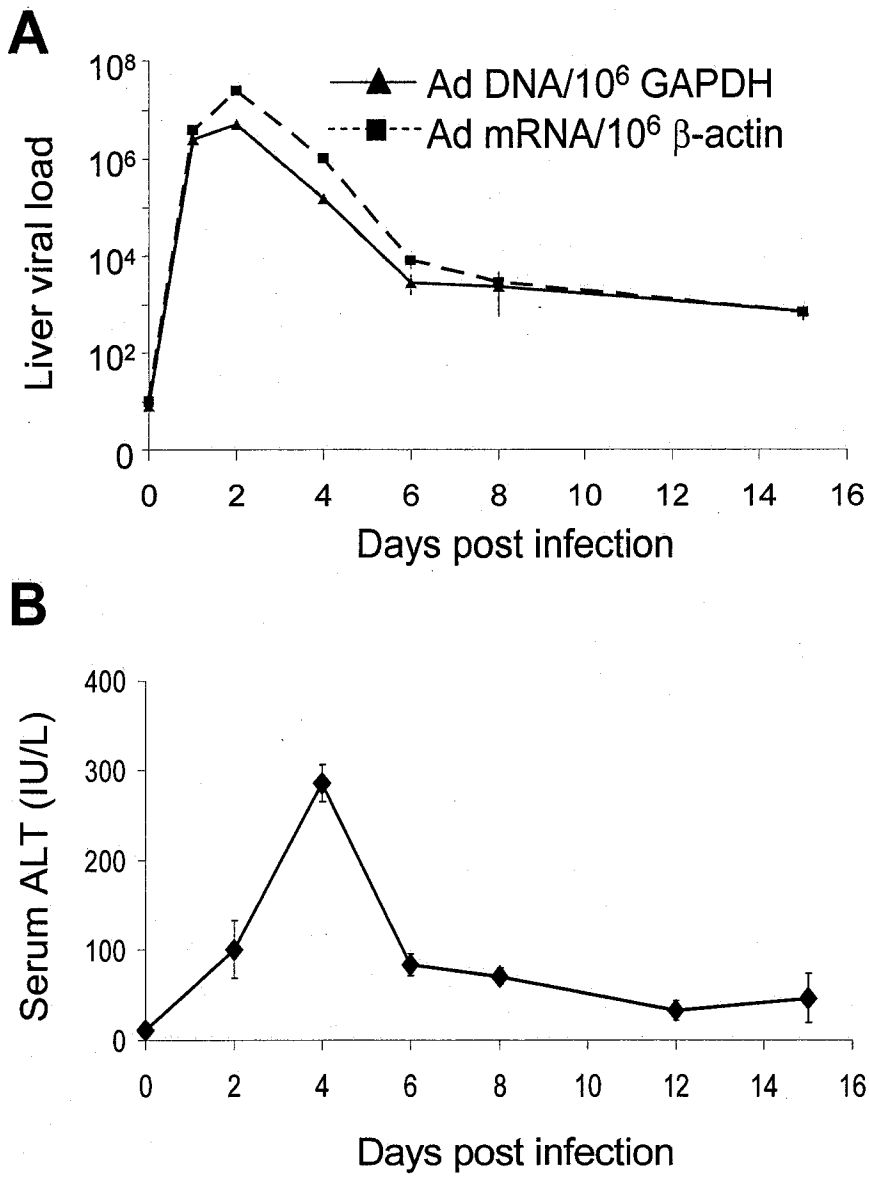


Figure 1 *Kinetics of adenoviral clearance from liver and serum ALT elevation. C57BL/6 mice were injected with $8x10^8$ i.u of wild-type Ad5. Livers of B6 mice were recovered at different times after Ad5 infection and processed for total DNA and RNA. Real time qPCR was performed to measure Ad5 genome number (▲) and E1B mRNA (■) (A). Serum samples were collected at different time points and serum ALT levels were assayed (B). Data shown are the mean ± s.e.m. of a minimum of 3 mice at each time point.*

peaked at day 4, followed by a significant decline by day 6 after virus administration (Figure 1B). These results indicate that, after Ad administration, the immunologic mechanism for clearance of Ad was initiated by day 2 after administration, peaked on day 4 and was largely down-regulated by day 6.

Characterization of the primary CD8 T cell response in Ad-primed B6 mice with a virus-specific peptide and class I MHC tetramer

To determine the role of the virus-specific CD8 T cell response in the clearance of adenovirus from the liver, we developed an adenovirus-specific tetramer that enabled detection of Ad-specific CD8 T cells after adenovirus immunization. The immunodominant H2-D^b restricted CTL epitope, E1Bp (*192 VNIRNCCYI*),[14-16] is contained in the protein encoded by the adenovirus *E1B* gene. The ability of this peptide to stimulate Ad-specific CD8 T cells in the context of class I D^b was first demonstrated using an IFNγ ELISPOT assay. The E1Bp peptide could specifically stimulate IFN-γ production by spleen and liver T cells from B6 mice after injection of Ad5 (Figure 2A). Interestingly, there was a two-fold increase in the number of E1B specific spot forming cells (SFCs) from the liver of Ad-infected B6 mice compared to the spleen of Ad-infected B6 mice. As a control, there were very few IFNγ spots produced by stimulation of spleen cells from Ad-primed B6 mice by an M1 flu peptide (*58 GILGFVFTL*), and no production of IFNγ spot by spleen or liver cells from naïve B6 mice after stimulation with the E1Bp (Figure 2A).

To determine if the E1Bp peptide could sensitize target cells for lysis by Ad-specific CTLs, spleen cells from naïve B6 mice were either pulsed with the E1Bp peptide and labeled with a high dose of 5,6-carboxysuccinimidylfluoresceine ester (CFSE; $CFSE^{hi}$) or unpulsed and labeled with a low dose of CFSE ($CFSE^{lo}$). The spleen cells

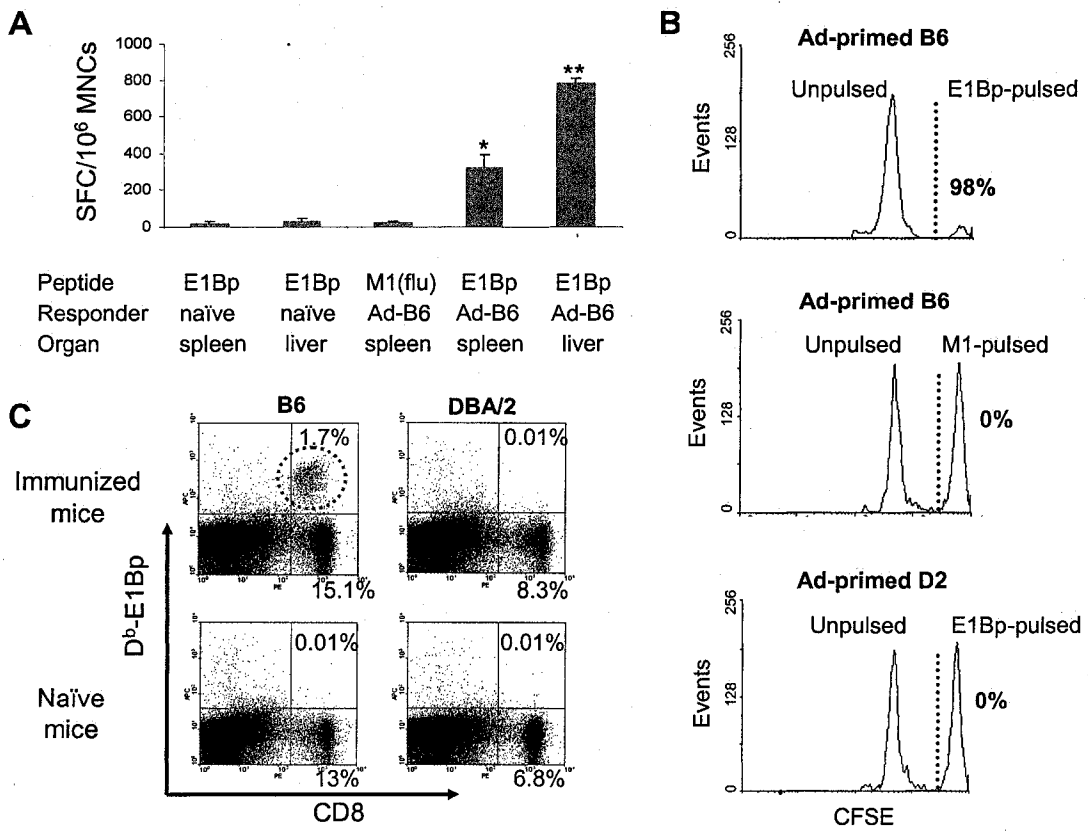


Figure 2 *Characterization of epitope peptide E1Bp and MHC tetramer. B6 mice and D2 mice were i.v. injected with $8x10^8$ i.u. of wild-type Ad5 and analyzed 8 days later. Mononuclear cells from spleens and livers of B6 mice or D2 mice were isolated and IFNγ ELISPOT assay were performed as described in Materials and Methods. The number of spot forming cells (SFC) per 10^6 cells was shown (A). Target cells from the spleen of naïve B6 mice were labeled with a high concentration of CFSE ($CFSE^{hi}$) and then pulsed either with E1Bp peptide (B; upper panel) or irrelevant flu peptide M1 (B; middle panel). Peptide-pulsed $CFSE^{hi}$ cells were cotransferred i.v. into Ad-primed B6 mice with unpulsed target cells that were labeled with a low concentration of CFSE ($CFSE^{lo}$). Target cells from the spleen of naïve D2 mice were treated similarly and transferred into Ad-primed D2 mice (B; bottom panel). Six hours later, single cell suspensions of the spleen were analyzed by flow cytometry (100,000 events) for CFSE fluorescence. Numbers in the histograms represent the percentage of target cells killed. MNCs from spleens were labeled with anti-CD8 antibody and D^b-E1Bp tetramer and analyzed by flow cytometry (C). The results are representative of 5-8 independent experiments. Data shown are the mean ± s.e.m of SFC/10^6 MNCs from 3-5 mice each group. * $p < 0.05$ and ** $p < 0.001$ compared to naïve mice.*

(10^7/mouse) were then injected *i.v.* into B6 mice that had been infected with adenovirus 8 days earlier. The spleens of the recipient mice were recovered 6 hr after injection, a single-cell suspension was prepared and the relative recoveries of $CFSE^{lo}$ (unpulsed) cells and $CFSE^{hi}$ (peptide-pulsed) cells were compared. In comparison with unpulsed $CFSE^{lo}$ cells, 98% of the E1Bp-sensitized $CFSE^{hi}$ target cells were deleted *in vivo* during this 6 hr period (Figure 2B). The lysis was specific for the E1Bp peptide since identical target cells pulsed with the M1 influenza peptide were not eliminated. Similarly, H2-D^b specific lysis was confirmed by the observation that there was no specific lysis of E1Bp pulsed syngenic target cells recovered from D2 (H-2^d) mice.

Using this Ad-specific peptide, a D^b-E1Bp MHC tetramer was then produced and used to characterize Ad-specific T cells in the spleen of Ad-injected mice. At eight days post-infection, 1.7% of spleen mononuclear cells (MNCs) in the Ad-primed C57BL/6 mice were D^b-E1Bp$^+$CD8$^+$ (Figure 2C). In contrast, less than 0.01% of the CD8$^+$ T cells in the spleen of naïve B6 mice, or Ad-primed D2 mice, were D^b-E1Bp tetramer positive. These results show that the E1Bp can be used to detect Ad5 specific CTLs by an ELISPOT assay, by an *in vivo* killing assay and by tetramer staining.

Adenovirus-specific CD8 T cells in adenovirus target tissues

Quantification of adenovirus-specific CD8 T cells in the blood and target tissues, including the liver and lung, of treated animals would provide valuable information regarding the tissue distribution of CTLs. Through the use of the D^b-E1Bp tetramer, we were able to evaluate the number of adenovirus-specific mononuclear cells in the peripheral blood (PBMCs) and from the liver and lung. Prior to treatment, less than 0.01% of

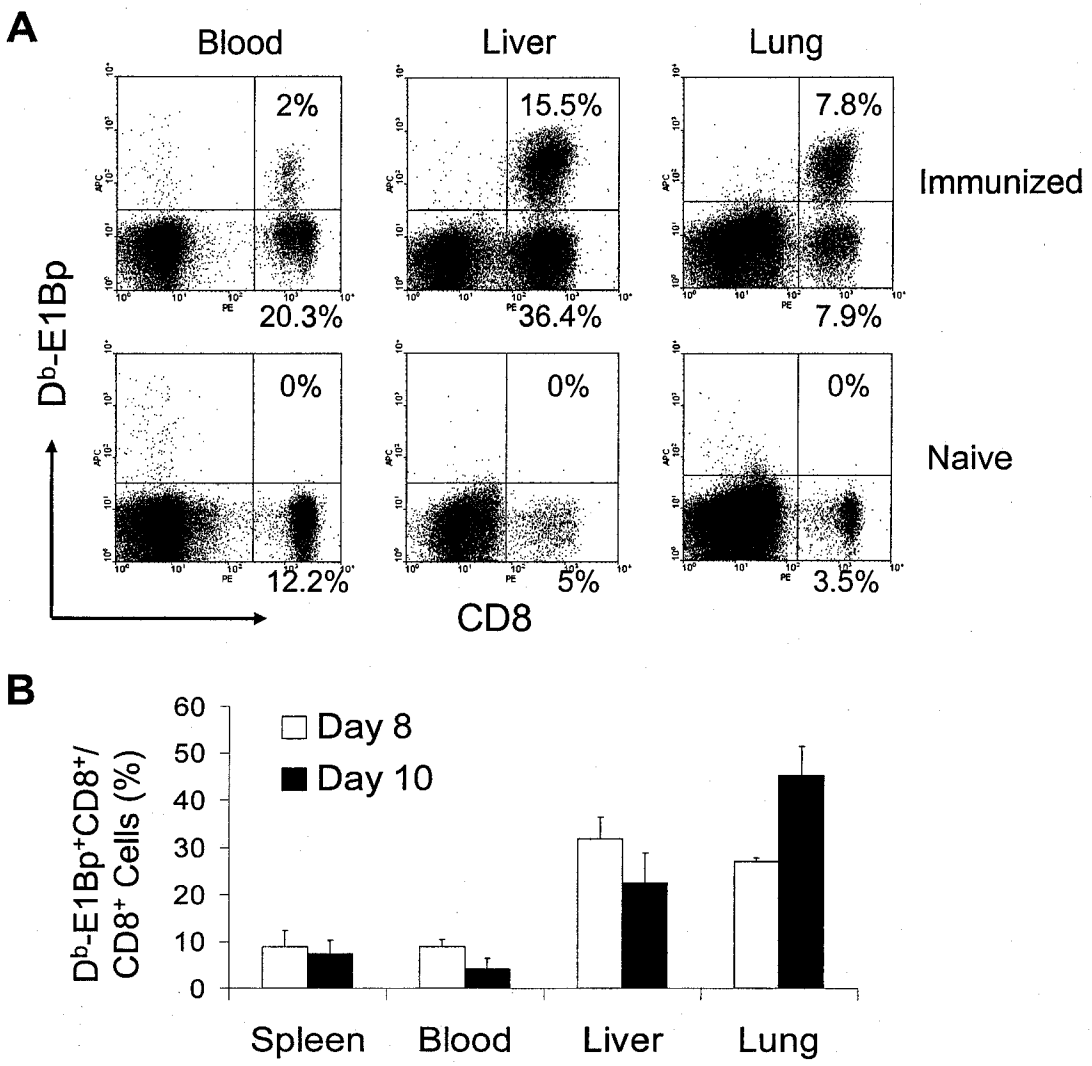


Figure 3 *D^b-E1Bp tetramer staining of MNCs from blood, liver and lung. MNCs from peripheral blood, liver and lung were isolated from B6 mice at day 8 post infection. All cells were labeled with D^b-E1Bp tetramer and anti-CD8 antibodies and analyzed by flow cytometry (A). The percentage of D^b-E1Bp$^+$ cells in total CD8$^+$ T cell population of the spleen, blood, liver and lung at days 8 and 10 post infection are shown in (B). Data shown are the mean ± s.e.m. of D^b-E1Bp$^+$ CD8$^+$ cells/total CD8$^+$ T cells from 3-5 mice each group.*

mononuclear cells from the blood, liver and lung of B6 mice were $CD8^+$ and reactive with the D^b-E1Bp-tetramer (Figure 3A, lower panels). Eight days after treatment, approximately 10% (2.0%/20.3%) of the $CD8^+$ T cells in the peripheral blood were D^b-$E1Bp^+$, consistent with the results obtained on analysis of the spleen on day 8 (Figure 3B). Surprisingly, there was extensive recruitment and accumulation of D^b-$E1Bp^+$ $CD8^+$ T cells in the liver and lung. These cells represented approximately 15% of the total mononuclear cells in the liver and 8% of the total mononuclear cells in the lung (Figure 3A, upper panels). We found that 33% of the total $CD8^+$ T cells that accumulate in the liver, and 45% of the total $CD8^+$ T cells that accumulate in the lung, after administration of adenovirus, were D^b-E1Bp tetramer positive (Figure 3B). These results indicated that there was marked recruitment of virus-specific $CD8^+$ T cell to the target tissues (liver and lung) following Ad administration.

D^b-E1Bp-tetramer$^+$CD8$^+$ T cells are cytotoxic effector cells

To determine the phenotype of the D^b-$E1Bp^+CD8^+$ T cells in the spleen, mononuclear cells were isolated at day 8 after Ad administration, stimulated *in vitro* with E1Bp peptide for 3 days, and then were intracellularly co-stained with anti-IFNγ and anti-granzyme B antibodies, respectively. Nearly 100% of the D^b-E1Bp-specific CD8 T cells were $IFN\gamma^+$ and granzyme B^+ (Figure 4A and 4B). At the same time, all of these D^b-E1Bp-specific CD8 T cells expressed elevated levels of CD44, an activation/memory marker for T cells (Figure 4C), and low levels of CD127 (Figure 4D), the IL-7 receptor that is essential for T cell survival and homeostatic maintenance of memory T cells [21]. On day 8, only 22% of D^b-E1Bp-specific CD8 T cells expressed high levels of CD127, indi

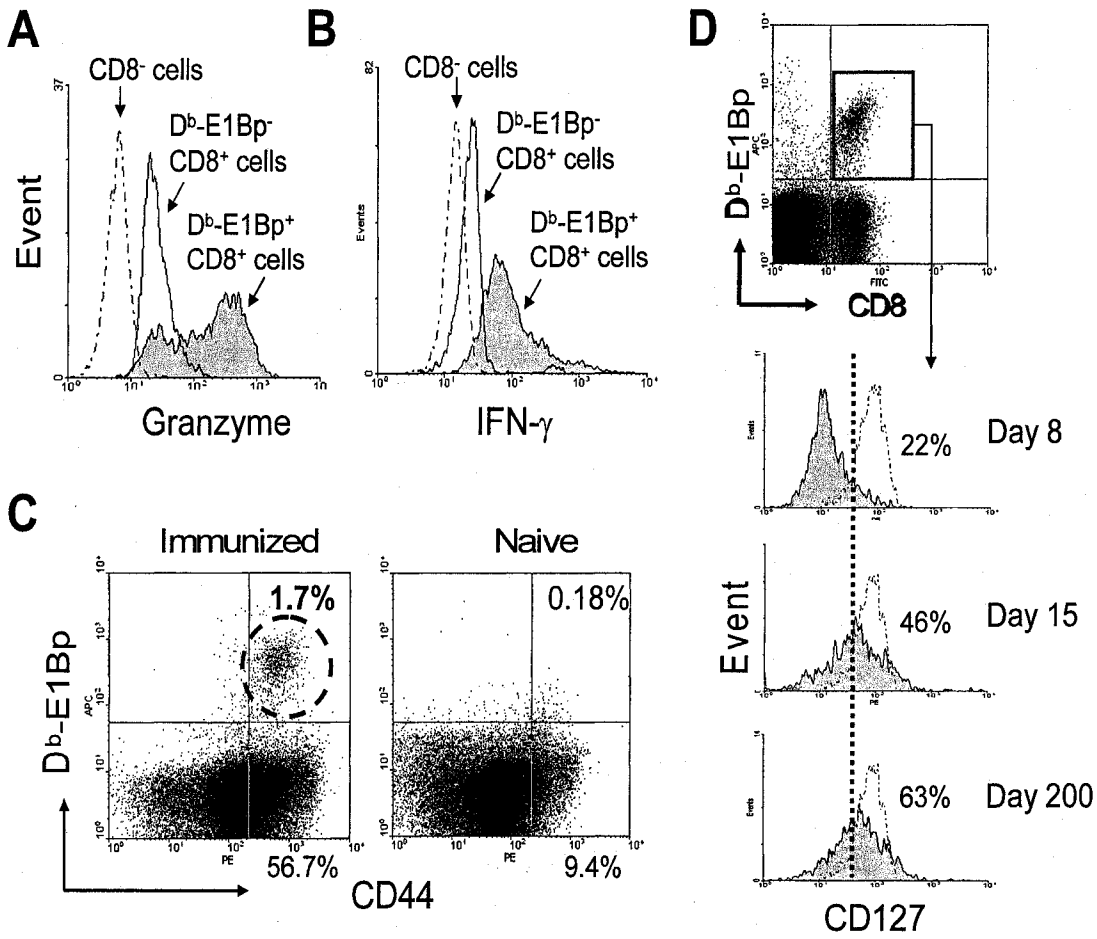


Figure 4 *Characterization of D^b-E1Bp$^+$ CD8$^+$ cells. B6 mice were i.v. injected with wild type Ad5 and sacrificed 8 days later. Spleen cells from B6 mice at day 8 were incubated in vitro with E1Bp-pulsed, irradiated spleen cells from naïve B6 mice for 3 days and then intracellularly labeled with anti-granzyme B (A) or anti-IFNγ (B) antibodies. Spleen cells were labeled with anti-CD44 along with anti-CD8 antibodies and D^b-E1Bp tetramer 8 days post infection. Spleen cells labeled with CD44 and tetramer were gated on CD8$^+$ cells (C). The numbers in the right upper quadrants indicate the percentage of CD8 T cells that were tetramer$^+$. CD127 (IL-7Rα) expression on spleen cells recovered from B6 mice at days 8, 15 and, 200 were analyzed (D). Histograms were gated on D^b-E1Bp$^+$ CD8$^+$ cells. The numbers in the histograms indicate the percentage of CD127^{hi} cells. The shaded area indicates the D^b-E1Bp$^+$ CD8$^+$ cells and dotted lines indicate the Db-E1Bp$^-$ CD8$^+$ cells recovered from naïve mice.*

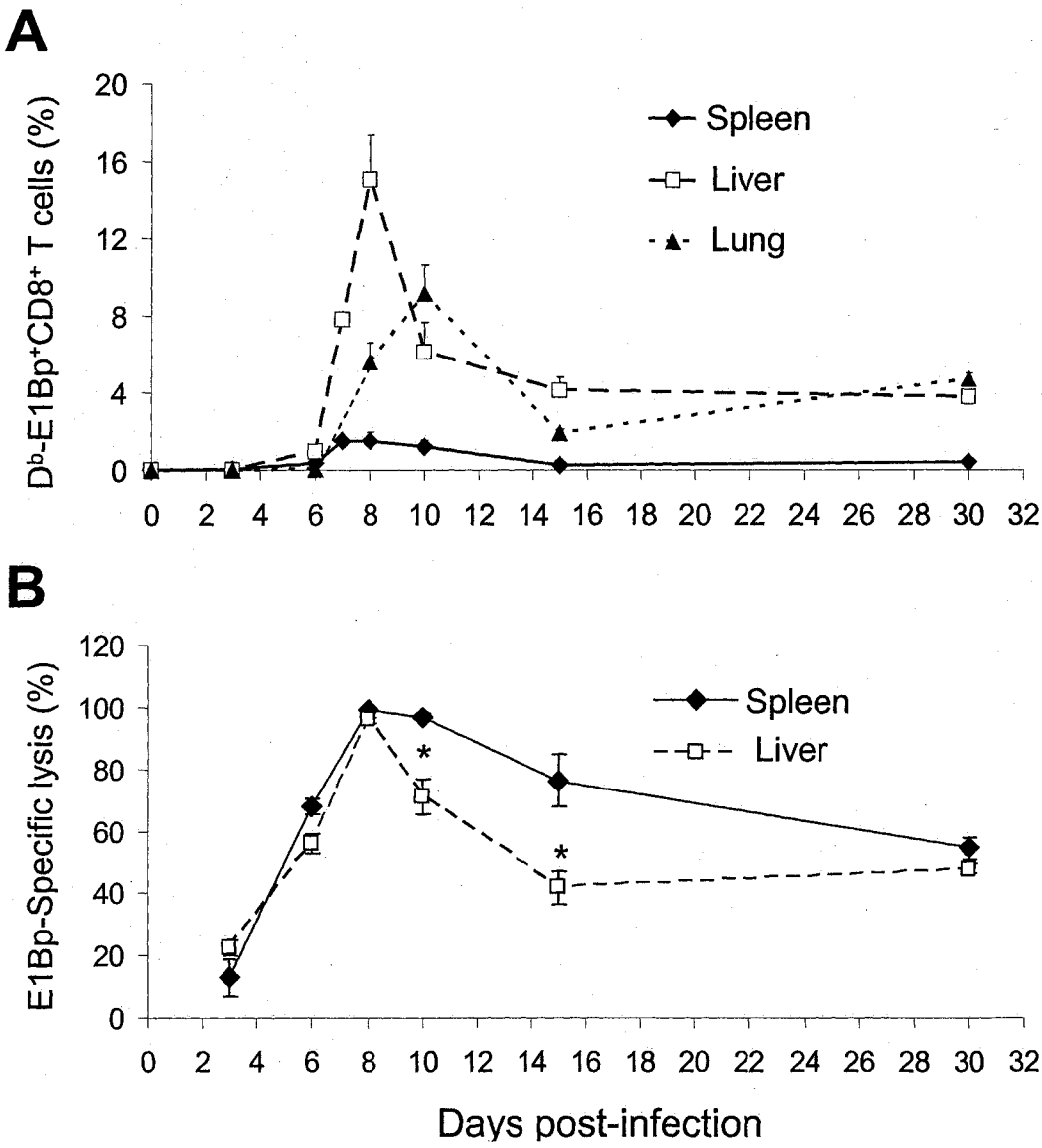
cating these cells were acute effector cells. After the acute CTL response, the $CD127^{hi}$ population gradually increased to 46% at day 15 and 63% at day 200 after infection (Figure 4D). Therefore, Ad administration is associated with the development of specific effector CD8 T cells and the subsequent generation of Ad-specific memory cells.

Adenovirus-specific CTL response occurs after liver toxicity and virus clearance

To address whether the Ad-specific CTL response correlates with the clearance of Ad or liver enzyme elevation, we utilized D^b-E1Bp tetramers and E1Bp-specific *in vivo* CTL assay. MNCs from the spleen, liver and lung were obtained on days 0, 3, 6, 7, 8, 10, 15, and, 30 after *i.v.* administration of Ad5. As shown in Figure 5A, there were no detectable tetramer-positive cells in the spleen, liver, and, lung at day 3 and a very low percentage at day 6, a time when the clearance of Ad had been substantially accomplished and after the peak of liver enzyme elevation. The tetramer-positive cells began to increase after day 6, sharply increased and peaked at day 8 and declined thereafter (Figure 5A). This did not correlate with a further reduction of Ad-viral clearance and liver enzyme elevation shown in Figure 1. Therefore, the primary peak response of $tetramer^+$ CD8 T cells does not correlate with the elimination of adenovirus or the liver cytotoxic response.

To determine if the cytotoxic activity in the spleen and liver correlated with the presence of the tetramer positive CD8 T cells, the kinetics of the specific *in vivo* CTL activity against CFSE labeled, E1Bp-pulsed target cells was measured. The E1Bp-specific CTL activity in spleen and liver was low at day 3 (Figure 5B). The CTL activity was detectable at day 6, peaked at day 8, and declined by day 10. These results indicate that the

Figure 5 *Kinetics of Ad-specific CTL response. B6 mice were i.v. injected with wild-type Ad5. The Ad-specific CTL response was analyzed by D^b-E1Bp tetramer and in vivo CTL assay at indicated days post infection. The frequencies of D^b-E1Bp$^+$ CD8$^+$ cells in spleen, liver, and lung are indicated for each time point (A). Target cells from the spleen of naïve B6 mice were labeled with a high concentration of CFSE (CFSE^{hi}) and then pulsed with E1Bp peptide. Peptide-pulsed CFSE^{hi} cells were cotransferred i.v. into adenovirus-primed B6 mice with unpulsed target cells that were labeled with a low concentration of CFSE (CFSE^{lo}). After 6 hr, single cell suspensions of the spleen and liver were analyzed by flow cytometry (100,000 events) for CFSE fluorescence. The E1Bp-specific lysis at the indicated time points was calculated as described in Materials and Methods and is shown in (B). The number of spleen D^b-E1Bp$^+$CD8$^+$ cells are plotted against the percent E1Bp-specific lysis in the spleen. Each point represents results from an individual recipient mouse (C). At frequencies of < 1%, the percent of E1Bp-specific lysis was correlated with the log number of D^b-E1Bp tetramer-positive CD8$^+$ T cells ($p < 0.001$; $r = 0.988$). The total number of spleen cells among animals was similar (data not shown). Data shown are the mean ± s.e.m. of 3-5 mice each group. * $p < 0.05$ for lysis in livers compared to spleens.*



peak of the Ad-specific CTL response at day 8 occurred after clearance of adenovirus and after the peak of liver cell damage.

Kinetics of Ad viral clearance and liver enzyme elevation in SCID mice

The above results indicate that after a primary challenge, the Ad viral clearance and liver cell toxicity occurs before the peak of the primary $CD8^+$ CTL response. This was further confirmed by administration of wild-type Ad5 to B6.SCID ($H\text{-}2^b$) mice and subsequent analysis of Ad-viral clearance and serum ALT levels at different times. The kinetics of Ad clearance and serum ALT elevation were identical in SCID mice and wild-type B6 mice. For both strains, Ad-virus genome and serum ALT peaked at day 2 and day 4, respectively, and declined thereafter (Figure 6A and 6B). As expected, there were no tetramer-positive T cells generated after administration of Ad5 into SCID mice (Figure 6C). There was also no *in vivo* CTL response to E1Bp-pulsed target cells after transferred into SCID 8 days after Ad5 administration (data not shown). The results above indicate that this early immune response might completely account for the clearance of Ad and liver toxicity.

Serum levels of IFNγ were analyzed at different times after Ad5 administration. The serum levels of IFNγ peaked at day 2 after Ad administration and declined gradually thereafter (Figure 6D). These results suggest that innate immune response, but not the virus-specific CD8 T cells, account for adenovirus clearance and liver cell damage.

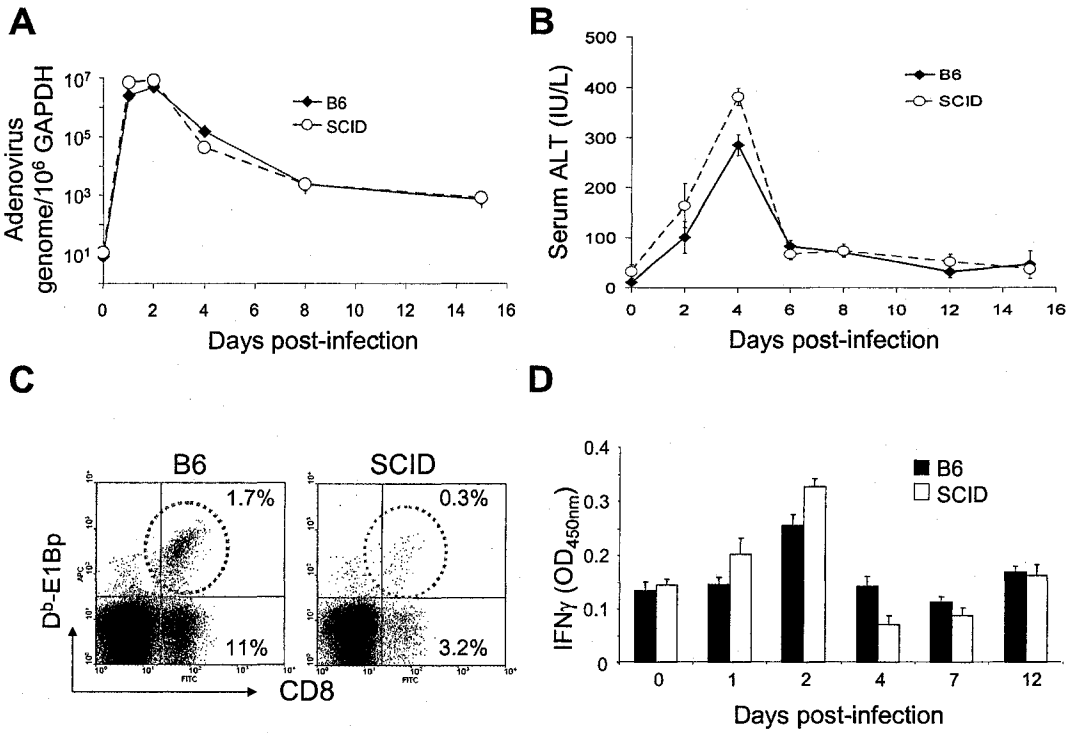


Figure 6 *Analysis of the immune response to Ad infection in SCID mice. SCID (H-2^b) and wild-type B6 mice were i.v. injected with 8x10^8 i.u. of wild-type Ad5 and analyzed at indicated days post infection. Livers of SCID and B6 mice were recovered at different times after Ad5 infection and processed for total DNA isolation. Real time qPCR were performed to measure Ad5 genome number (A). Serum samples of SCID and B6 mice were collected at indicated days and serum ALT was assayed (B). Spleen MNCs of SCID and B6 mice were labeled with anti-CD8 Ab and D^b-E1Bp tetramer 8 days post infection and analyzed by flow cytometry (C). Serum IFNγ levels in SCID and B6 mice at each time point post-infection were measure by ELISA (D). The results are representative of 5-8 independent experiments. Data shown are the mean ± s.e.m. of 3-5 mice at each time point.*

Discussion

The cytotoxic T lymphocyte response to adenovirus vectors has been proposed to play a key role in the elimination of transduced cells, thus limiting transgene expression.[5,22] Immunosuppressive strategies have been proposed to prolong therapeutic gene expression and prolong gene therapy efficacy, which have been reviewed elsewhere.[1,2] However, until now, the analysis of the CTL response could be explored only using *in vitro* assays or through indirect approaches [23-26]. Consequently, the mechanisms underlying CTL generation and effector functions after adenovirus infection have not been clarified completely, making it difficult to correlate specific CTL generation with Ad virus clearance and liver toxicity. The primary limitation of the *in vitro* CTL assay is that *in vitro* killing activity can be induced by several specific effector mechanisms including the cytolytic molecules TNFα and IFNγ, in addition to the perforin/granzyme-B lysis induced by effector cells that act on specific and/or bystander targets. Thus, it is not possible to obtain a precise relationship between the numbers of effector cells which can execute specific cytotoxicity based on the results of *in vitro* assays. Since the MHC class I/peptide tetramer technology was first introduced in 1996,[27] it has been widely applied in the quantification and characterization of $CD8^+$ T cells induced by viral and bacterial infections.[28] We now have used this tetramer binding method to gain a better understanding of the role of CTL response after Ad administration. We also applied an *in vivo* CTL assay to directly investigate and distinguish the generation and effector functions of Ad-specific CD8 T cells.

The adenovirus *E1B* gene-encoded protein contains the immunodominant CTL epitope peptide (*192 VNIRNCCYI*), referred to as E1Bp, that is presented by the $H2\text{-}D^b$

molecule.[14-16] We developed the tetramer, D^b-E1Bp, consisting of H2-D^b, E1Bp, and β_2-microglobulin that is conjugated with the flurochrome, allophycocyanin (APC). Analysis of the specificity of this D^b-E1Bp tetramer in Ad-immunized B6 mice (H-2^b), naïve mice and Ad-immunized D2 mice (H-2^d) indicated that eight days after immunization, approximately 10% of $CD8^+$ T cells in the spleen were specific for E1Bp epitope in B6 mice. The tetramer-positive $CD8^+$ T cells expressed elevated levels of CD44, an activation/memory marker of lymphocytes, and were nearly 100% IFNγ^+ and granzyme-B^+. The numbers of effector cells exhibited similar kinetics in the liver. It has been proposed that IL-7 is essential for both naïve and memory T cell survival,[29,30] and IL-7 receptor deprivation in effector CTLs ensures their death after viral clearance.[31] IL-7R α-chain (CD127) has been used as a marker to distinguish primary effector cells from memory T cell precursors.[21] In the present study, 78% of D^b-E1Bp^+ CD8 T cells expressed low levels of CD127 during the peak of the CTL response indicating these cells would not develop into memory effector CTLs, but rather undergo apoptosis.[19] The peak of the virus clearance and liver enzyme elevation occurred by day 4, before a significant number of Ad-specific CTLs were generated, indicating that the innate immune response is responsible for most of the virus clearance after a primary response. These results are consistent with previous findings that there was an identical decrease in adenoviral DNA in the liver and lung of both immunocompetent and immunodeficient mice.[9,32,33] In those cases, adenoviruses were replication-defective due to deletion of the E1 and/or E4 genes, and it was not surprising that more than 80% of viral DNA was eliminated in the first hours following infection. In the present study, replicative wild-type adenovirus was used and the vi-

ral DNA and E1B mRNA in the liver was greater on day 2 than day 1 indicating moderate replication of the virus *in vivo*.

There were detectable, stable levels of virus and virus DNA in the liver during and after the peak of the CTL response. This is consistent with a previous report showing that Ad infected cells can escape CTL lysis *in vivo*.[34] It was also noted that after influenza virus infection, the number of apoptotic $CD8^+$ T cells was higher in the liver, suggesting that antigen-specific $CD8^+$ T cells were destroyed in the liver.[35] Down regulation of antigen-presentation in the liver would make these liver cells resistant to CTL response.[36] We propose that viral persistence in the liver during and after the peak CTL response is due to the inability of infected liver cells to form good targets for CTL attack. We also observed that the killing efficiency of CTLs in the liver for peptide-pulsed target cells was lower compared to the spleen. This suggests that other mechanisms were involved in limiting the duration of antigen specific cytotoxicity in liver. This was supported by our previous findings that soluble Fas could inhibit the CTL response in the liver.[37] $CD4^+CD25^+$ regulatory T cells have also been proposed to play an inhibitory role in suppression of CTLs in the liver.[38]

The clearance of the adenovirus and associated transgene from the liver has been proposed to be due to the effects of cytokines, such as TNFα and IFNγ, which may be associated with both the CTL response and the innate immune response.[39] In the present study, the peak of the CTL response occurs after viral clearance and liver enzyme elevation, indicating viral clearance and enzyme elevation are primarily due to the innate immune response. We therefore propose that the development of the CTL response was set in motion by early immune response mechanisms, but the presence of detectable antigen

was not necessary when the CTL response reached its peak.[40] In perforin knockout mice, we and others have shown that there was a poor viral clearance in the absence of perforin,[41,42] indicating a key role for perforin release from CTLs to mediate viral clearance. The lack of a significant role for the CTL response in the clearance of the Ad5 in the present study may be due to the relatively low replication of the virus. The virus reached a high titer on day 1, peaked on day 2, then declined to a maintenance level by day 6. The kinetics of viral clearance is consistent with previous work with the E1-deleted, first generation Ad vector or helper-dependent Ad vector.[11] It was also shown that after exposure to hepatitis C by a cutaneous route, the CTL response was not required to eliminate hepatitis C, whereas after *i.v.* acquisition, the CTLs were required.[43] Together, the results suggest that under conditions of low virus load or low viral replication, as in these experiments, the CTL response may not play a critical role in clearance of the virus during the primary immune response.

One of the major obstacles facing gene therapy is not only to permit long-term expression of the transgene by minimizing the primary immune responses but also to predict the pre-existing immune response of the individual to gene therapy. A recent survey including Europe, Japan, and the United States showed a > 80% pre-exposure to the most commonly used Ad5 virus [44] in the adult population (age range from 20 to 70 years old). This will present a problem for Ad5 based gene therapy in these individuals. Therefore, one important application of tetramer technology would be to predict the magnitude of the CTL response that might occur upon administration. As an example, the possible epitope peptide from Ad5 hexon protein were predicted to be $_{913\text{-}921}$ *TLLYVLFEV* for HLA A0201* with two prediction programs BIMAS [45] and SYFPEITHI.[46] Its antigenicity had

been shown to specifically stimulate IFNγ production of CD8 T cells from HLA-A0201* donors.[47] In combination with ever-increasing knowledge of T cell epitopes of adenovirus, the present results demonstrate the feasibility of development of class I MHC/peptide tetramers in humans with different MHCs.

The second potentially clinical application of the present work is the demonstration that the use of a tetramer combined with the analysis of CD127 (IL-7 receptor α-chain) expression can predict the magnitude of the CTL response. Following acute exposure of naïve mice, there is homeostatic maintenance of memory CD8 T cells that are derived from the $CD127^{hi}$ population.[21] This population is predicated to give rise to the highest number of CTLs upon re-challenge. Therefore, the combined analysis of PBMCs from humans with an Ad-specific tetramers and the IL-7R antibody, anti-CD127, will provide important predictive information as to the magnitude of the CTL response. We would predicted that patients with the highest number of tetramer-positive and $CD127^{hi}$ T cells would be at especially high risk for forming a brisk antivirus CTL response upon re-challenge. Armed with this knowledge, gene therapy could be individually tailored to patients with different HLA haplotypes to avoid a potential dangerous memory T cells response.

Acknowledgements

We thank Dr. Fiona Hunter for reviewing the manuscript, Dr. Rafi Ahmed for providing critical suggestions, and Carol Humber and Jennifer Allen for excellent secretarial work. This work was supported in part by National Institutes of Health Grants

RO1-AI-42900 (to J.D. Mountz) and RO1-CA-86881 (to D.T.Curiel); by a VA Career Development Award and Merit Review Award (to J.D.Mountz).

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ROLE OF CD28, FASL, AND PERFORIN IN ACTIVATION AND EFFECTOR FUNCTION OF CD8 T CELLS DURING PRIMARY ADENOVIRUS INFECTION

by
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Submitted to *Journal of Immunology*

Format adapted for dissertation

Abstract

Although the cytotoxic T lymphocyte (CTL) response induced by adenovirus (Ad) vectors has long been recognized as the major limitation for long-term transgene expression, there is still a lack of efficient strategies to suppress it. This is mainly due to a poor understanding of the mechanisms underlying CD8 T cell activation and effector functions after Ad infection. In this study, we examined the requirement for CD28-B7 interactions in the primary CD8 T cell response and the effector functions of activated CTLs after Ad infection. We also assessed the role of effector molecules, including perforin and Fas ligand, in mediating target cell lysis. During the primary response, there was a substantial defect in both the generation and effector function of Ad-specific CD8 T cells in CD28 knockout mice. Defects in FasL resulted in a significant decrease in the generation of Ad-specific CD8 T cells, but the effector function was not impaired. Perforin deficiency resulted in a 28% decrease in the cytolysis mediated by Ad-specific CTL cells. However, the most significant effect was found when the perforin and the FasL pathways were both deficient, indicating a synergistic role of these two pathways.

Introduction

Cytotoxic CD8 T cells are generated in response to Ad gene therapy and play a key role in limiting transgene expression.[1,2] To improve the efficacy of virus-mediated gene transfer, the CTL response has been reduced through manipulation of the vector or modulation of the host immune response. Due to its central role in T cell activation, the CD28 signaling pathway has become a key target for specific immunomodulation.[3,4] The

role of CD28 in regulation of the activation and effector function of CD8 T cells is still unclear.

The requirement for CD28-B7 interactions in eliciting anti-viral $CD8^+$ CTL responses has been examined both *in vivo* and *in vitro*. [5-10] Interactions between CD28 and B7 are not essential in priming naïve CD8 T cells *in vitro* through TCR engagement by peptide/MHC tetramers.[5,6] Similar to these *in vitro* studies, functional CTL responses can be generated in *CD28*$^{-/-}$ mice.[8,11,12] In contrast, CD28 signaling is required for optimal $CD8^+$ CTL responses following influenza and vesicular stomatitis virus infections [9,10] or in HY-specific TCR transgenic mice.[7] These results indicate that, depending on the model systems being analyzed, inhibition of CD28 activity may result in either no decrease or a two-fold decrease in the number of CTLs.

Despite these studies, several questions remain unclear. The first set of questions are associated with the cell type(s) that expresses CD28, since both CD4 and CD8 T cells may require the costimulatory signal provided by CD28 for optimal upregulation of IL-2 and IFN-γ.[13,14] A second set of questions is related to the effect of CD28 deficiency. First, does CD28 deficiency lead to a decreased number of CTLs that are generated? Second, does CD28 deficiency lead to a decrease in CTL function? And finally, does CD28 deficiency lead to a decrease in number and/or function either at the site of generation in the spleen or at the infected sites where the CTLs migrate *in vivo* to kill virus infected target cells?

These questions become practically important in relation to viral delivery of gene therapy. Blocking of CD28-B7 by CTLA4-Ig has been shown to prolong transgene expression in the context of Ad gene therapy.[3,4,15] In these experiments, a reduced T cell

dependent antibody response was observed, but the role of CD8 T cells was not determined. In response to virus infection, activated CD8 T cells produce perforin, FasL, as well as IFN-γ and TNFα to clear virus.[16,17] Previous studies have shown that signaling through Fas and TNFR, but not perforin, mediated Ad vector infected hepatocyte death.[18,19] However, other studies have indicated that the perforin/granzymes pathway is essential for the control of many viral infections and that the Fas pathway plays only a minor role in control of virus.[20] One major limitation thus far has been the reliance on FasL knockout *gld* mice or Fas-mutant *lpr* mice, which are known to exhibit autoimmune disease. As in the case of CD28, it is not certain if the decrease in the CTL response in FasL mutant mice is due to a decrease in number or a decrease in function.

To address these questions, we applied an immunodominant epitope peptide of Ad, E1Bp, which could specifically bind to the class I MHC molecule H2-D^b antigen groove on target cells or confer recognition of the E1B specific $CD8^+$ T cells. This enabled an accurate correlation between the number of tetramer-positive T cells and the specific *in vivo* cytotoxic capacity of these T cells. We found that there was a decrease in both the number of CTLs as well as the function of *CD28*$^{-/-}$ CTLs after Ad administration. Importantly, this decrease was not due to a decrease of CD4 T cell function since elimination of $CD4^+$ cells by treatment with the anti-CD4 antibody resulted in a lesser decrease in a number of CTLs generated and no substantial decrease in CTL function. We also found that perforin deficiency resulted in a significant decrease in the cytotoxic activity of CTLs on a per cell basis; whereas FasL deficiency alone had little effect on CTL function except for a defect in generating a sufficient number of effector CTLs. An-

other intriguing finding is the synergistic roles of perforin and Fas pathways that may act together to produce the optimal target cell death.

Materials and Methods

Mice

Female C57BL/6 (B6; H-2^b), B6.CD28$^{-/-}$ (*CD28*$^{-/-}$; H-2^b), B6.perforin$^{-/-}$ (*prf*$^{-/-}$; H-2^b), B6.FasL$^{-/-}$ (*gld*; H-2^b), and B6.Fas$^{-/-}$ (*lpr*; H-2^b) mice at 6-8 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Groups of at least 3-5 mice were analyzed in each experiment or at each time point. All animal protocols were approved by the Institutional Animal Care and Use Committee at University of Alabama at Birmingham.

In vivo CD4$^+$ T cell depletion

For *in vivo* CD4$^+$ T-cell depletion, mice were injected intraperitoneally with anti-CD4 mAb (GK1.5) at 200 μg per mouse on days -3, -1, 3, and 7 where mice were injected with Ad5 on day 0. The efficiency of CD4 T-cell depletion was monitored by flow cytometry analysis of CD4$^+$ as well as CD3$^+$ and CD8$^+$ peripheral blood mononuclear cells (PBMCs) on days 0, 3 and, 8. For each time point, the CD4$^+$ cells represented < 1% of the total T cells in the depleted mice as measured by a different clone of anti-CD4 mAb (RM4-5).

Adenovirus and infection

Wild-type Ad type 5 was obtained from the American Tissue Culture Collection (Rockville, MD) and propagated in the HEK293 cells as previously described.[21] Infected cells were harvested and lysed by 3 freeze-thaw cycles to release the virus, which was then purified through two cesium chloride gradients. The purified wild-type Ad5 was then titrated by a plaque assay,[22] aliquoted, and stored at -80°C until used. Mice were administered $8x10^8$ i.u. per mouse through tail vein injection on day 0 and sacrificed at different time points for CTL analysis.

Cell preparation

Single-cell suspensions of spleen and lung were prepared as previously described.[21] Briefly, spleens and lungs were disrupted using wire mesh screens, and erythrocytes were lysed by treatment with buffered-NH_4Cl (8.29 g of NH_4Cl, 1.0 g of $KHCO_3$, and 0.037 g of EDTA/liter). The livers were strained through a 70 μm nylon cell screen (BD Falcon, Bedford, MA). Cells were collected and mononuclear cells were centrifuged at 1500 rpm for 5 min. The cell pellets were resuspended in 8 ml 40% Percoll, pipetted onto 3 ml 70% Percoll and centrifuged at 2500 rpm for 20 min at room temperature. The cells were washed by PBS containing 2% (w/v) fetal bovine serum (FBS) and 0.2% (w/v) NaN_3 before staining with fluorescent antibodies.

Preparation of MHC class I tetramers

MHC class I tetramers were prepared, as described previously.[23] Briefly, β_2-microglobulin as well as recombinant H-$2D^b$ H chains fused to a BirA substrate peptide

was produced in *Escherichia coli* BL21 (DE3). Monomeric MHC-peptide complexes were refolded with the Ad5 peptide epitope E1Bp (*192 VNIRNCCYI*) and subsequently enzymatically biotinylated using BirA. Tetramers were formed by the stepwise addition of allophycocyanin-conjugated streptavidin (Molecular Probes, Eugene, OR).

MHC class I tetramer staining

Mononuclear cells from spleen, lung, liver, and peripheral blood were stained with allophycocyanin-conjugated MHC class I tetramer, D^b-E1Bp, anti-CD8 FITC, and anti-CD44 PE (BD PharMingen, San Diego, CA). Staining procedures were performed at 4°C in PBS containing 2% (w/v) FBS and 0.2% (w/v) NaN_3. After incubation with the antibodies, the samples were washed and then fixed in PBS containing 2% (w/v) para-formaldehyde. At least 100,000 events were acquired using a FACSCalibur (BD Biosciences, San Jose, CA) and analyzed using the CellQuest software.

In vivo CTL assay

For target cells, 10^7 spleen cells from naïve B6, lpr or $TNFR1^{-/-}$;$TNFR2^{-/-}$ mice were incubated with either 2 μM (high dose) 5,6-carboxysuccinimidylfluoresceine ester (CFSE; $CFSE^{Hi}$) or 0.2 μM (low dose) CFSE ($CFSE^{Lo}$) in PBS, at 25°C for 12 min. CFSE labeling was then quenched by the addition of FBS to a final concentration of 20% (v/v). Cells were washed with RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 50 μM β-mercaptoethanol and 25mM HEPES. The $CFSE^{hi}$ cells were then incubated in the presence of 1 μM E1Bp peptide at 37°C for 1 hr, whereas $CFSE^{lo}$ cells were incubated in medium only. Human influenza virus M1 peptide

(*58 GILGFVFTL*) was used as control to pulse a separate population of $CFSE^{hi}$ cells. After labeling and peptide pulsing, both populations of target cells were washed and mixed together in 0°C PBS such that recipient mice received 10^7 cells of each population in a single *i.v.* injection. As a control, naïve B6 mice were adoptively transferred with an identical number of target cells. Recipient mice were sacrificed 6 hr following cell transfer, spleen and liver mononuclear cells were prepared as described above and then analyzed by flow cytometry. Percent specific lysis was determined using the following formulas: [24]

The ratio of recovery of nonpeptide-treated control spleen cells to peptide-sensitized spleen cells = (percentage of $CFSE^{lo}$ cells)/(percentage of $CFSE^{hi}$ cells).

The percent specific lysis (%) = 100 x (1 – (ratio of cells recovered from naive mice/ratio of cells recovered from infected mice)).

Statistical analyses

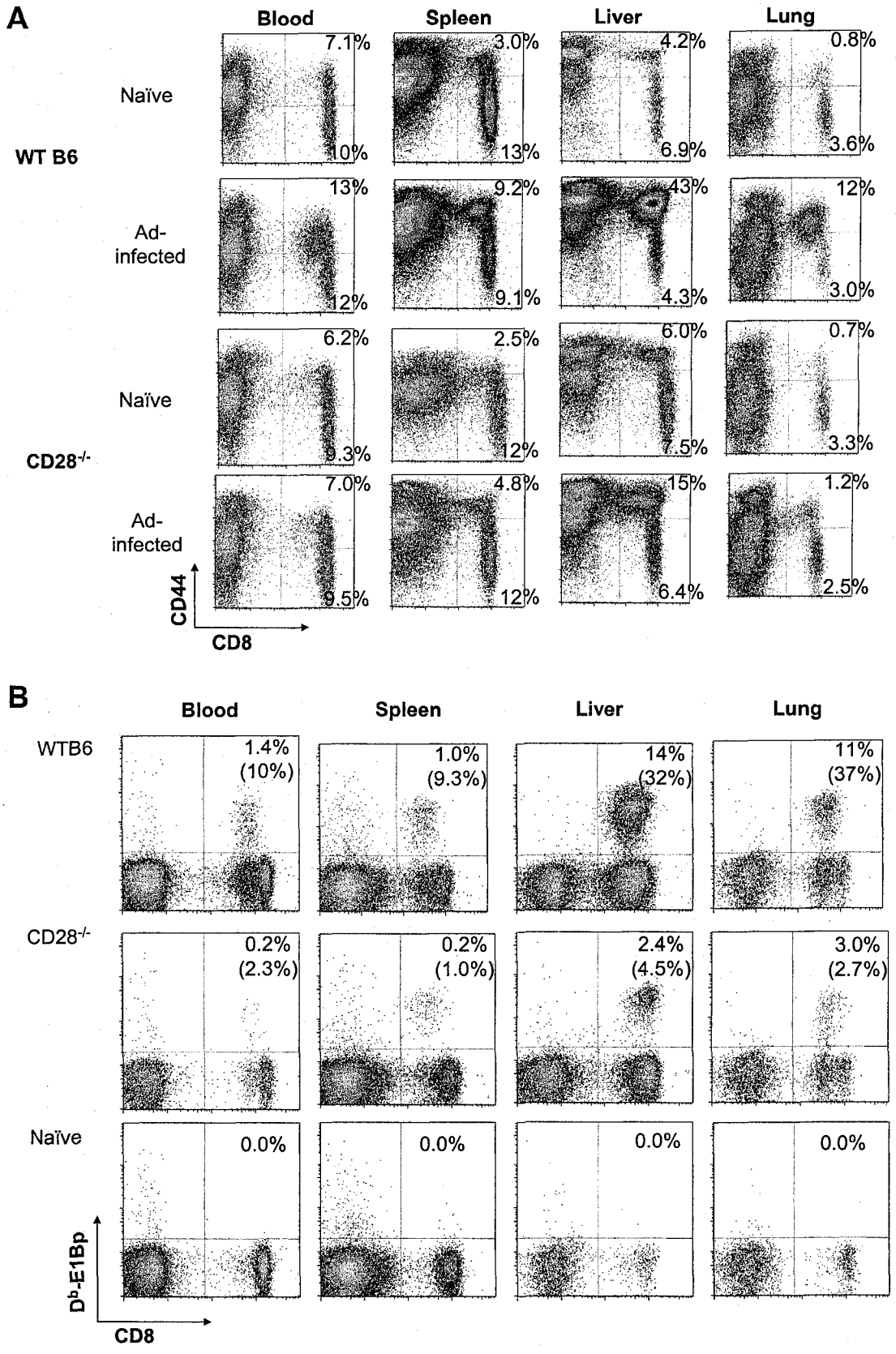
Significant differences between mean values were determined by Student's *t* test. $p < 0.05$ was considered statistically significant. Curve estimation for the regression of *in vivo* lysis to tetramer positive cells was conducted using the Statistical Package for Social Sciences version 10.0 for Windows.

Results

Defective generation of the primary CTL to Ad infection in $CD28^{-/-}$ mice

To determine the role of the CD28 signaling pathway in the activation of Ad specific CTLs, *$CD28^{-/-}$* mice as well as wild-type B6 mice were infected with Ad. Eight days

Figure 1 *Expansion of Ad-specific $CD8^+$ T cells in $CD28^{-/-}$ mice. To monitor activation of CD8 T cells, 8 days following infection with Ad, MNCs from peripheral blood, spleen, liver, and lung of WT B6 and $CD28^{-/-}$ mice were stained with anti-CD8 and anti-CD44 Abs and were analyzed by flow cytometry. Naive CD8 T cells are $CD44^{low}$ and activated/memory CD8 T cells are $CD44^{high}$ (A). On the eighth day after infection, the number of Ad-specific CD8 T cells was determined by staining with anti-CD8 Abs and D^b-E1Bp tetramers (B). The numbers represent percentages of tetramer-binding CD8 T cells among total lymphocytes. Numbers in parentheses represent percentages of tetramer-binding cells of total CD8 T cells. The mean of tetramer binding CD8 T cells are compared (C). The results are representative of 5-8 independent experiments. Data shown are the mean ± s.e.m. of 3-5 mice at each time point. ** $p < 0.001$ compared to WT B6 mice.*



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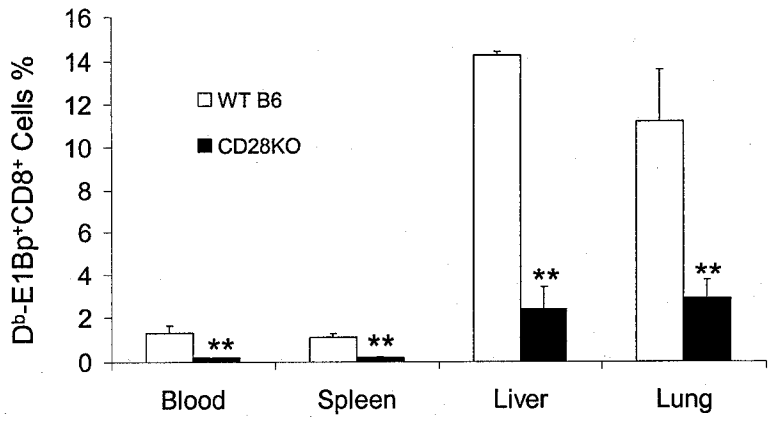


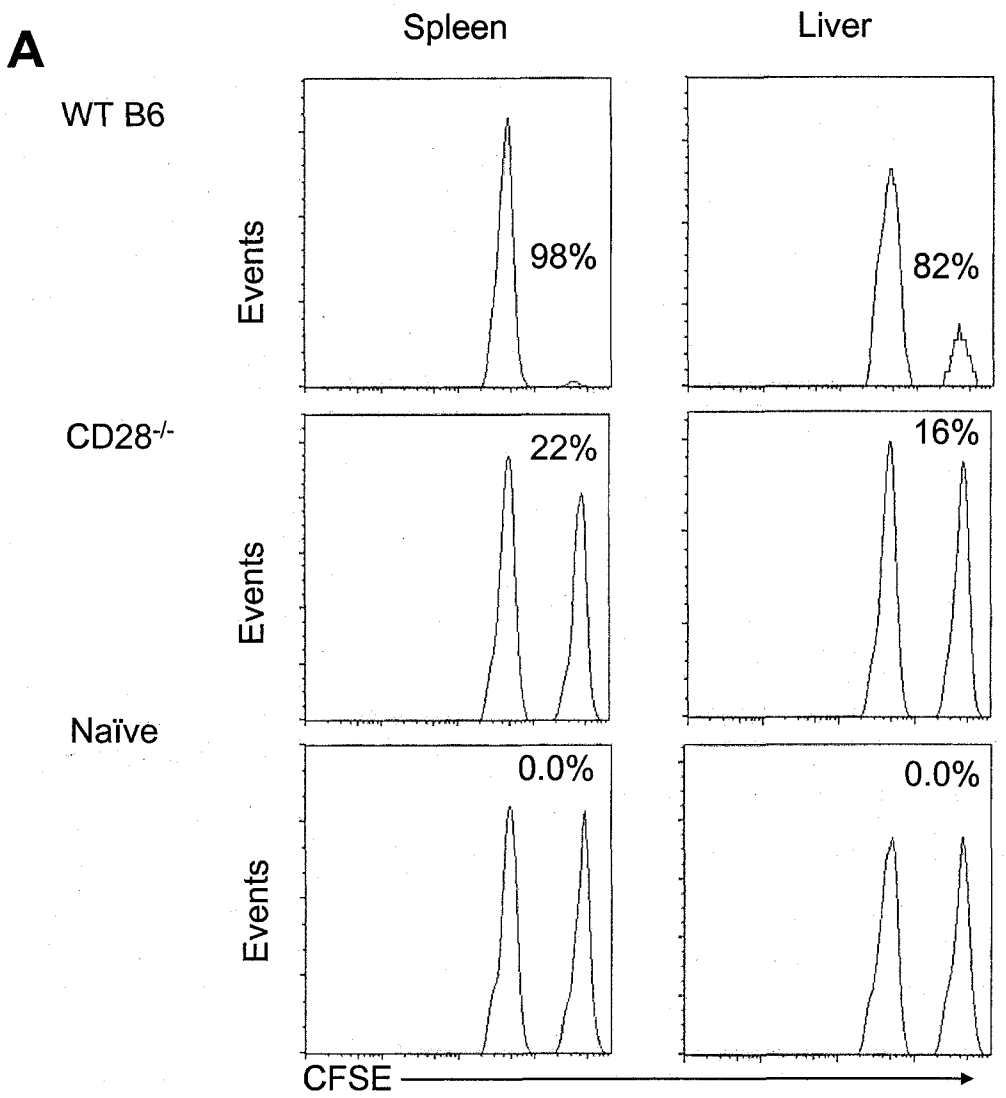
after administration of Ad by tail vein injection, CD8 T cells extensively infiltrated into the liver and lung of WT B6 mice. Approximately 90% and 80% of these infiltrating CD8 T cells in the liver and lung, respectively, were $CD44^{high}$ (activated/memory) (Figure 1A, upper panels). In contrast, there was only a moderate increase in number of infiltrating as well as $CD44^{high}$ CD8 T cells in the liver and lung of *CD28*$^{-/-}$ mice at day 8 after infection (Figure 1A, Lower panels). The numbers of Ad specific CTLs in these target tissues as well as in the peripheral blood and spleen were determined with the D^b-E1bp tetramers. There was a 4-7 fold lower percentage of D^b-$E1Bp^+CD8^+$ cells in different target tissues of *CD28*$^{-/-}$ mice compared with that in WT B6 mice 8 days after administration of Ad ($p < 0.05$) (Figure 1B and 1C).

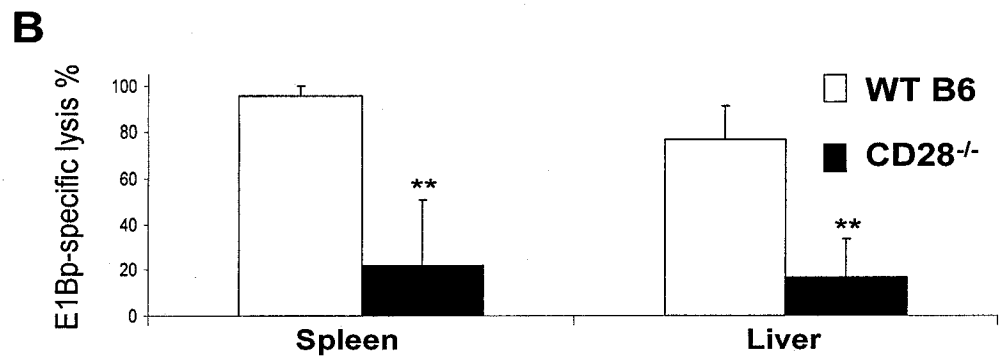
Defective CTL effector function to Ad infection in CD28$^{-/-}$ mice

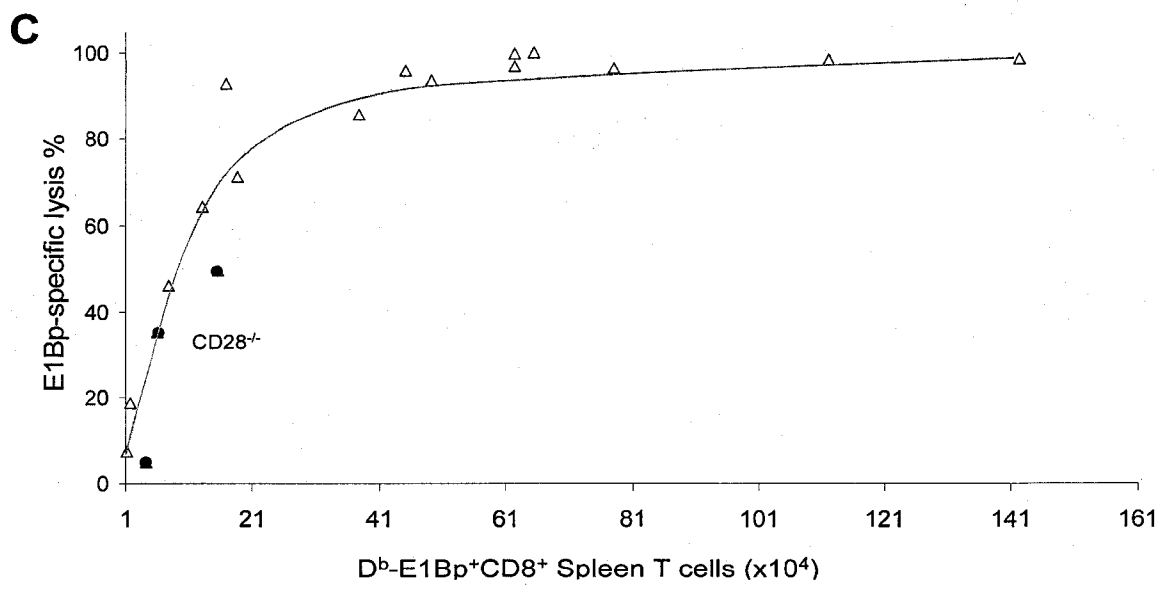
The lytic activity of Ad specific CTLs were measured using an *in vivo* killing assay in which target cells were first labeled with CFSE, pulsed with the E1Bp peptide, and then administered into either the wild-type B6 or *CD28*$^{-/-}$ mice that had been injected with adenovirus eight days earlier. *CD28*$^{-/-}$ mice showed a significant decrease in the specific killing of E1Bp peptide-pulsed target cells in the spleen and liver compared with that in WT B6 mice (22 vs 95% and 16% vs 82%, respectively, $p < 0.05$) (Figure 2A and 2B).

Although *CD28*$^{-/-}$ mice exhibited both a decrease in target cell killing and a decrease in CTL generation as determined by tetramer staining, it is impossible to conclude that the defective generation alone could account for the reduced target cell killing. To address this question, we first examined the correlation between the number of D^b-

Figure 2 *Ad-specific CTL responses in CD28-deficient mice. $CD28^{-/-}$ mice and WT B6 mice were i.v. injected with $8x10^8$ i.u. of wild-type Ad5 and Ad-specific CTL response was analyzed 8 days later. Target cells from the spleen of naïve B6 mice were labeled with a high concentration of CFSE ($CFSE^{hi}$) and then pulsed either with E1Bp peptide. Peptide-pulsed $CFSE^{hi}$ cells were cotransferred i.v. into Ad-primed B6 mice with unpulsed target cells that were labeled with a low concentration of CFSE ($CFSE^{lo}$). Six hours later, single cell suspensions of the spleen (A; left panels) and liver (A; right panels) were analyzed by flow cytometry (100,000 events) for CFSE fluorescence. Numbers in the histograms represent the percentage of target cell killed (A). The mean of E1Bp specific killing in the spleen are compared (B). Data shown are the mean ± s.e.m. of 3-5 mice at each time point. ** $p < 0.001$ compared to WT B6 mice. The cytotoxic effector function of individual CTL from the spleen of $CD28^{-/-}$ (●)mice and WT B6 (Δ) mice is compared (C). Each data point represents an individual mouse.*







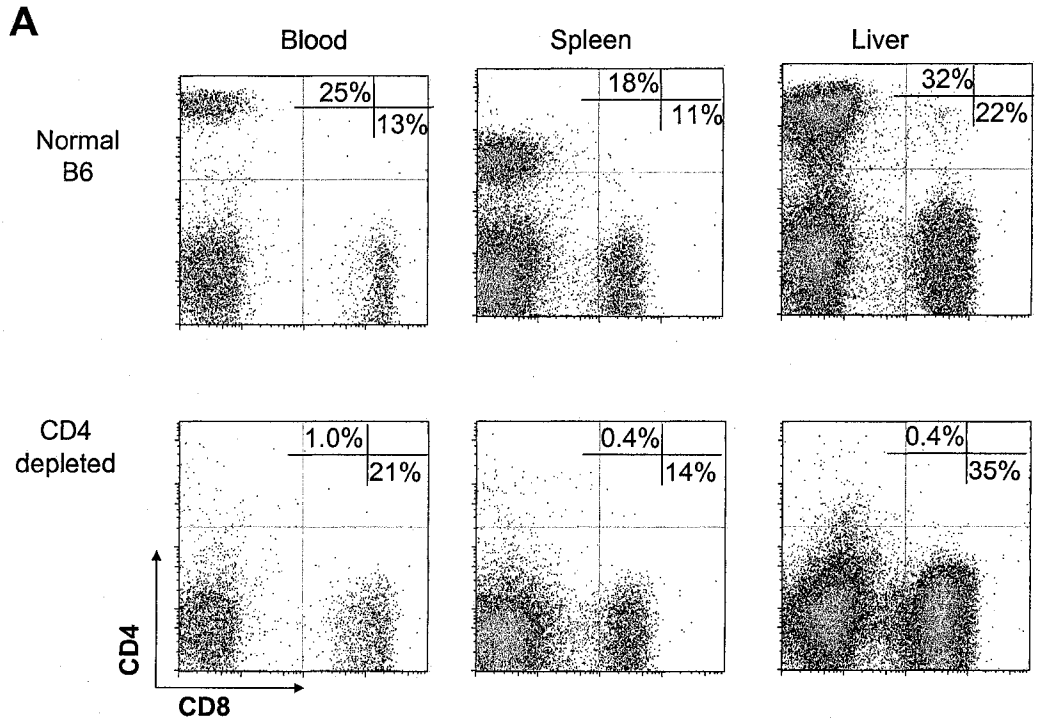
E1Bp^{+}CD8^{+} T cells and the E1Bp-specific killing of target cells in the spleen in B6 mice during the primary response after Ad administration (Figure 2C). There was a statistically significant correlation of the percentage of the specific killing with the number of tetramer-positive cells ($p < 0.001$; $r = 0.988$).

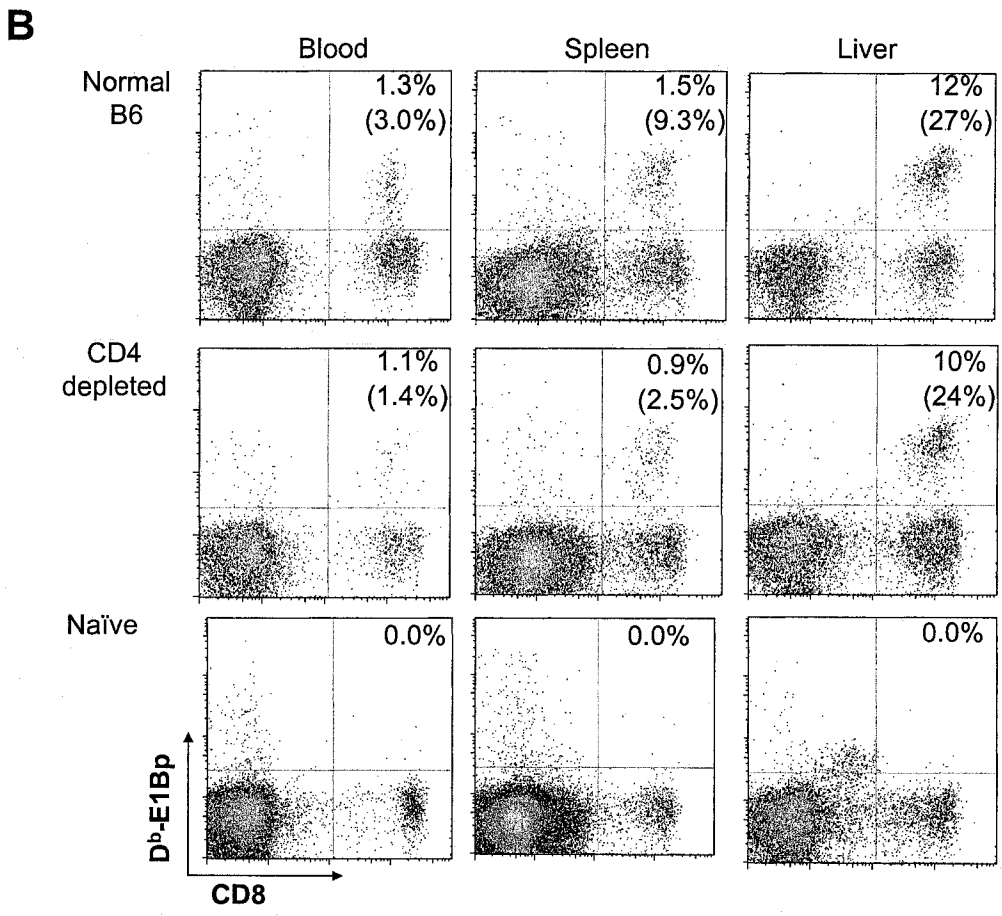
The correlation of the E1Bp specific killing of target cells versus the number of E1Bp specific CD8 obtained from *CD28*$^{-/-}$ mice was then compared with that obtained from WT B6 mice. The relativity of killing versus the number of CTL plot indicate that two out of the three *CD28*$^{-/-}$ mice tested exhibited lower *in vivo* killing efficiency per cell compared with that from B6 mice. Taken together, our results suggest that CD28 deficiency not only caused a decrease in the generation of Ad specific CD8 T cells, but also a decrease in cytotoxic effector function per cell.

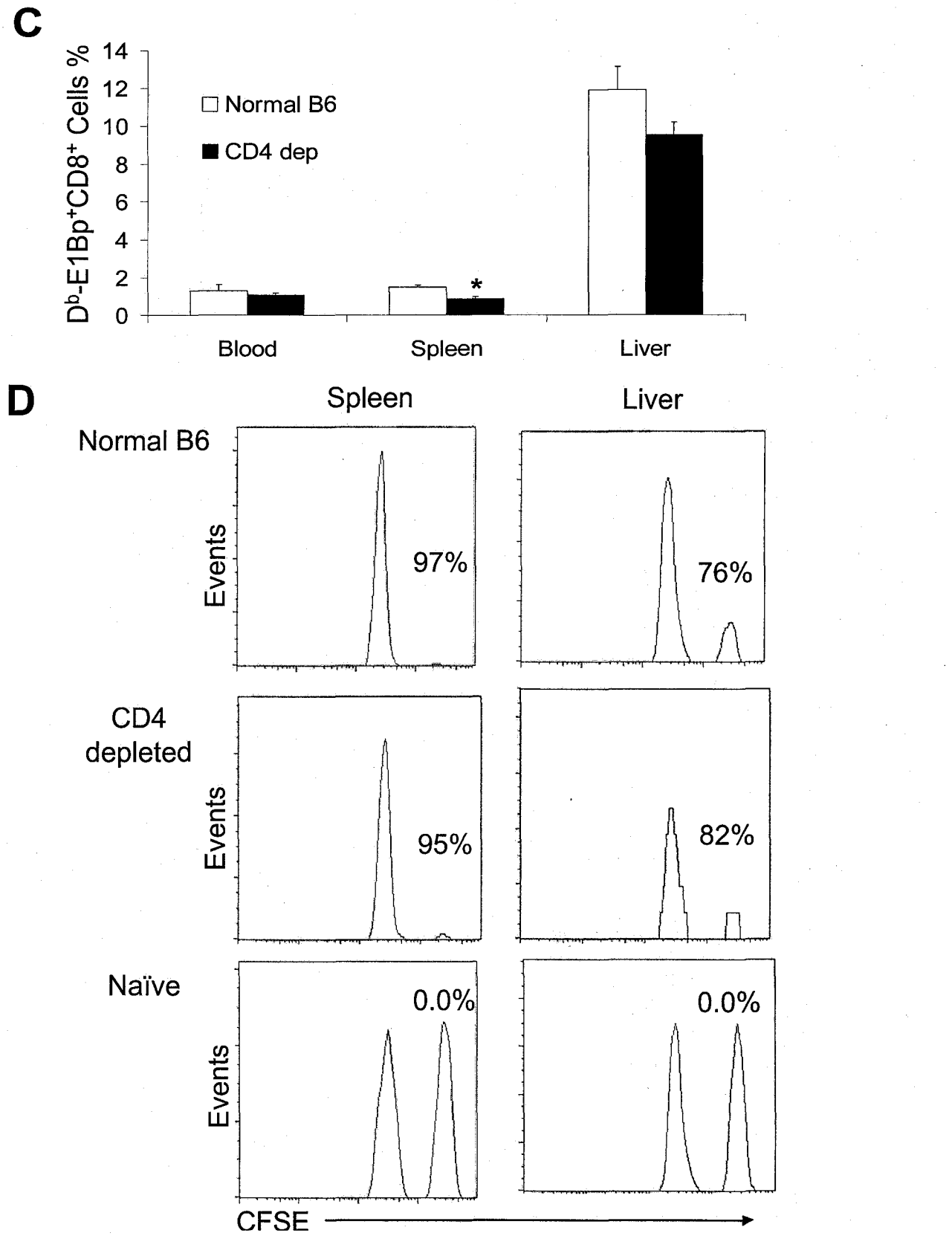
Deficiency of CD4 T cells does not alter CTL effector function – Expression of CD28 on CD8 T cells is critical for induction of Ad-specific CTL.

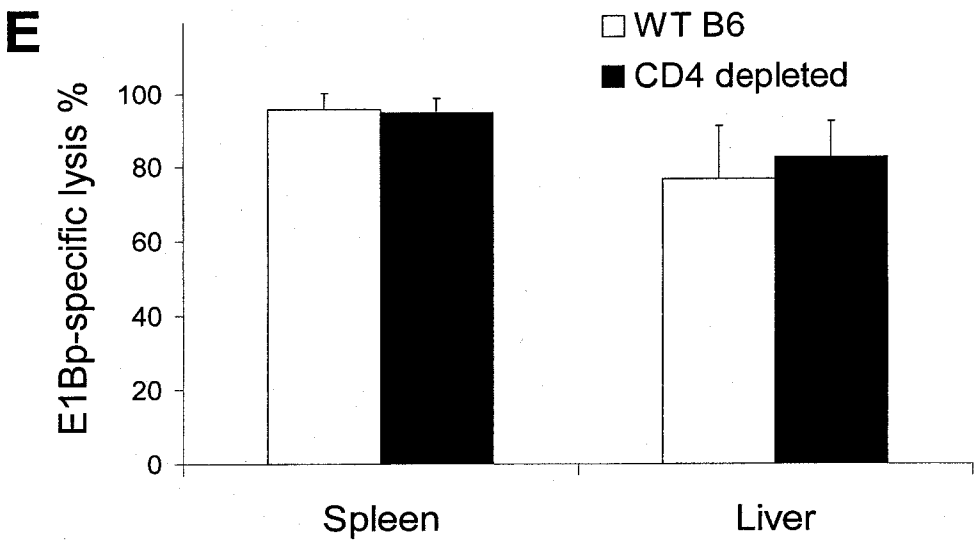
CD28$^{-/-}$ mice exhibit deficiency in T cell-dependent antibody responses and in other responses dependent on CD4 T helper-cell activity.[11] To assess if the lower number and function of CTL in response to Ad-E1Bp that was observed in *CD28*$^{-/-}$ mice can be explained by a lower CD28 induced CD4 T-helper cell function, WT B6 mice were depleted of CD4 T cells using anti-CD4 antibody (GK1.5) before and after administration of Ad. The treatment eliminated 99% of the CD4^{+} T cells (Figure 3A). To our surprise, depletion of CD4 T cells resulted in a 40% decrease in the number of D^{b}-E1Bp^{+} CD8^{+} CTLs in the spleen, but only 15% and 16% decrease in the same CTLs in peripheral blood and the liver, respectively (Figure 3B and 3C). Importantly, there was no decrease in Ad E1Bp specific *in vivo* CTL response in either the spleen or liver (Figure 3D and

Figure 3 *Expansion and effector function of Ad-specific $CD8^+$ T cells in CD4 depleted mice. B6 mice were depleted of $CD4^+$ T cells by intraperitoneal injection of anti-CD4 mAb (GK1.5) before and after adenovirus infection as described in Materials and Methods. Depletion efficiency was monitored by staining MNCs from the periphery blood, spleen and liver with anti-CD4 MAbs (RM4-5) (A). The CTL response in the spleen and liver was analyzed 8 days after Ad administration. The number of Ad-specific CD8 T cells was determined by staining with anti-CD8 Abs and D^b-E1Bp tetramers (B). The numbers represent percentages of tetramer-binding CD8 T cells among total lymphocytes. Numbers in parentheses represent percentages of tetramer-binding cells of total CD8 T cells. The mean of tetramer binding CD8 T cells are compared (C). E1Bp-specific lysis in CD4-depleted mice and normal mice was shown in (D). Numbers in the histograms represent the percentage of target cell killed. The mean of E1Bp specific killing in the spleen and liver in CD4-depleted mice and normal B6 mice are compared (E).The results are representative of 5-8 independent experiments. Data shown are the mean ± s.e.m. of 3-5 mice at each time point. * $p < 0.05$ compared to normal B6 mice.*









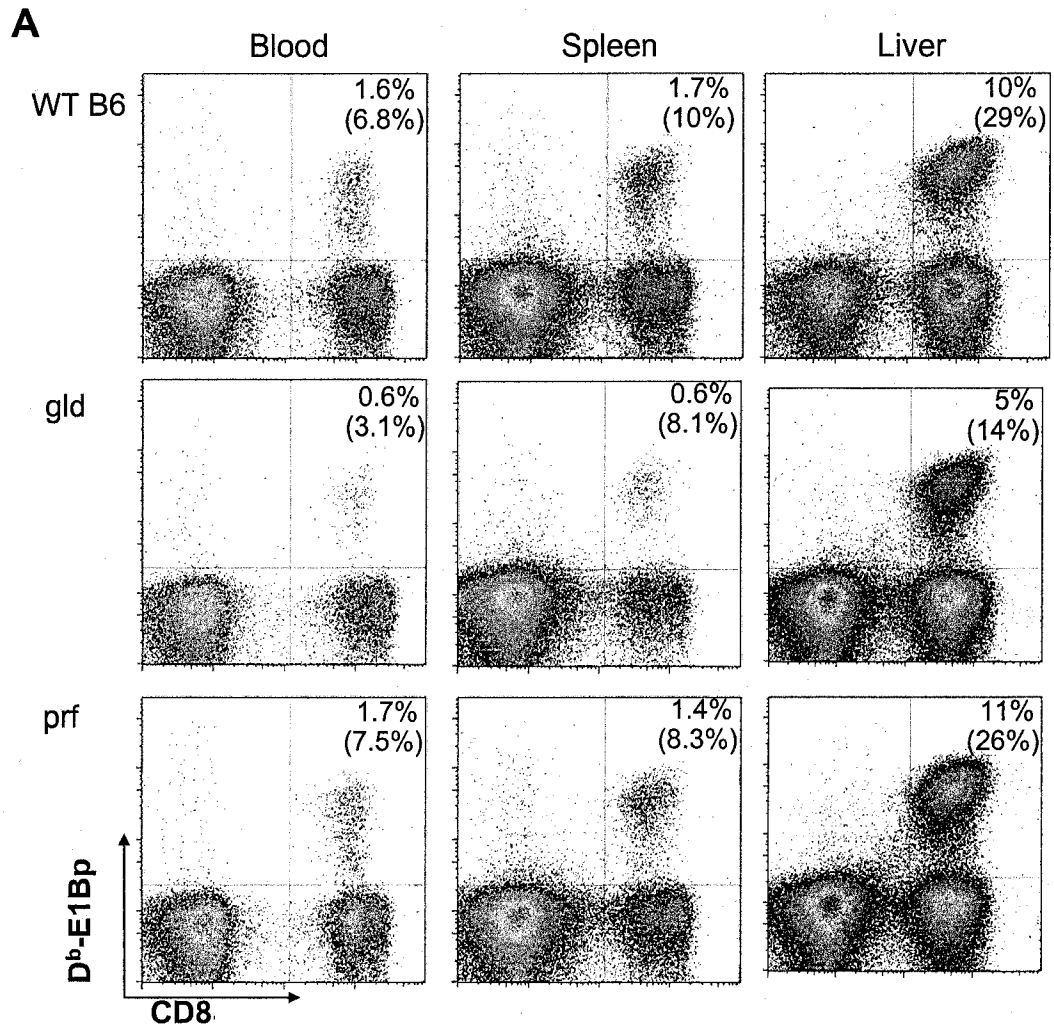
3E). The results indicate that depletion of CD4 T cells results in only a minor effect on the generation of E1Bp-specific CTLs and had no effect on the killing efficiency of E1Bp-specific CTLs. These results further suggest that the expression of CD28 on the CD8 T cells, but not on the CD4 T cells, plays an important role in the generation of both CTL effector number and function.

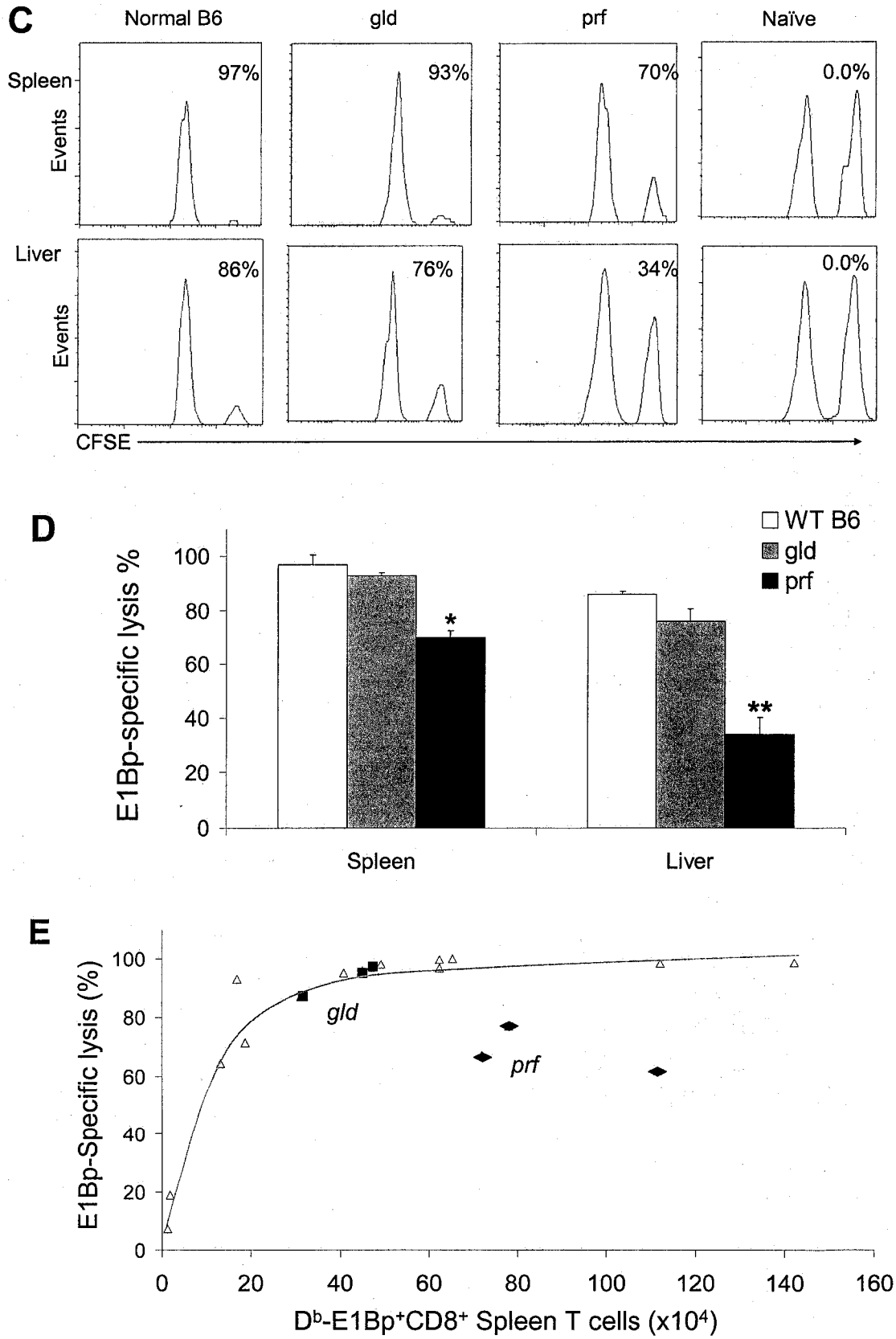
Compromised CTL cytotoxic function in prf$^{-/-}$ mice and decreased CTL generation in gld mice

The effector molecules that mediate infected target cell lysis by activated CD8 T cells are predominantly perforin/granzymes and FasL.[25] However, the major effector molecules that mediate the CTL response after administration of Ad vectors remain controversial.[18-20,26] To determine the relative roles for perforin/granzymes and FasL-Fas pathways in CTL response against Ad, perforin KO (*prf*$^{-/-}$) mice and FasL mutant (*gld*) mice were infected by Ad5 and the number of Ad specific CTLs was assessed by the D^b-E1Bp-tetramer. The function of the Ad-specific CTLs was determined by the E1Bp specific *in vivo* killing assay eight days later. As expected, the numbers of D^b-E1Bp-tetramer$^+$ CD8$^+$ T cells in the blood, spleen, and, liver of *prf*$^{-/-}$ mice were similar to those in normal B6 mice (Figure 4A and 4B). However, there was a 28% and 60% reduction in E1Bp-specific lysis in the spleen and liver, respectively, in *prf*$^{-/-}$ mice compared with that in normal B6 mice ($p < 0.05$; Figure 4C and 4D).

Compared to perforin, the lack of FasL in *gld* mice reduced both the number and function of CTLs. There was not only a 62% decrease in the number of D^b-E1Bp-tetramer$^+$ CD8$^+$ T cells in the blood and spleen, but also a 50% decrease in these CD8$^+$ T

Figure 4 *Primary $CD8^+$ T cells response against Ad in $prf^{-/-}$ and gld mice. Perforin knockout ($prf^{-/-}$) mice and FasL mutant gld mice as well as WT B6 mice were i.v. injected with $8x10^8$ i.u. of wild-type Ad5. The number of Ad-specific CD8 T cells in the periphery blood, spleen, and liver 8 days after Ad injection was determined by staining with anti-CD8 Abs and D^b-E1Bp tetramers (A). The numbers represent percentages of tetramer-binding CD8 T cells among total lymphocytes. Numbers in parentheses represent percentages of tetramer-binding cells of total CD8 T cells. The means of tetramer-binding CD8 T cells in different group of mice are compared (B). E1Bp-specific lysis in $prf^{-/-}$, gld and, WT B6 mice is shown in (C). Numbers in the histograms represent the percentage of target cell killed. The means of E1Bp specific killing in the spleen and liver in CD4-depleted mice and normal B6 mice are compared (D). The results are representative of 5-8 independent experiments. Data shown are the mean ± s.e.m. of 3-5 mice at each time point. * $p < 0.05$ compared to WT B6 mice. The cytotoxic effector function of individual CTLs from the spleen of $prf^{-/-}$, (♦) gld (■) mice, and WT B6 (Δ)mice are compared (E). Each data point represents an individual mouse.*





cells in the liver of FasL defective *gld* mice (Figure 4A and 4B). However, the E1Bp-specific lysis was either not decreased in the spleen or was decreased by only 11% in the liver of *gld* mice compared to that in WT mice (Figure 4C and 4D).

To distinguish the decrease in CTL function from its generation in these gene knockout mice, the E1Bp specific lysis against the D^b-E1Bp-tetramer$^+$ $CD8^+$ T cell numbers in the spleen of individual *prf*$^{-/-}$ or *gld* mice was analyzed in comparison with that WT B6 mice based on the function/number graph (Figure 4E). The deficiency in perforin resulted in a dramatic decrease in the CTL function on a per-cell basis whereas FasL deficiency caused a significant decrease in the generation of CTLs, but had no impact on the target cell lysis.

Synergistic function of perforin and Fas in mediating target cell lysis

Although there was a 28% decrease in specific lysis in the spleen of perforin deficient mice compared to WT B6 mice, there was still 70% lysis of target cells in *prf*$^{-/-}$ mice (Figure 4C), indicating that other mechanisms may play a role in CTL effector function in the absence of perforin. Furthermore, previous studies had shown that deficiency in the Fas pathway had no effect on the role of viral clearance, but elimination of both Fas and perforin led to uncontrolled infection.[27] To further address the relative importance of perforin and Fas pathways in target cell lysis by CTL, target cells were prepared from Fas mutant *lpr* mice or *TNFR1*$^{-/-}$*TNFR2*$^{-/-}$ mice and were transferred into *prf*$^{-/-}$, *gld* or WT B6 mice 8 days after administration of Ad. Compared with WT target cells, there was no decrease in the specific lysis of the Fas-deficient *lpr* or TNFR1$^{-/-}$TNFR2$^{-/-}$ target cells in spleen of WT B6 mice or *gld* mice (Figure 5A). However, there was a 60% decrease in

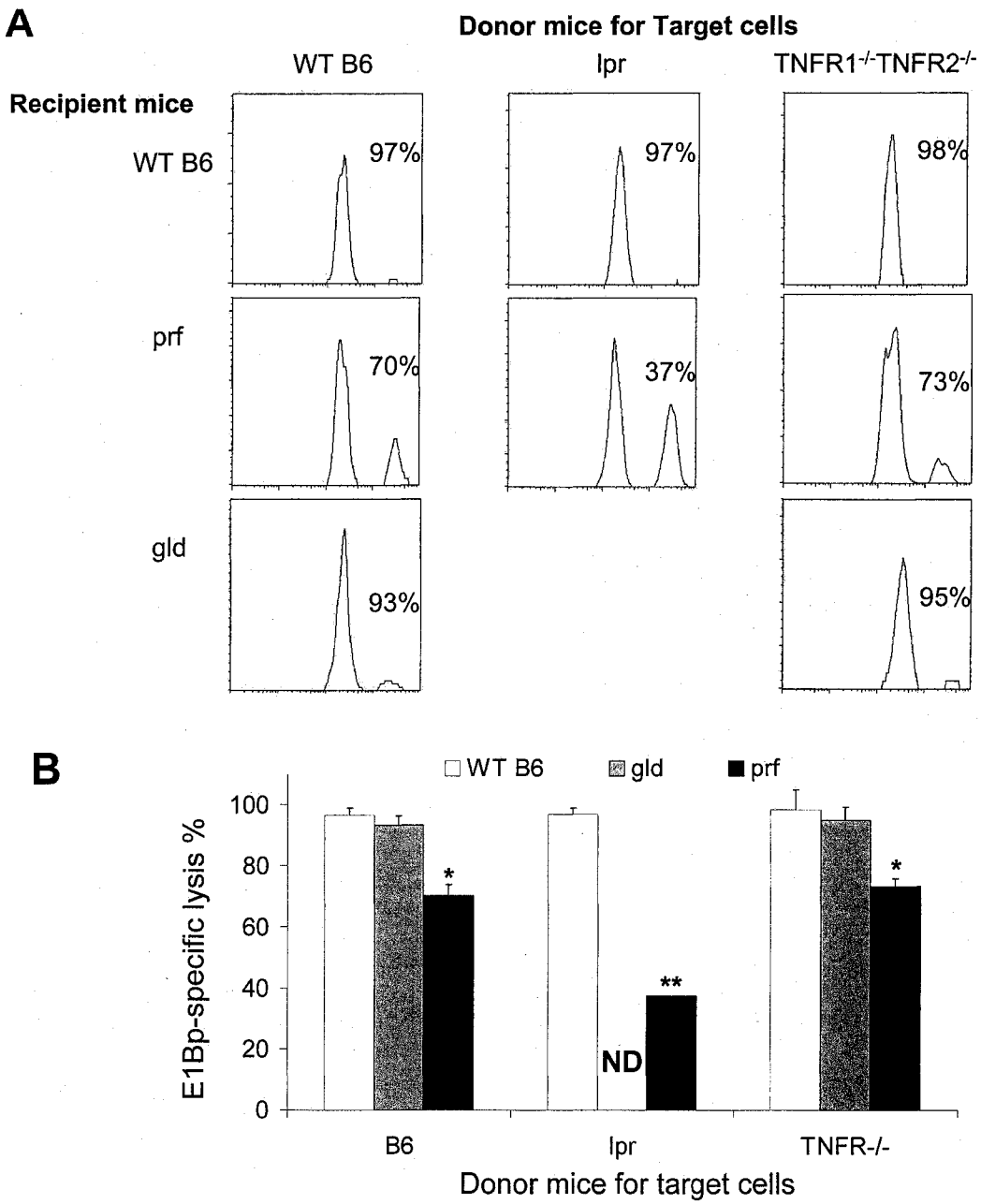


Figure 5 *Synergistic role of perforin and Fas in mediating cytolysis. Spleen cells from WT B6 mice, Fas deficient lpr mice and TNFR1$^{-/-}$TNFR2$^{-/-}$ mice were labeled with CFSE and pulsed with Ad E1Bp peptide. Target cells and control populations of cells not pulsed with peptides were then adoptively transferred into WT B6, prf$^{-/-}$, or gld mice that had been immunized with wild-type Ad5 eight days earlier (A). In vivo CTL activity in the spleen was evaluated by flow cytometry. Numbers in the histograms represent the percentage of target cell killed. The means of E1Bp specific killing in the spleen and liver in prf$^{-/-}$, or gld mice and WT B6 mice are compared (B). The results are representative of 5-8 independent experiments. Data shown are the mean ± s.e.m. of 3-5 mice. * $p < 0.05$ and ** $p < 0.001$ compared to WT B6 recipient mice. ND indicates not determined.*

specific lysis when the effector cells were deficient in perforin and the target cells were deficient in Fas (Figure 5B). These results indicated that perforin/granzymes are the major effector molecules for the induction of an anti-Ad CTL response and that FasL-Fas pathway deficiency alone has low effect on target cell lysis. However, these two pathways may play synergistic roles in mediating target cell death.

Discussion

In this study, we first examined the requirement for CD28-mediated costimulation in the activation, expansion, and effector function of Ag-specific CD8 T cells during the primary response against Ad infection. We show that the activation and expansion of Ad-specific CD8 T cells is largely dependent on the CD28 expression on CD8 T cells. We also determined the relative role of the effector molecules of Ad-specific CD8 T cells. Our results show that perforin/granzymes mediated target cell lysis is the major pathway for CTL effector function. Specific cytolysis by activated CD8 T cells could be independent of Fas-medicated cell death, however, Fas pathway exhibits synergistic activity with the perforin pathway in mediating target cell cytolysis.

The role of the CD28 co-stimulatory pathway in T cell activation has been extensively studied both *in vivo* and *in vitro*. Previous studies have shown that *CD28*$^{-/-}$ mice are deficient in T cell-dependent antibody response and other responses that depend on CD4 T-helper activity,[11,28] indicating the importance of CD28 signaling in the CD4 T cell system. In contrast, much less is known with regard to the role of CD28 in the activation of CD8 T cells. Although a number of *in vitro* and *in vivo* studies have demonstrated that CD28-B7 interactions are not essential for CD8 T cell activation,[5,6,10-12,29] others have

shown that the primary CD8 T cell response was affected in absence of CD28-B7 interaction.[7,9,10]

Thummala et al. [30] have previously shown that coexpression of CTLA4-Ig permits prolonged expression and repeatable gene transfer by an adenoviral vector. CD28 exhibits a number of possible functions that may become a limiting factor for prolongation of Ad gene therapy. First, the costimulatory function of CD28 on CD4 T-helper cells may help the production of IL-2 for the generation of CD8 T cells.[31] Second, CD28 on precursor CD8 T cells may sustain CD8 expansion and effector function.[28,32] Third, the costimulatory function of CD28 on CD4 T-helper cells may enhance the production of IL-4 and CD40L that may enhance the production of Ad-specific B cells to prevent subsequent Ad challenge.[33,34] Due to this complexity, the present study focused on addressing the role of CD28 in the primary CTL response of Ad infection. These analyses have not previously been possible since there have been no tetramers available to measure the precise number of CTLs and to correlate this number with the Ad-specific CTL effector functions. These limitations are overcome by the present studies in which an Ad-specific peptide/MHC tetramer and an *in vivo* Ad-E1Bp-specific killing assay were applied.

Activated/memory CD8 T cells express elevated levels of CD44 ($CD44^{high}$). The activation and expansion of CD8 T cells following Ad infection was first determined by flow cytometry after staining lymphocytes from WT B6 and *CD28*$^{-/-}$ mice for CD8 and CD44. Compared with WT mice, there was a very weak activation of $CD8^+$ T cells in the spleens of *CD28*$^{-/-}$ mice. The total number of activated CD8 T cells in the liver and lung of *CD28*$^{-/-}$ mice was 3- and 10-fold lower, respectively. A more precise analysis of the activation and expansion of Ad-specific CD8 T cells was performed using the D^b-E1Bp

tetramer. On day 8 after infection, there was 4~5 fold decrease in the D^b-E1Bp$^+$CD8$^+$ T cells in different tissues of *CD28*$^{-/-}$ mice compared to that generated in WT B6 mice. These data are similar with the decrease in LCMV-specific [9] and VSV-specific CTL response in *CD28*$^{-/-}$ mice.[10]

The CTL function *in vivo* was determined using CFSE labeled, E1Bp-pulsed target cells. *CD28*$^{-/-}$ mice exhibited significant defect in E1Bp-specific lysis of target cells in the spleen and liver at day 8 as compared with WT B6 mice. The impaired CTL response *in vivo* could be a result of either a defect in generation of sufficient numbers of effector CTLs, defects in the cytotoxic function of CTL, or a combination of both events. In the present studies, the relative decrease in number versus function was addressed using the CTL function/number plot. This is made possible since the dominant immunologic epitope E1Bp is used to specifically bind to the class I MHC molecule H2-D^b antigen groove on the labeled target cells or confer recognition of the D^b-E1Bp$^+$CD8$^+$ T cells. Interestingly, we have estimated that the *in vivo* CTL function is lower than that expected for the decrease in CTL number in *CD28*$^{-/-}$ mice. This result indicates that there is both a decrease in the number of CTLs and a decrease in cytotoxic CTL function per cell in the absence of CD28. These data are in agreement with the observations made by Vermeiren et al. [35] who showed that T cells in which activation had been blocked during the priming phase with anti-CD40 and anti-B7 antibodies were unable to affect the production of IFNγ by Ad-mIFNγ-transduced cells or to kill the Ad-GFP-transduced cells.

The cellular and molecular mechanism for the decrease in the CTL response in *CD28*$^{-/-}$ mice has not been characterized. The present results show that depletion of CD4$^+$ T cells in B6 mice has minimal impact on generation and effector function of the primary

CTL response to Ad infection. These data are consistent with observations by Harding et al. [28] who showed that the primary CTL response is independent of CD4 help. This indicates that loss of CD28 expression on $CD4^+$ T cells cannot account for the decrease in CTL generation and function in *CD28*$^{-/-}$ mice. Our results thus suggest that the expression of CD28 on precursor CTL may exhibit direct effect on CTL generation and function. Our results also demonstrate that deficiency of CD28 exhibited differential effects on the percentage of Ad-E1Bp-specific CTL in different tissues. The percentage of Ad-E1Bp-specific CTLs was most impaired in lung compared to PBMC, spleen, and liver; results suggesting that CD28 may promote a tissue-dependent effect on CTL migration. It remains to be determined what effector molecules are affected under conditions of CD28 deficiency.

The primary mediators of CTL function after virus infection have previously been found to be FasL and perforin/granzyme B.[16] Nakamoto et al. [36] showed that hepatitis B-mediated CTL was reduced in *gld* mice. Chirmule and colleagues have further demonstrated that Fas-FasL interaction plays a major role in the liver [37] but plays no role in effector functions of cytotoxic T lymphocytes in the lung [38] after Ad vector-mediated gene transfer. However, these previous investigations did not consider the possibility that fewer CD8 T cells were being generated. One major concern has been the reliance on FasL knockout *gld* mice or Fas-mutant *lpr* mice, which are known to spontaneously develop autoimmune diseases, exhibit a low capability to produce IL-2,[39] and did not produce a normal CTL response.[40] Therefore, a likely mechanism for the decreased CTL response in *gld* mice may be a decrease in cell number rather than a decrease in function. In most situations, the role of perforin is less controversial, since *prf*$^{-/-}$ mice have normal

numbers of CTLs after virus infection as determined by virus MHC/peptide tetramers, but exhibit decreased CTL activity and impaired viral clearance.[41-44] However, it has previously been reported that after Ad infection, perforin was dispensable, nevertheless, FasL and TNFα play a major role in immune mediated clearance of Ad vectors.[18] To resolve these controversies, we applied the MHC class I tetramer staining method and an *in vivo* killing assay and correlated the results from these two methods in analysis of the CD8 T cell response in *prf*$^{-/-}$ and *gld* mice. In *gld* mice, the generation of E1Bp-specific CD8 T cells reduced by about 60% and the infiltrating D^b-E1Bp$^+$CD8$^+$ T cells in the liver also reduced by 50% at day 8 after infection compared with WT B6 mice. To our surprise, the E1Bp-specific lysis in the spleen and liver in *gld* mice were equivalent to that in WT B6 mice, indicating that FasL deficiency alone has no impact on the cytotoxic function of CTLs, but causes a defect in the generation of CTL in response to Ad infection. These data are similar with the observations made by Topham et al. [44] and Parra et al.[27]

As opposed to the observations in *gld* mice, *prf*$^{-/-}$ mice exhibited a 27% and a 60% decrease in E1Bp specific lysis in the spleen and liver, respectively, whereas the number of D^b-E1Bp$^+$CD8$^+$ T cells remained the same as in WT B6 mice at day 8 after Ad infection. Noticeably, in the absence of perforin, there was still 70% lysis of target cells in the spleen. Because the adoptively transferred target cells only present one epitope peptide, E1Bp, the lysis of target cells can only result from contact-dependent effector mechanisms which are mainly mediated by perforin, Fas, or both. Previous studies by others provided evidence showing that T cells from *prf*$^{-/-}$ mice can utilize death receptor-mediated lysis, which is usually dependent on caspase activation, to delete target cells

and that the *in vitro* lysis of A20 cells mediated by *prf*$^{-/-}$ T cells could be blocked by neutralizing antibodies to FasL.[45]

To determine if *prf*$^{-/-}$ T cells utilized Fas medicated cell death pathway for the lysis of Ad-E1Bp infected target cells *in vivo*, we employed target cells isolated from Fas deficient *lpr* mice and *TNFR1*$^{-/-}$*TNFR2*$^{-/-}$ mice and separately transferred these cells into *prf*$^{-/-}$, *gld*, or WT B6 mice. Our results indicate that TNFR deficiency has no effect on the lysis of target cells in WT B6 mice or *gld* mice. However, there was a 60% reduction in killing efficiency of Fas-deficient target cells by *prf*$^{-/-}$ mice. Our results support the previous findings by Walsh et al. [26] and suggest that the Fas-mediated lytic pathway is the most critical alternate pathway for effector cytolytic response after a primary virus infection.

In summary, our findings have important implication for Ad gene therapy since not only is the CTL response a major limitation for long-term efficacy of Ad gene therapy, but also CTL can directly induce hepatotoxicity in certain susceptible recipients. Previous studies have shown that transient blockade of CD28-B7 interaction by CTLA4-Ig suppressed the activation of CD4 T cells and antibody response and prolonged transgene expression.[3,4,46,47] However, these experiments did not demonstrate if CD28 costimulation exhibits direct impact on the primary CTL response to Ad-infected target cells. Therefore, until now, it has been unclear if the prolonged transgene expression is due to a reduced antibody response or an inhibition of CTL response. The present results provide evidence to demonstrate the importance of CD28 costimulation on Ad-E1Bp-specific CTL generation and effector function. These findings may help to define more

precise targets for immunosuppressive modulation as well as for elimination of undesirable cytotoxicity induced by the host CTL response.

Acknowledgements

We thank Carol Humber and Jennifer Allen for excellent secretarial work. This work was supported in part by National Institutes of Health Grants RO1-AI-42900 (to J.D. Mountz) and RO1-CA-86881 (to D.T.Curiel); by a VA Career Development Award and Merit Review Award (to J.D.Mountz).

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DISCUSSION

The main goals of this dissertation were three-fold: first, to identify and validate CTL specific epitopes from Ad proteins; second, to apply these specific epitopes to the analysis of the CTL response against Ad infection; and third, to determine the mechanisms underlying Ad-specific CTL generation and effector function. Data were obtained for each of these goals and described in the papers that are presented in this dissertation. Here the implications of these data are discussed.

The immune response to Ad gene therapy has been a major factor limiting long-term gene expression.[7,16,28] The immune response also contributes to systemic toxicity and is one of the factors that limit the safety of gene therapy delivery.[19,71]

Nonspecific immunosuppressive drugs including cyclophosphamide,[72] cyclosporine,[73] and FK506,[74,75] have been shown to reduce the T cell immune response. Other specific strategies include blocking of the co-stimulatory signaling activity delivered by CD28 using the CTLA4 immunoglobulin Fc (CTLA4-Ig) and blocking CD40-CD40 ligand interaction with the MR1 anti-CD40L antibody.[49] This treatment resulted in a decrease in the immune response after Ad administration and led to a prolonged expression of the Ad transgene in the presence of either sCTLA4-Ig or in the presence of an Ad that expresses CTLA4-Ig.[49,51,76] Although the decreased immune response has been documented by decreased anti-Ad antibodies or decreased killing of Ad-infected targets *in vitro*, the mechanism and specificity of the lower immune response was not determined. Therefore, it remains uncertain if the decreased immune response was due to a decrease

in the innate immune response or a decrease in the primary T cell immune response. Also, it is not clear if these immunosuppressive agents also suppress the response to the vector or the transgene. Finally, the mechanism of the decreased specific CTL activity was not determined. This is because the assays are not specific for the vector or transgene, and the vector delivery system can affect the response to both the vector and transgene.

These limitations are partly due to a poor understanding of the Ad-specific CTL response. Until now, the analysis of the CTL response could only be explored using indirect approaches, i.e., the duration of transgene expression, infiltration of $CD4^+$ and $CD8^+$ T cells in the infected tissues, *in vitro* CTL assay using spleen cells, or hepatic toxicity assayed by serum metabolic enzymes.[69,70,77,78] This has made it difficult to investigate the Ad CTL response and the mechanisms underlying its generation and effector functions.

Thus, there is a need for the development of specific methods that can enable detection of CTL function by a specific killing assay or the number of the specific CTLs by an MHC/peptide tetramer. To accomplish these objectives, it is first necessary to identify antigen epitopes of the viral proteins as well as the transgene products that are capable of binding to the class I MHC and specific stimulation of T cells.

The antigen peptide epitopes that are presented in the context of the class I MHC molecule and recognized by the specific TCRs, could be derived from both the transgene and viral gene encoded proteins. The T cell receptor on CD8 T cells recognize these epitopes that are presented in the cleft of MHC class I molecules at the surface of the antigen presenting cells or infected target cells. Thus, identification of the antigen peptides is essential for the study of the specific CD8 T cell response.

In previous studies, two MHC restricted CTL epitopes have been identified from Ad E1A and E1B encoded proteins that bind to mouse H2-D^b.[33-35] These previously identified peptide epitopes of E1A and E1B were used to analyze the response to wild-type Ad.[79] However, most Ad gene therapy vectors, except some conditionally-replicative Ad vectors, have deletions of E1A and E1B regions.[6] Therefore, to derive epitopes that are more applicable to Ad gene therapy vectors, the present studies have aimed at defining epitopes for protein components of Ad capsid, including hexon, penton and fiber.

Computer-driven algorithms are now routinely employed to predict potential CTL epitopes from antigenic proteins as reviewed by Martin et al. [80] and Nussbaum et al.[81] These prediction programs have been developed based on the following features of binding peptides: (a) charge, (b) hydrophobicity, (c) 3-D structure of the individual amino acid, and (d) available cleavage sites of the linear polypeptides. These tools offer a significant advantage over other methods of epitope selection because high-throughput screening can be performed, which is followed by confirmatory studies *in vitro* and *in vivo*. CTL epitopes discovered using these tools have been used to develop novel vaccines and therapeutics for the prevention and treatment of infectious diseases and some cancers.[32,36,39,82,83]

In this study, two prediction programs, BIMAS [84] and SYFPEITHI [85], which are based on different algorithms have been used to predict CTL epitopes from Ad capsid proteins. Two sensitive methods, IFN-γ ELISPOT assay and *in vivo* CTL assay, were employed to test and validate these predicted peptides. These two specific methods provide complementary results for each other in that the ELISPOT detects the ability of peptide/MHC complex to activate CD8 T cells via the detection of IFN-γ secretion by acti-

vated T cells,[86] whereas the *in vivo* CTL assay detects the ability of the specific effector cells to kill the peptide-bearing target cells. The present method used for the identification of CTL epitopes provides an additional advantage in that the specific genetic loci for K, D and, L encoding each MHC class I molecule can be analyzed for potential binding. Knowledge of the specific class I MHC locus responsible for the CTL response to a particular antigen is important for subsequent tetrameric MHC class I/peptide complex development.[87] Therefore, in the present experiments, the K^b and D^b class I MHC structures were analyzed for the predictive binding to all 8-mer, 9-mer, and 10-mer peptides contained in the hexon, penton, and fiber. This lead to the analysis of 29 predicted peptides by IFNγ ELISPOT assay and *in vivo* CTL assay. This resulted in the identification of an H2-D^b-restricted epitope peptide (*391 VGNKNNDKL*), from Ad fiber, which will enable us to develop MHC/peptide tetramers and then to further assess Ad vector-induced CTL response.

Since the MHC class I/peptide tetramer technology was first introduced in 1996,[87] it has been widely applied in the quantification and characterization of CTL response induced by viral or bacterial infections and tumors.[88] Analysis with the tetramers has demonstrated very clearly that antigen-specific CD8 T cells concentrate at foci of maximum antigen production. The prevalence of CD8 T cells specific for an immunodominant epitope can range from 15% (influenza virus infection) [89] to 70% (Sendai virus infection) [90] of the CD8 T cells obtained from the pneumonic lung. Yee et al. [91] used a tetramer staining method to show that tumor-specific CD8 T cells can be found at frequencies of 2–5% in some melanoma patients. The MHC/peptide tetramer can also be utilized to sort monoclonal CTL lines that can recognize tumor cells. These tetramer-sorted cells can be grown

to large numbers, suitable for immunotherapy for cancers.[92] In this dissertation, the tetramer binding method has been employed to analyze the primary CTL response after Ad administration. An *in vivo* CTL assay was also applied to directly investigate and distinguish the generation and effector functions of Ad-specific CD8 T cells.

A major focus of this work was to carry out a comprehensive analysis of Ad-specific CTL response. Administration of Ad gene therapy vectors results in several aspects of inflammatory response including: (a) capillary leaking, (b) viral infection of endothelium, parenchymal tissues and antigen-presenting cells, (c) release of innate response factors including cytokines, chemotactic factors, (d) upregulation of adhesion molecules that enable subsequent specific cell infiltration, contact, and interactions, (e) initiation of the onset phase of the adaptive response, and (f) CTL generation in the spleen. Prior to the studies in this dissertation, it has been difficult to differentiate the specific versus the bystander CD8 T cell response. To address this question, the Ad-specific tetramer, D^b-E1Bp, was used to enumerate the number of Ad-specific CD8 T cells, and an *in vivo* CTL assay was performed to determine the CTL function. With these two specific methods, I was able to present the first analysis of and correlation between the number of E1Bp tetramer-positive CD8 T cells and the ability of these CTLs to specifically kill E1Bp-labeled target cells *in vivo* in the spleen and liver, respectively. In these experiments, bystander toxicity was minimized since killing of E1Bp-pulsed target cells is compared to that of unpulsed target cells. Correlation of these two specific and sensitive approaches can be used to distinguish a low number of highly cytolytic T cells from a higher number of T cells with lower cytolytic capacity per cell. This enables separate determination of the regulatory mechanisms involved in the generation and function

of CTLs. The implication of different molecular pathways to regulate the generation and/or function of CTLs based on this study is discussed below.

In agreement with previous reports,[93-95] the results of the present studies clearly showed that the peak of the virus clearance and liver enzyme elevation occurred before a significant number of Ad-specific CTLs were generated, indicating that the innate immune response accounts for most of the virus clearance after a primary response. Surprisingly, in spite of the clearance of the majority of virus after even a moderately high dose of Ad vectors (10^{10} infection unit/mouse), there are still more than 95% of hepatocytes transduced by Ad vectors.[96] These cells can survive the innate immune response but become the targets for $CD8^+$ CTLs. The importance of this CTL response was further supported by the observation that in immunocompetent mice, transgene expression rapidly declines to baseline levels by 2 to 3 weeks after infection.[7,97]

Our observation of the detectable, stable levels of virus in the liver during and after the peak of the CTL response was unexpected, and indicates that during a primary response, some of the Ad-infected cells that survive the innate immune response are resistant to the CTL lysis *in vivo*. This is consistent with a previous report that an Ad vector expressing human alpha1-antitrypsin administered by intravenous injection stimulated an Ad-specific cellular immune response but this response failed to abolish vector-directed gene expression *in vivo*.[98] There are several mechanisms that may account for this resistance (Figure 1). First, there may be a production of factors that can inhibit CTL-mediated apoptosis of liver cells as reported by Belz et al.[99] This was supported by our previous findings that soluble Fas could inhibit the CTL response in the liver.[100] Second,

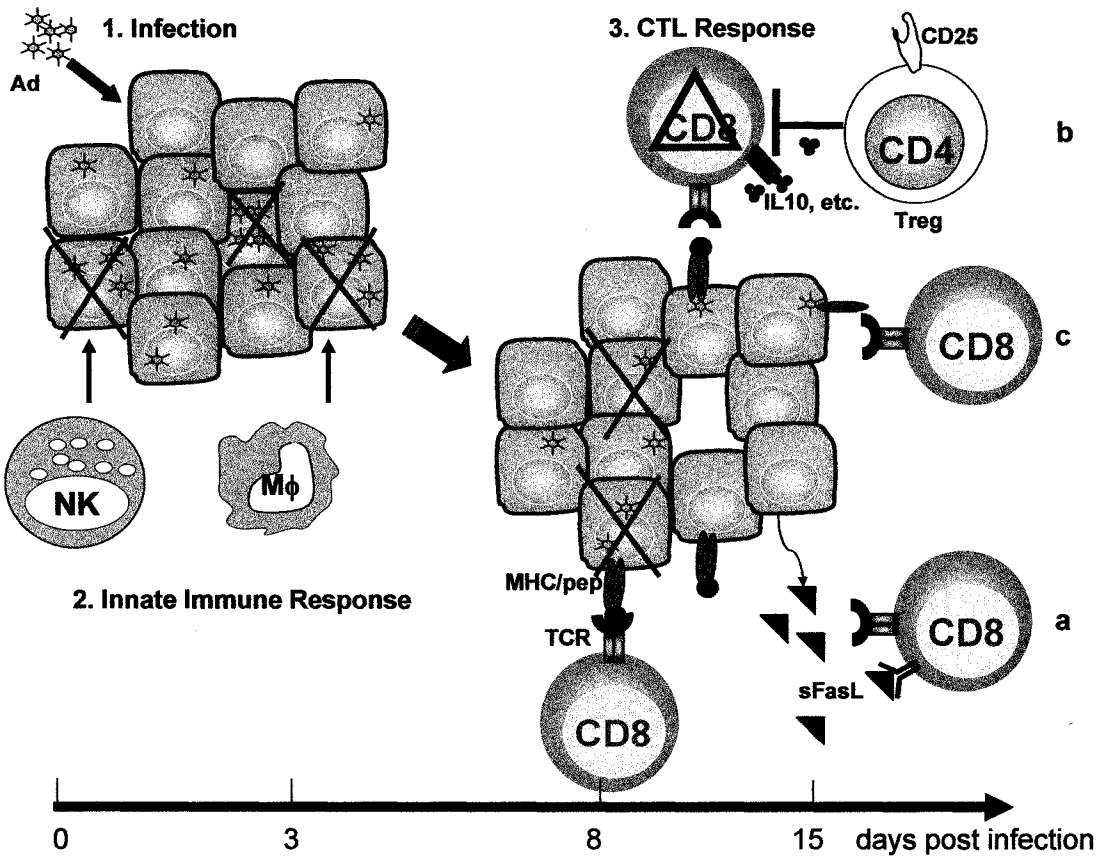

Figure 1 *The innate immune response leads to development of CTL resistant hepatocytes. Infected cells that have survived the innate immune response are susceptible to CTL induced lysis. However, some cells are resistant to CTL killing. The possible mechanisms include: (a) liver cells secretion of soluble FasL (sFasL) to induce activated cytotoxic CD8 T cell apoptosis; (b) development of $CD4^+CD25^+$ T regulatory cells that inhibit the CD8 T cell response by secreting inhibitory cytokines, such as IL-10, TGFβ, and unknown mechanisms; and (c) infected liver cells down regulation of class I MHC antigen presentation.*

$CD4^+CD25^+$ regulatory T cells have also been proposed to play an inhibitory role in suppression of CTLs in the liver.[101] Another mechanism that contributes to development of CTL-resistant liver cells is through down-regulation of antigen-presentation.[102] This failure of infected cells to be eliminated may have beneficial effects since it might promote an even stronger CTL response or promote the generation of memory CTLs since complete clearance of antigen might actually diminish subsequent CTL response. It would be important to know if there are residual viruses in tissues other than the spleen and liver, such as the lung.

With the ultimate goal of determining the major factors that regulate the specific CTL response to Ad infection, the requirement for CD28-B7 interaction in CTL generation and function was assessed. The results showed that there was a very weak activation of $CD8^+$ T cells in the spleens of *CD28*$^{-/-}$ mice along with an impaired CTL response *in vivo*. These data are similar to those previously reported for the decrease in lymphocytic choriomeningitis virus-specific [46] and vesicular stomatitis virus-specific CTL response in *CD28*$^{-/-}$ mice.[47]

The impaired CTL response *in vivo* could be a result of either a defect in generation of sufficient numbers of effector CTLs, defects in the cytotoxic function of CTL or a combination of both events. Previous work by Vermeiren et al. [103] showed that T cells in which activation had been blocked during the priming phase with anti-CD40 and anti-B7 antibodies were unable to induce transcription of an IFNγ promoter-reporter by Ad-mIFNγ or to kill the Ad-GFP transduced cells. In the present study, this question was addressed with an MHC I/peptide tetramer staining method to determine the precise number of CTLs and use of the antigen-specific peptide to assess the CTL function *in vivo*. In

agreement with previous studies, the *in vivo* CTL function is lower than that expected for the decrease in CTL numbers in *CD28*$^{-/-}$ mice, indicating that there is both a decrease in the number of CTLs and a decrease in cytotoxic function per cell in the absence of CD28. Thus, we proposed that the blockade of CD28-B7 interaction may result in both a defect in the proliferation and the differentiation of Ad-specific CTLs. However, it remains to be determined what effector molecules are affected under conditions of CD28 deficiency.

Another important target for immunomodulation of the CTL response is the CD4 T cell. Based on the knowledge that CD4 T cell help is necessary for CTL generation, Wilson et al. and others have depleted CD4 T cells to prolong transgene expression.[52,53] However, the mechanism of prolonged transgene expression after CD4 T cell depletion is not clear. Many studies have shown that CD4 T cell help is not necessary for the primary CTL response. Shedlock et al. found that in CD4 T cell-depleted or MHC class II$^{-/-}$ mice, *L. monocytogenes* infection-induced CD8 T cell activation and primed epitope-specific CD8 T cells to levels commensurate with those in normal B6 mice.[104] Matloubian et al. also showed that CD4 T cell-depleted mice were capable of generating a lymphocytic chorio meningitis virus-specific CTL response and eliminated virus with kinetics similar to those for wild-type mice.[105] In agreement with these previous reports, the present studies showed that depletion of CD4 T cells in B6 mice had a minimal impact on the generation and effector function of the primary CTL response to Ad infection. This also indicates that the loss of CD28 expression on CD4 T cells cannot account for the decrease in CTL generation and function in *CD28*$^{-/-}$ mice. Our results thus suggest that the expression of CD28 on CTL precursors may be necessary for optimal CTL generation and function.

FasL has been proposed to be an important mediator of the CTL response.[106] FasL acts through Fas to activate caspase 8 and caspase 3 and induce apoptotic cell death. FasL is extensively produced by CTLs and is therefore a strong candidate for potent CTL activities. However, it has been difficult to quantitate the killing activity of FasL compared to other CTL effector molecules present in the CTL, such as perforin/granzymes, TNFα and IFNγ, which potentiate CTL pathway and induce lytic activity. Zhou et al.[107] previously showed that the absence of Fas-ligand has no significant effect on the kinetics of proliferation and functional inactivation of virus-specific CD8 T cells in the onset of chronic lymphocytic chorio meningitis virus infection. However, Tamar et al.[108] and Suzuki et al.[109] have shown that FasL is not only an inducer of death, it is also a costimulator of peripheral T cell activation and an accessory molecule in positive selection of thymocytes. The major obstacle to understand if Fas is essential for CTL function is that it is also essential for homeostatic maintenance of normal immune function. Mice deficient in FasL not only develop autoimmune disease, but exhibit a defect in specific immune response.[107] Suzuki et al.[110] showed that CD8 T cells derived from gld mice are depressed in antigen-specific proliferation compared with normal CD8 T cells. To determine the relative role of these effector molecules, perforin, FasL and TNFα, in CTL-mediated target cell lysis, the CTL function in gene knockout mice was determined. The results showed that blocking of the Fas-mediated apoptosis pathway by removal of either FasL or Fas, has very little impact on the cytotoxic function of CTLs. However, a deficiency in FasL resulted in a defect in the generation of CTLs in response to Ad infection. These data are in agreement with the observations made by Suzuki et al.[110]

As opposed to the observations in *gld* mice, *prf*$^{-/-}$ mice exhibited a compromised effector function, whereas the number of CTLs remained the same as in wild-type B6. To determine if *prf*$^{-/-}$ T cells utilized the Fas-medicated cell death pathway for the lysis of Ad-E1Bp-infected target cells *in vivo*, we performed the *in vivo* CTL assay by using *prf*$^{-/-}$ CTLs to kill *Fas*$^{-/-}$ or *TNFR1*$^{-/-}$*TNFR2*$^{-/-}$ target cells. Our results indicate that there was a further reduction in killing efficiency of Fas-deficient target cells in *prf*$^{-/-}$ mice. Our results support the previous findings by Walsh et al.[111] and suggest that the Fas-mediated lytic pathway is an alternate pathway for the effector cytolytic response after a primary virus infection.

The present work has shown the potential of the class I MHC/peptide tetramer and *in vivo* CTL assay in the analysis of vector-induced CTL response. We next plan to extend our expertise to the following studies.

One of the major obstacles facing gene therapy is not only to permit long-term expression of the transgene by minimizing the primary immune responses, but also to decrease toxicity associated with gene therapy. The importance of this goal has been understood by the death of a 18-year old patient in an Ad trial [112], who succumbed to the Ad vector related toxicity. It would be a major advance to be able to predict the pre-existing immune response of an individual to a particular gene therapy. Therefore, one important application of tetramer technology would be to predict the magnitude of the CTL response that might occur upon administration. In combination with ever-increasing knowledge of T cell epitopes of Ad, the present results demonstrate the feasibility of the development of class I MHC/peptide tetramers in humans with different MHCs to determine the percent and phenotype of T cells specific for a proposed gene therapy.

The second potential clinical application of the present work is the demonstration that the use of a tetramer combined with the analysis of CD127 (IL-7 receptor α-chain) expression can predict the magnitude of the CTL response. Following acute exposure of naïve mice, there is homeostatic maintenance of memory CD8 T cells that are derived from the $CD127^{hi}$ population.[113] We would predict that patients with the highest number of tetramer-positive and $CD127^{hi}$ T cells would be at especially high risk for forming a brisk antivirus CTL response upon re-challenge. Armed with this knowledge, gene therapy could be individually tailored to patients with different HLA haplotypes to avoid a potential dangerous memory T cells response.

The third potential clinical application of the present work is to develop a novel immunoablation strategy by alternating the viral immunogenic epitope to influence the presentation of the viral epitope to its specific CTLs. This would be a potential way to suppress the specific gene therapy vector CTL response without inducing a non-specific immune suppressive effect. It has been proposed that the host genetic factors play an important role in differentiating the susceptibility of host-immune responses to viral infection and that the MHC (or HLA locus in humans) have been shown to be one of the major clusters of genes influencing this susceptibility.[114-116] On the other hand, previous studies demonstrated that viruses can escape immune attack by changing their structural features.[117-120] Ossendorp et al.[121] showed that a single amino acid change from lysine (K) to arginine (R) in a Friend Moloney-Rauscher viral peptide aborted antigen processing. Therefore, it is conceivable that modification of viral antigenic protein can ablate the antigen presentation by the MHC molecule, thus attenuating the immune response. Previous investigations have shown that the mutation of the viral proteins do not affect their capa-

bility to replicate or infect the hosts, suggesting the possibility to alter the Ad antigenic epitope to suppress the host-immune response without affecting its efficiency of inducing the transgene expression.[122-125] As an example, we used the prediction programs to estimate the binding affinity of a previously identified Ad hexon peptide (*913 TLLYVLFEV*) to human HLA A0201 molecule (Table 1). After substitution of the second amino acid L (leucine) to I (isoleucine), which has a similar electro-chemical feature with L, the binding affinity of this peptide is reduced by 85% as predicted by BIMAS. The binding affinity is further reduced by substituting L with a small, hydrophobic amino acid, G (glycine). Future proof-of-principle studies will help to develop new specific immunomodulation strategies to help prolong the Ad-mediated gene therapy transgene expression.

Table 1 Predicated binding affinity of mutant adenovirus hexon peptide to HLA-A0201 by BIMAS program

Protein	Peptide sequence	Score*
Wild-type		
	TLLYVLFEV	3142
Mutant		
	TILYVLFEV	472
	TGLYVLFEV	22

* The score indicates the estimate of half-time of dissociation of a molecule containing this subsequence.

CONCLUSIONS

In this dissertation, several advanced technologies were employed to analyze the Ad specific CTL response. Ad capsid protein sequences were screened using computer-driven algorithms for MHC binding peptides followed by experimental validation with two sensitive methods: IFNγ ELISPOT assay and *in vivo* CTL assay. An Ad fiber-derived peptide was identified as the CTL epitope restricted by mouse H2-D^b molecule.

In the studies described here, an Ad-specific MHC/peptide tetramer, D^b-E1Bp, was developed and applied in the analysis of the primary Ad-specific CTL response in combination with the *in vivo* CTL assay.

After the primary infection, a strong CTL response was developed against Ad. There was a significant increase in D^b-E1Bp positive cells in the liver and lung, respectively, at the peak of the response which was 8 days after intravenous infection. These D^b-E1Bp$^+$CD8$^+$ T cells produced an elevated level of IFNγ and granzyme B and exhibited CD44^{high} phenotype, indicating that the majority of these cells were activated and exhibited the typical features of functional CTLs. The present studies also indicated that the number of D^b-E1Bp positive CTLs correlated with the *in vivo* CTL function.

Although a strong CTL response was detected by day 8 after infection, the majority of viral clearance and liver damage occurred before the CTL response became detectable, suggesting that the innate immune response is the major immune factor associated with the early and massive virus clearance.

CD28-B7 interaction was necessary for both the CTL generation and its full cytolytic function. $CD28^{-/-}$ CTLs showed a compromised cytolytic function compared with normal CTLs. $CD4^{+}$ cell depletion resulted in a decrease in CTL generation in the spleen. However, the CTL response in the liver was unaffected, indicating that the help from CD4 T cell plays a minor role in the primary CTL response against Ad infection.

The relative role of the two major cytotoxic pathways, perforin pathway and Fas pathway, were also assessed. FasL or Fas deficiency had no effect on the effectiveness of target cell killing, but when perforin was also deficient, blocking of the Fas pathway reduced CTL-induced target cell lysis. In contrast, perforin deficiency alone caused defective *in vivo* killing. These results indicate that the perforin pathway is the predominant mechanism for target cell lysis and the Fas pathway plays a synergistic role.

These findings have important implications for human gene therapy. First, the application of human MHC tetramers will help to assess the CTL response in patients receiving Ad gene therapy. Second, classification of major mechanisms underlying Ad CTL generation and effector function may help to define potential targets for immunomodulation. Finally, identification of new CTL epitopes from Ad capsid proteins not only enables development of specific methods for studies related to the Ad CTL response, but also provides a target for Ad capsid modification to ablate its immunogenicity.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

UAB ANIMAL USE SAFETY INFORMATION

This project must be registered and authorized by UAB OH&S if you will be using biohazards, radioisotopes, carcinogens, or toxic chemicals in the animal or animal facility.

OH&S Administrative Use Only
Project # 03-608 Amendment
Authorization Date 5-26-04

PI INFO

Name John D. Mountz, MD, PhD Phone 4-8909 Emergency # _____
Department Medicine Alternate Contact Hui-Chen Hsu, PhD. Alt. Phone 4-8874

PROJECT

Project Title Ablation of the Immune Response to Adenovirus Vectors
Species Mice
Funding Source _____
IACUC Administrative Use Only
APN: 3721

POTENTIALLY HAZARDOUS MATERIALS (Excluding Anesthetics)

Agent/Material is potentially hazardous for:
☑ Humans
☐ Animals (Species _____)

Agent(s)	Route of Administration	Excretion (e.g., urine/feces)	Human Health Risks or Other Concerns
Adenovirus	IV; intratumor	None	During the first 2 days, the blood may contain virus, which is potentially hazardous for operators.

SPECIAL PRECAUTIONS/INSTRUCTIONS (check all that apply)

☑ The PI or his/her technicians are responsible for the feeding and care of these animals (must receive IACUC approval)
☒ The following may be contaminated with potentially hazardous material and must be handled only by authorized personnel:
☐ Cage ☐ Pen ☐ Cage/pen accessories ☐ Water Bottle ■ Animal Carcasses ■ Bedding ☐ Other _____
☐ Cages/Pen must be decontaminated before cagewash (Method _____)
☒ Cages/Pen/Bedding must be autoclaved before cleaning or disposal
■ Animal carcasses must be disposed of as follows:
☐ Rad. Contaminated (Package, Store, and Manifest as per Radiation Safety Procedures)
■ Chem. And/or Bio. Contaminated (Red barrel incineration) ☐ Other _____
☒ All contaminated waste (soiled bedding and other animal waste) must be properly labeled and disposed of as follows:
☐ Chem. Contaminated (Yellow barrel incinerate) ■ Bio. Contaminated (Autoclave/ Red barrel)
☐ Rad. Contaminated (Package, Store, and Manifest as per Radiation Safety Procedures) ☐ Other _____
☐ Other (incl. special tests or immunizations) _____

REQUIRED PERSONAL PROTECTIVE EQUIPMENT (PPE) (check all that apply)

☒ The following Personal Protective Equipment (PPE) must be worn/used in the room:
■ Lab Coat ■ Disposable Gloves ☐ Face Shield ☐ Safety Glasses ☐ Goggles
☐ NIOSH Certified Dust Mask ☒ Head/hair (beard) Cover ☒ Closed front gown with long sleeves and elastic cuffs
☒ Shoe Covers/Booties ☐ Disinfectant Foot Spray ☐ H_2O Repellant Coveralls/Jumpsuit
☒ Biosafety Cabinet req. ☐ N-95 or Equivalent Fitted Respirator
☐ Other _____
☑ PPE must be removed before leaving the room.
☐ PPE must be discarded or decontaminated after each use.
☐ Other _____

☐ Check here if additional information is attached.

Form Must Be Posted On Animal Room Door
Where Animal is Used or Housed

IACUC Revised 11/10/00

Jian Chen

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

UAB Institutional Biosafety Committee Review of Investigator's Report of Research Activities Involving Recombinant DNA and Biohazardous Agents

PI: John D. Mountz, M.D., Ph.D.
LHRB 473, -0007

Project Title: Ablation of the Immune Response to Adenovirus Vectors

Agency: NIH

OH&S Project #: 03-608 (modification)

Evaluation: The Project Registration Document of Recombinant DNA Research and supporting documents submitted by Dr. Mountz indicate that the proposed experiments require IBC approval since replication defective adenovirus encoding B-galactosidase and adeno-associated virus encoding Factor IX protein will be injected into mice. Replication competent and wild type adenoviral vectors will also be injected into mice.

The PI states that BSL2 will be used for *in vitro* vector preparation and manipulation and ABSL2 for *in vivo* animal studies. Animals receiving AAV should not be housed in the same animal space as those receiving adenovirus. The Animal Resources Program should be notified prior to project initiation. The cages/bedding used to house mice receiving replication competent adenovirus should be autoclaved prior to cleaning/disposal. Under these conditions, IBC approval to proceed with the project is granted.

This IBC review addresses the use of microbial and/or recombinant agents only. If chemical agents, radioactive material, or animal or human subjects are proposed in this research, other reviews and approvals may be required.

Suzanne M. Michalek 5-28-04
Suzanne M. Michalek, Ph.D, Chair Date
UAB Institutional Biosafety Committee

xc: IBC File
IACUC

SMM/dsw

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DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Jian Chen

Graduate Program Pathology

Title of Dissertation Cytotoxic T Lymphocyte Response to Adenovirus Gene Therapy

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name		Signature
John D Mountz	, **Chair**	
Peter D. Burrows		
Paul A. Goepfert		
Jay M. McDonald		
Selvarangan Ponnazhagan		

Director of Graduate Program

Dean, UAB Graduate School

Date