Prime-boost immunization generates a high frequency, high-avidity CD8+ cytotoxic T lymphocyte population

Marie J. Estcourt, Alistair J. Ramsay¹, Andrew Brooks², Scott A. Thomson, Coralie J. Medveckzy and Ian A. Ramshaw

John Curtin School of Medical Research, Australian National University, Canberra, ACT 2610, Australia.
¹Gene Therapy Program, LSU/Tulane Gene Therapy Consortium, LSU Health Sciences Center, New Orleans, LA 70112, USA.
²Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3052, Australia.

Keywords: antigens, cytotoxic, DNA, immunologic memory, immunotherapy, T lymphocytes, vaccines

Abstract

Development and expansion of high-avidity T cell populations may be important for the success of immunization strategies against HIV and other pathogens that have presented major problems for vaccine development. We have used tetrameric–MHC complexes *ex vivo* and lytic assays to show that ‘prime-boost’ immunization with DNA vaccines and recombinant poxvirus vectors generates high frequencies of cytotoxic T lymphocytes (CTL) that recognize target cells expressing very low levels of specific antigen. These cells persist for at least 6 months at levels representing ~10% of the CD8+ T cell population. Using a novel *in vivo* assay, we also found that prime-boost immunized animals were capable of eliminating target cells expressing 10- to 100-fold less immunogenic peptide than mice given either vector alone. In addition, viral challenge led to rapid expansion of CTL effectors in prime-boost groups, to levels representing >30% of total CD8+ T cell numbers. Strategies that generate specific T cells of high avidity, optimizing early detection of infected cells, offer new hope for effective prophylaxis and immunotherapy.

Introduction

Clearance of intracellular pathogens relies on the generation of potent cell-mediated immune responses (CMI) characteristically mediated by CD8-bearing cytotoxic T lymphocytes (CTL) and CD4-bearing T<sub>H</sub>1 cells. New vector delivery systems, such as DNA vaccines and recombinant viral vectors, that in many ways mimic the antigenicity of infectious organisms, appear to represent effective strategies to elicit strong CMI, with numerous animal studies demonstrating their effectiveness in stimulating immune responses towards encoded foreign antigens (1–3). However, the levels of immunity induced by these vectors has usually been insufficient to protect against challenge with highly pathogenic organisms (4). In earlier studies, we showed that priming with DNA vaccines and boosting with recombinant poxviruses encoding similar vaccine antigens generated high levels of CMI (3,5) and induced protective CMI responses against challenge with HIV-1 in macaques (6). Greatly enhanced immune responses have now also been demonstrated in prime-boost immunization protocols against SIV (7), influenza (8) and *Plasmodium berghei* (9), illustrating the general nature of these observations.

Reasons for the efficacy of prime-boost vaccination are unclear, although several features of the vaccine vectors may underlie their capacity to induce heightened immune responses, particularly CMI (10). A recently recognized and important property of T cells is their ability to recognize and kill target cells with low densities of surface peptide–MHC determinants (11,12), suggesting that the T cell avidity may be an important factor for virus clearance *in vivo*. The recent development of tetrameric MHC–peptide complexes has facilitated the direct enumeration of antigen-specific T cells(13). Moreover these reagents may also be used to identify high-avidity CD8+ CTL within a heterogeneous population (12,14,15). In the present study, we have used tetrameric peptide–MHC complexes, together with assays for cytolytic function, to evaluate CD8+ T cell responses generated by prime-boost immunization. We report that this approach stimulates high levels of antigen-specific CTL that recognize targets expressing very low levels of specific antigen.
Spleens were harvested 24 h later for immunization with DNA-MP, VV-MP or both, as described above. CTL activity was measured in standard 51Cr-release assays using splenocytes. Stimulated for T cell avidity experiments were maintained by Dr J. Bennink (National Institutes of Health). CTL assays and tetramer staining were performed with 5×106 irradiated (3000 rad) autologous splenocytes pulsed with 100 μM SIINFEKL peptide (Auspep, Melbourne, Australia). CTL assays and tetramer staining were performed using cells from 6-day cultures. Cells repetitively stimulated for T cell avidity experiments were maintained by weekly re-stimulation in culture with irradiated, peptide-pulsed autologous splenocytes.

**In vitro cytotoxic T cell assay**

CTL activity was measured in standard 51Cr-release assays as described elsewhere. Using MC57G target cells (ATCC, Marnasas, VA) labeled with 150 μCi of Na251CrO4 and peptide. Direct ex vivo CTL assays were performed using splenocytes harvested from animals challenged with replicating VV-OVA 7 days earlier.

**Assay for in vivo CTL activity**

Splenocytes from naive mice were labeled with CFSE or SNARF (Molecular Probes, Eugene, OR) at 2.5 mM as described elsewhere. SNARF-labeled cells were incubated with SIINFEKL for 90 min at 37°C in PBS, washed twice in 5% FCS in HBBS and twice in PBS, and then added to an equal number of CFSE-labeled cells. Then 4×107 cells in 400 μl PBS per mouse were injected i.v. to animals previously immunized with DNA-MP, VV-MP or both, as described above. Spleens were harvested 24 h later for flow cytometry analysis.

**Flow cytometry**

Cells suspended in PBS with 2% FBS (Gibco/BRL, Gaithersburg, MD) and 0.1% sodium azide were stained with phycoerythrin (PE)-labeled tetramer for 50 min at 37°C before addition of CyChrome-conjugated anti-CD8α (PharMingen, San Diego, CA) for 20 min on ice. PE-conjugated tetrameric H-2Kb/SIINFEKL complexes were synthesized as previously described. Cells were then washed 3 times and analyzed on FACSscan (Becton Dickinson, Mountain View, CA). Calibration of the cytometer was done using CaliBRITE Beads (Becton Dickinson) and the software program FACSComp (Becton Dickinson). WinMDI (J. Trotter) software was used for analysis of FACS data and generation of FACS plots. In all plots viable lymphocytes are shown after gating on forward and side scatter.

**Results**

**Prime-boost immunization generates large numbers of CD8+ T cells with high lytic capacity**

The role of CMI in the efficacy of prime-boost immunization warrants investigation as the mechanism by which this strategy facilitates protection remains unclear. To study the characteristics of immune responses induced by prime-boosting we immunized mice with the peptide SIINFEKL from OVA (amino acids 257–264) encoded in DNA vectors (DNA-MP) or recombinant vaccinia vectors (VV-MP). In particular, we sought to evaluate the lytic activity of CTL from mice immunized consecutively with DNA-MP and VV-MP or with either vector alone. Initially, splenocytes harvested from immunized mice were assessed for cytotoxic activity following short-term in vitro re-stimulation with SIINFEKL (Fig. 1A). Prime-boost immunization resulted in significantly greater killing than VV immunization alone, while DNA immunization induced little or no response. We next used H-2Kb/SIINFEKL tetramers to monitor the development of specific CD8+ CTL. Figure 1(B) shows that ~13% of the CD8+ T cell population in these animals was tetramer-positive by direct, ex vivo staining at 28 days post-immunization. Importantly, these heightened numbers of peptide-specific CD8+ T cells were seen to persist for at least 6 months (data not shown). This figure was well in excess of the frequencies of tetramer-binding CD8+ T cells from mice given either DNA-MP or VV-MP alone, the latter being detected only at background levels. We next evaluated the ability of these CTL populations to kill targets pulsed with varying levels of SIINFEKL peptide following short-term in vitro re-stimulation of splenocytes harvested from vaccinated mice (Fig. 1C). The prime-boost immunization gave rise to CTL that displayed high levels of killing against targets coated with very low levels of peptide, unlike those generated by DNA-MP or VV-MP alone. Indeed, targets incubated with peptide at only 1 nM were efficiently lysed by CTL induced by prime-boosting; levels 100- to 1000-fold lower than those recognized by VV-MP-primed CTL. Cells from mice primed with DNA-MP alone experienced only background levels of lytic activity even at high peptide concentrations. Thus, our data show that prime-boost immunization generates high frequencies of CTL that both persist and can detect target cells expressing
very low levels of specific antigen, unlike immunizations with DNA or VV vaccines alone.

An interesting finding was that, whereas VV-derived CD8\(^+\) CTL were able to kill peptide-coated targets, albeit at lower levels than those induced by prime-boosting, only low levels of H-2K\(^{b}\)/SIINFEKL tetramer-positive cells were found in this population (Fig. 1B). This contrasts with the high levels of tetramer-positive cells present in the prime-boost CTL population and raises the question as to whether prime-boost immunization induces effector populations with higher avidity for the immunizing antigen.

In order to study the relationship between avidity and tetramer staining in our system, we have established a model system that preferentially induces CTL of differing avidities, using techniques based on those described by Berzofsky et al. (11). In these assays, VV-primed spleen cells were re-stimulated \textit{in vitro} with different concentrations of SIINFEKL peptide and responding T cell populations were tested for their lytic activity and their tetramer binding capacity. Figure 2(A) shows that CTL populations re-stimulated with very low levels of peptide were able to recognize antigen-coated target cells much more efficiently than those re-stimulated with higher peptide concentrations. Thus, the former were able efficiently to kill targets incubated with SIINFEKL at only 1 nM, whereas the latter required peptide concentrations of 1000 nM in order to lyse targets effectively. These differences are not due to variations in the levels of expression of TCR or CD8 co-receptors in the CTL populations (data not shown). Figure 2(B) shows the shift in tetramer staining intensity of cells derived from these cultures. CTL that were re-stimulated at high peptide concentrations stained only marginally above background levels, although they were capable of killing target cells pulsed with high levels of peptide. In contrast, T cells stimulated with lower peptide concentrations displayed far greater tetramer staining intensities (i.e. 2 log) and were capable of killing target cells expressing significantly lower peptide concentrations (as shown in Fig. 2A). These data are suggestive of correlation between the tetramer staining intensities of responding CTL and their capacity to kill target cells expressing low densities of peptide determinants.

\textbf{Challenge with vaccine antigen following prime-boost immunization leads to rapid expansion of tetramer-positive CD8\(^+\) CTL and expression of lytic activity}

We next evaluated CTL responses in vaccinated animals following live virus challenge, using recombinant VV expressing OVA. Both H-2K\(^{b}\)/SIINFEKL tetramer-positive CD8\(^+\) T cell profiles and direct lytic activity of splenocyte

![Graph A](image1.png)

**Fig. 1.** Prime-boost immunization generates high frequencies of CTL that kill target cells expressing low levels of antigen. Splenocytes from vaccinated animals were harvested at 4 weeks after the final immunization and re-stimulated with peptide for 6 days \textit{in vitro}. (A) Lytic activity of re-stimulated splenocytes pooled from groups of four mice given DNA-MP (■), VV-MP (●) or both in prime-boost combination (▲) against targets coated with \(10^5\) nM SIINFEKL peptide. (B) Prime-boost vaccination generates high numbers of antigen-specific CD8\(^+\) T cells. Splenocytes were harvested from mice immunized with DNA-MP, VV-MP or both in prime-boost combination and direct \textit{ex vivo} analyses of CD8\(^+\) T cell populations were carried out by flow cytometry using H-2K\(^{b}\)/SIINFEKL tetramers. Numbers of tetramer-positive cells are shown as a percentage of CD8\(^+\) T cells stained 28 days after the final immunization. Data represent mean values for groups of four mice ± SD. (C) Lytic activity of re-stimulated splenocytes pooled from groups of four mice given DNA-MP (■), VV-MP (●) or both in prime-boost combination (▲) against targets coated with different concentrations of SIINFEKL. E:T ratios were 60:1. Data in (A)–(C) are representative of three independent experiments.
Prime-boost immunization generates high-avidity CTL

Fig. 2. Avidity of CTL populations correlates with tetramer staining intensity. (A) Splenocytes were taken from mice primed with VV-MP 14 days earlier and repetitively stimulated over 4 weeks with splenocytes coated with different concentrations of peptide [10^2 nM (○), 100 nM (△) and 0.1 nM (■)]. Cell populations were then tested for CTL activity against target cells pulsed with SIINFEKL over a range of concentrations. The E:T ratio was 6:1. (B) The re-stimulated T cell populations were also tested for tetramer staining intensity by FACS (unstained cells (shaded), 10^2 nM (dotted line), 100 nM (dashed line) and 0.1 nM (solid line)). Data in (A) and (B) are representative of three independent experiments.

isolates were monitored in these studies. In prime-boost immunized mice, which had a ‘resting’ population of ~13%, significant increases in the proportion of tetramer-positive cells were seen within 5 days of challenge, peaking at ~30% of the total CD8^+ T cell population (Fig. 3A). Interestingly, heightened numbers of positive cells were also observed after challenge of mice that had been immunized with DNA-MP only, reaching 10% of total CD8^+ T cells by day 3 from a low baseline level. This rapid increase in CTL numbers, albeit at significantly lower levels than seen following prime-boost immunization, illustrates the efficacy of DNA vaccination for the generation of CTL capable of rapid expansion upon re-exposure to antigen in vivo. Further analysis showed that the cytolytic activity stimulated following challenge was of sufficient strength in both prime-boost or DNA immunized mice to be directly detected ex vivo (Fig. 3B). In this case, challenge of DNA immunized animals with VV-OVA may be viewed as a ‘booster’, which follows multiple priming inoculations with DNA-MP. The rapidity with which significant numbers of antigen-specific CD8^+ T cells are then expressed following prime-boosting is impressive.

In vivo activity of high-avidity CTL generated by prime-boost immunization

Finally, we sought to demonstrate the effectiveness of prime-boost vaccine-induced CTL populations in vivo using a novel assay to monitor depletion of target cells labeled with immunogenic peptide and vital dye. Thus, vaccinated mice were inoculated with SNARF-labeled target cells coated with different concentrations of SIINFEKL (1–100 nM) and clearance of these cells was compared to that of targets labeled with CFSE but no peptide (Fig. 4). The ratio (r) of the percentage of unlabelled cells and the percentage of antigen-coated cells

Fig. 3. Rapid expansion of peptide-specific CD8^+ T cells following in vivo challenge of prime-boost vaccinated mice. Mice immunized with DNA-MP (■), VV-MP (○) or both in prime-boost combination (△) were challenged with 10^7 p.f.u. VV-OVA and their splenocytes were stained with H-2K^b/SIINFEKL tetramers (A) and lytic activity was assessed at day 7 (B), as described in Methods. Figures represent mean values for groups of four mice within each group ± SD for (A) and pooled values for (B). Data in (A) and (B) are representative of three independent experiments.
Fig. 4. Prime-boost immunization generates high-avidity CTL activity in vivo. Vaccinated mice were inoculated with SNARF-labeled splenocytes coated with different concentrations of SIINFEKL (1–100 nM) at 14 days after their final immunization and clearance of these cells at 24 h was monitored by FACS. Ratios in the top left corner of each scatter plot represent the percentage of peptide-pulsed SNARF-labeled cells to the percentage of CFSE-labeled splenocytes retrieved from inoculated mice. A ratio of 1 indicates little or no clearance of peptide-labeled cells. Figures are representative values for groups of four mice. Data are representative of three independent experiments.

represents the relative cytotoxic CD8+ CTL activities. It was apparent that prime-boost immunized animals cleared the great majority of targets which expressed higher concentrations of SIINFEKL (100 and 10 nM), with r values of 52 and 20 respectively. An elevated r value was also observed at the lowest (1 nM) concentration of peptide, indicative of some clearance of labeled cells. In marked contrast, animals immunized with either vector alone were able to clear significant numbers of targets expressing only the highest concentration of peptide (100 nM). Thus, effectors generated following prime-boost immunization were capable of eliminating target cells expressing 10- to 100-fold less immunogenic peptide than those induced by the other vaccination strategies. The greater in vivo efficacy of prime-boosting at low antigen levels may be due to both the magnitude and avidity of the CTL population that is induced. These characteristics of prime-boost vaccination may prove to be critical for early recognition of infected cells, or of cells expressing low levels of target antigen, and for early resolution of infection.

Discussion

Intracellular pathogens, including HIV, have presented major problems for vaccine development, not least because of the inability of many immunization strategies to elicit protective immune responses. It is now thought likely that the most effective form of immunity against these agents is a strong CMI response directed towards antigenic targets which may be expressed early in infection (21,22). Whilst new vaccine delivery systems, such as recombinant vectors, are capable of inducing CMI responses against encoded antigens, in many cases these have been of limited protective efficacy. In contrast, the recent development of prime-boost vaccination strategies, particularly where DNA vaccines are used consecutively with recombinant poxvirus vectors, has proven to be highly effective in generating CMI responses that are protective against diverse pathogenic challenge (3,6–10). In this study, we have attempted to delineate mechanisms underlying the efficacy of DNA/poxvirus prime-boost vaccination. Overall, it appears that this approach generates high frequencies of CD8+ CTL that both persist and recognize target cells expressing very low levels of vaccine antigen. Indeed, tetramer analysis revealed that antigen-specific CTL comprised ~10% of the total CD8+ T cell population following prime-boosting, but <4% of these cells in animals given DNA vaccine and below the limits of detection in VV-immunized mice. Furthermore, challenge with replicating vaccine antigen was followed by rapid expansion of these cells to >30% of CD8+ T cell numbers in the prime-boost immunized mice. Of equal significance was the finding that CTL induced following prime-boosting efficiently lysed targets expressing 100- to 1000-fold less specific antigen in vitro than CTL from mice...
given DNA or VV alone and were far more efficient than the latter in eliminating antigen-bearing target cells in vivo.

The generation of CTL populations capable of recognizing targets expressing low levels of antigen, i.e. which display higher avidity towards the immunogen, may be crucial for the efficacy of an antiviral vaccination strategy. Indeed, high-avidity CTL, such as those generated here following DNA/poxvirus prime-boosting, should be particularly effective early in the course of virus infection of each cell, when lower antigen densities are expressed. Cell transfer studies have recently shown that CTL of high avidity are more effective than those of lower avidity for the eradication of tumor cells (12,23) or for clearance of infection by LCMV (24) or vaccinia virus (11,25) and that their greater efficacy could not be reproduced simply by transferring greater numbers of low-avidity CTL (25). Another important feature of effective vaccination is the capacity of a primed population to respond rapidly upon exposure to infectious antigen. In this respect, the expansion of prime-boost vaccine-induced CTL to levels representing 20 and >30% of the total CD8+ T cell population within 3 and 5 days respectively of challenge with replicating virus, together with their ability to rapidly eliminate antigen-bearing targets in vivo, is impressive. These factors may be particularly relevant in the case of HIV infection where the presence of CTL populations capable of recognizing target cells early in the infectious process may be critical for delay or prevention of the establishment of disease. The capacity of DNA/poxvirus prime-boost immunization to generate long-lived, high-avidity CTL populations capable of rapid expansion is, therefore, a highly attractive feature of this strategy.

Several features of the vectors used in prime-boost immunization may underlie their ability to generate the high-avidity CTL populations described in this study. In general terms, both DNA vaccines and poxvirus vectors efficiently introduce encoded proteins into MHC class I and II antigen-processing pathways, leading to the efficient induction of specific CD4+ and CD8+ T cells. More particularly, the presence of short dinucleotide sequences in the bacterial plasmid backbone of the DNA vector strongly stimulates IL-12 production that may favor the development of CMI responses (1), whilst its non-replicating nature means that the immune response is likely to be focused almost entirely on encoded vaccine antigens. Another element crucial for the ability of DNA vaccines to prime high-avidity T cells for greatly enhanced secondary responses may be their persistent, low-level expression of immunogenic proteins in vivo. Indeed, the relationship between limiting antigen dose and selection of T cells with receptors of high affinity is now well established (11,26,27). It may also be instructive to note that reversal of the order of delivery of DNA and poxvirus vectors resulted in failure of protection (9), whilst recent studies indicate that priming with a poxvirus vector leads to induction predominantly of low-avidity CTL against encoded vaccine antigen (28). Thus, the generation of high-avidity T cell populations by DNA vaccination which are greatly and efficiently expanded following boosting by poxvirus vectors expressing higher levels of the same antigen may account, at least in part, for the efficacy of this immunizing strategy.

In conclusion, this study shows that DNA/poxvirus prime-boost immunization induces high levels of CTL that efficiently recognize cells expressing low levels of specific antigen and that expand rapidly upon infection. The activity of high-avidity populations such as these may be of crucial importance for the early elimination of cells expressing low levels of target antigen. Our findings help to explain the protective efficacy of this approach and have significant implications for the development and optimization of effective prophylactic strategies against pathogens such as HIV and immune-based treatments for cancer.

Acknowledgements

The authors would like to thank Christopher R. Parish and Bernadette Scott for valuable advice. The DNA–MP and VV–MP were kindly provided by The CRC for Vaccine Research, Australia. This work is funded by the Centre for HIV Virology, Australia.

Abbreviations

CMI cell-mediated immune responses
CTL cytotoxic T lymphocyte
PE phycoerythrin
OVA ovalbumin
VV vaccinia virus

References


