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Review Article

Mycobacterium bovis Bacille Calmette-Guérin as a Vaccine Vector for Global Infectious Disease Control

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Mycobacterium bovis bacille Calmette-Guérin (BCG) is the only available vaccine for tuberculosis (TB). Although this vaccine is effective in controlling infantile TB, BCG-induced protective effects against pulmonary diseases in adults have not been clearly demonstrated. Recombinant BCG (rBCG) technology has been extensively applied to obtain more potent immunogenicity of this vaccine, and several candidate TB vaccines have currently reached human clinical trials. On the other hand, recent progress in the improvement of the BCG vector, such as the codon optimization strategy and combination with viral vector boost, allows us to utilize this bacterium in HIV vaccine development. In this paper, we review recent progress in rBCG-based vaccine studies that may have implications in the development of novel vaccines for controlling global infectious diseases in the near future.

1. Introduction

Mycobacterium bovis bacille Calmette-Guérin (BCG) is the only licensed vaccine that has substantially helped controlling tuberculosis (TB) for more than 80 years. This vaccine affords ~80% protection against TB meningitis and miliary TB in infants and young children [1]. However, the BCG-induced protective effects against pulmonary diseases over all ages are variable; the escalation of the worldwide TB epidemic is evidence that the vaccine does not work well to prevent pulmonary TB [2]. Recently, studies on the advanced molecular biology and genomics of mycobacteria have revealed that the BCG genome has various mutations and deletions compared with the original virulent strain of *Mycobacterium tuberculosis* and *M. bovis* [3]. Interestingly, there are substantial differences in the genomic DNA even among BCG substrains [4, 5] that can cause biological differences in the population of BCG vaccines.

Since a host-vector system in mycobacteria was developed in 1987 [6], recombinant BCG (rBCG) technology has been extensively applied in the development of vaccines against a variety of infectious diseases, including bacterial, viral, and parasitic infections in addition to TB [7, 8]. BCG is attractive as a vaccine vector because of its extensive safety record in humans, heat stability, low production cost, induction of long-lasting type 1 helper T cell (Th1) immunity, $CD8^+$ T-cell triggering, adjuvant activity, usability in newborns and its mucosal immune induction by oral administration. Taking the current situation of serious epidemics of emerging and reemerging diseases mainly in developing African and Asian countries into account, a new global vaccine should be affordable in such areas. Therefore, the low price and heat stability of BCG-based vaccines would be desirable. In this paper, we review various efforts to develop novel BCG vector-based vaccines mainly for controlling TB and HIV/AIDS.

2. Immunological Properties of BCG Vector

The immune responses induced by BCG are outlined in Figure 1. The most characteristic response to BCG is the induction of innate (nonspecific) immunity by cell wall components through toll-like receptors (TLRs) 2 and 4 on dendritic cells and macrophages [9]. After phagocytosis,

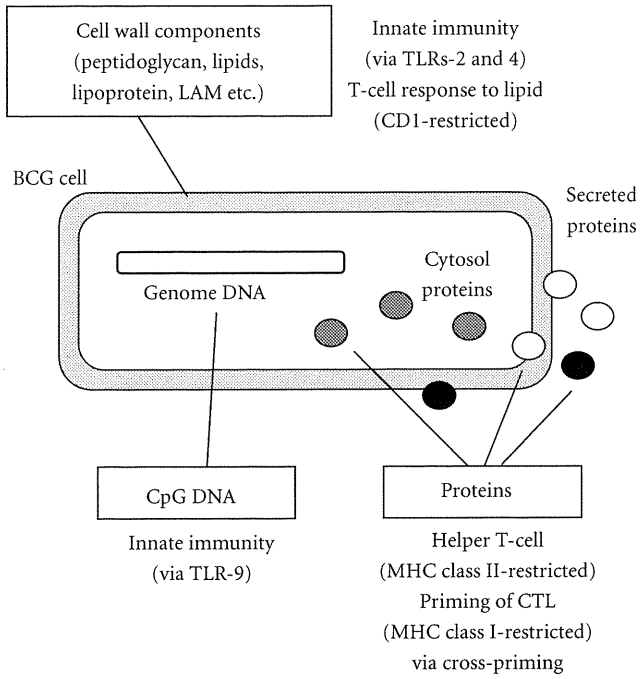

FIGURE 1: Outline of immune responses by BCG. Both innate immunity via TLRs and antigen-specific immunity via MHC- or CD1-restricted antigen presentation to T cells are induced by various BCG cell components.

BCG is degraded by lysosomal enzymes, and the processed antigen can be presented to the host immune system via various pathways. DNA fragments containing the CpG motif may activate innate immunity via the TLR9 route [10]. Lipids such as mycolic acid presented by CD1 stimulate CD1-restricted $CD8^+$ T cells [11]. Protein antigens, such as antigen 85 complex produced by BCG, induce Th1 response through presentation by major histocompatibility complex (MHC) class II. This pathway is the major route of BCG-induced responses and is indispensable for protective immunity against *M. tuberculosis* infection via protective cytokine interferon (IFN)-γ production. On the other hand, the processing and presentation of protein antigens via the MHC class I pathway are also elicited in the BCG-infected antigen presenting cell (APC). As reported by Goonetilleke et al. [12], immunizing BCG-sensitized animals with recombinant vaccinia virus MVA expressing antigen 85A greatly enhances the MHC class I-restricted CTL response against antigen 85A, indicating that BCG priming could be a novel type of prime-boost vaccine. This immunological feature of BCG vector allows its application in vaccines against chronic viral infectious diseases such as HIV/AIDS. In addition, the strong Th1 induction by BCG would be favorable to aid the maturation and maintenance of CTL [13]. Thus, the BCG vector is expected to induce effective cell-mediated immunity against a targeted antigen.

3. TB Vaccine

3.1. Background of the Global TB Epidemic. TB kills 1.7 million people worldwide each year; someone dies from TB every 19 seconds [14]. Although the TB treatment protocol was established a long time ago, the recent increase of multidrug-resistant *M. tuberculosis* infection has generated a serious situation. New vaccines are urgently needed to eliminate TB as a public health threat and should be a major global public health priority. TB is a disease that is spread from person to person through the air. Furthermore, the terrible synergy between TB and HIV makes this disease even more dangerous, especially in sub-Saharan African countries. For instance, according to the World Health Organization's (WHO) Global TB report 2010 [14], South Africa had nearly 400,000 new TB cases in 2009 with an incidence rate of an estimated 806 cases per 100,000; TB is one of the leading causes of death in both adults and children of this country. The case fatality rate has increased from 3% in 1993 to 24.3% in 2007. A major reason for the increased fatality rate is South Africa's concurrent HIV epidemic. The prevalence of HIV infection in South Africa in 2009 was approximately 7%, which has been decreasing as a result of various efforts toward prevention. TB is a common opportunistic infection among people living with HIV, and 60% of new TB cases occurred in persons who were also infected with HIV in 2009 [14]. We can observe similar critical situations in the countries surrounding South Africa. Regarding the vaccination, such situation has raised concerns about the safety of using BCG vaccine in HIV-infected infants because between 10 and 30% of pregnant women are HIV infected in many sub-Saharan African countries.

3.2. Current Efforts toward New TB Vaccine Development. The global plan to stop TB 2011–2015 report [15] offers 7 objectives as follows: (i) to maintain a robust TB vaccine pipeline by supporting research and discovery, (ii) to conduct research to identify correlates of protection and preclinical studies to assess new TB vaccine candidates, (iii) to ensure the availability of vaccine production capacity by expanding manufacturing facilities for TB vaccines, (iv) to build capacity for large-scale clinical trials (phases II and III) of TB vaccine candidates at field sites in TB-endemic countries, (v) to conduct phase I, II, and III clinical trials of TB vaccine candidates, (vi) to develop delivery, regulatory, and access strategies for new TB vaccines, (vii) to build support for TB vaccine development and uptake through advocacy, communications, and resource mobilization. All these objectives are important to realize new TB vaccine development.

The main goal of vaccine development in the Global Plan to Stop TB 2006–2015 is for 2 vaccines to be in proof-of-concept trials by 2010 and that 1 new and safe vaccine is available by 2015. As of 2009, 12 TB vaccine candidates had entered clinical trials. Of these, 9 are still being tested (Table 1) : 5 are in phase I clinical trials, 2 are in phase II trials, and 2 are in phase IIb proof-of-concept trials [15]. One vaccine has produced estimates of safety and effectiveness in a targeted HIV-infected population. At least 6 TB vaccine candidates are in preclinical development, and at least 21 additional next-generation candidates are in the vaccine discovery phase [15]. As mentioned earlier, the current BCG vaccine has limited and variable effectiveness against TB.

TABLE 1: Summary of candidate TB vaccines in clinical trials 2009. Nine candidate preventive TB vaccines are currently in clinical phases.

Status	Products	Product description	Sponsor
Phase IIb	MVA85A/AERAS-485	Vaccinia virus MVA	OETC/AERAS
Phase IIb	AERAS-402/Crucell Ad35	rBCG/adenovirus 35	Crucell/AERAS
Phase II	Hybrid-I + IC31	Ag85B/ESAT6 + adjuvant	SSI/TBVI
Phase II	M72	Fusion protein + adjuvant	GSK/AERAS
Phase I	AdAg85A	adenovirus 5/Ag85A	McMaster Univ.
Phase I	VPM 1002	rBCG/listeriolysin::ΔureC	Max Planck/TBVI
Phase I	Hyvac 4/AERAS-404	Fusion protein + adjuvant	SSI/Sanofi/AERAS
Phase I	RUTI	Fragmented Mtb cell	Archivel Farma
Phase I	Hybrid-I + CAF01	Ag85B/ESAT6 + adjuvant	SSI

Abbreviations in the sponsors: AERAS, AERAS Global TB Vaccine Foundation; GSK, GlaxoSmithKline; OETC, The Oxford-Emergent Tuberculosis Consortium Ltd.; SSI, Staten Serum Institute; TBVI, Tuberculosis Vaccine Initiative.

Therefore, the first choice of strategy may be improving BCG by using recombinant DNA technology even though it may imply safety issue of vaccination in HIV-infected individuals. Overproduction against a protective antigen of TB in BCG (rBCG30) exhibited enhanced immunogenicity in humans [16]. Moreover, the expression of the listeriolysin gene in BCG (rBCG/*hly*$^+$::Δ*ureC*) is proven to be more potent in the induction of TB-specific cellular immune responses [17]. Another strategy for improving BCG vaccines is boosting BCG immunity with protein [18, 19] or viral vector vaccine such as modified vaccinia virus Ankara (MVA) strain [20] and adenovirus type 35 [21]. BCG-prime and recombinant MVA-antigen 85A boost regimen [22] exhibited efficient immune responses in humans and have entered the first phase IIb trial in newborns. Furthermore, a combination of such strategies in which 3 major antigens are overproduced and the perfringolysin gene is incorporated into BCG and boosted with a recombinant adenovirus vaccine has been developed [23]. However, it is unknown whether such strategies are relevant for developing vaccines that are effective against adult pulmonary TB. It is necessary to test whether these candidate vaccines effectively induce mucosal immunity and protect against lung disease.

4. HIV/AIDS Vaccine

4.1. Background of the Global HIV Epidemic. In 2009, there were an estimated 2.6 million people who became newly infected with HIV. This is more than 21% less than the estimated 3.2 million who became infected in 1997, the year in which annual new infections peaked. In 33 countries, the incidence of HIV has decreased by more than 25% between 2001 and 2009; 22 of these countries are in sub-Saharan Africa. This trend reflects a combination of factors including the impact of HIV prevention efforts and the natural course of HIV epidemics [24].

Although highly activated antiretroviral therapy apparently contributes to control HIV replication in infected individuals [25], several problems remain to be resolved. These problems include: (i) the following viral load recovers soon after the interruption of treatment; (ii) chronic toxicities cause abnormalities in lipid metabolism and mitochondria; (iii) drug-resistant viruses increase during long period of treatment; (iv) long-term treatment carries a risk of carcinogenesis [26]; (v) expensive drugs are still difficult to access in developing countries. Even in developed countries, the high cost of antiretroviral drugs produces a sense of impending crisis in public health policy [27]. In such circumstances, although the rate of new infections with HIV-1 is gradually decreasing, an effective preventive vaccine is still urgently needed to stem further spread of the virus [28]. Even though considerable recent progress has been made in the development of an HIV vaccine [29, 30], the immune correlate of viral protection is not fully elucidated due to the complicated interaction of viral, immunological, and genetic factors [31, 32]. Since it is known that some populations of HIV-1-infected people do not present disease progression when HIV-1 replication is regulated by host immunity [33, 34], targeted vaccine immunogens are designed to closely mimic the long-lasting protective immunity induced in the long-term human survivors of natural infection [35, 36]. Due to safety issues, a live-attenuated HIV vaccine is not practical. This inevitably led the trend of HIV vaccine development to component- and vector-based vaccines.

4.2. Current Trends in HIV/AIDS Vaccine Research. The first large-scale efficacy trial of an HIV/AIDS vaccine was conducted by a US company, Vaxgen Co., in which a genetically engineered surface envelope (Env) glycoprotein, gp120, vaccine was tested in humans. Although the vaccine was targeted toward inducing effective virus-neutralizing antibodies, the phase III efficacy trial revealed its ineffectiveness [37, 38]. The failure of the gp120 vaccine changed the trend of HIV/AIDS vaccine research from an antibody-targeted strategy to a cell-mediated immunity-targeted strategy. Because HIV-1 causes chronic infection due to its cell-associated features, cellular immunity especially virus-specific cytotoxic T lymphocyte (CTL) should be a more important arm of the host immune system. Indeed, immune deficiency virus-specific cell-mediated immunity has been suggested to effectively control viral replication during the natural course of viral infections [39–41]. Based on these findings, various vaccine modalities, including live viral vectors and DNA vaccines, have been used to elicit strong CTL and Th1 type

responses in nonhuman primate models. Although single-vaccine delivery systems sometimes exhibit insufficient immune responses, boosting with viral vector vaccines such as vaccinia virus [40, 41], adenovirus [42, 43], and Sendai virus [44] in DNA-primed individuals strongly amplified CTL responses and resulted in the effective control of simian immunodeficiency virus (SIV) replication. Among such viral vectors, adenovirus type 5 (Ad5) had the strongest CTL enhancement effect, and the DNA-prime and recombinant Ad5 boost vaccine strategy is recognized as the most promising. However, in 2007, Merck Co. reported that a recombinant Ad5 vaccine expressing HIV-1 Gag, Pol, and Nef antigens did not demonstrate any protective efficacy in a phase IIB clinical trial [45]. Surprisingly, the vaccinated group exhibited a significantly higher HIV-1 infection rate than the placebo group [45], suggesting that the recombinant Ad5 immunization may have some unknown effect in enhancing HIV-1 infection. Thus, we were aware that T-cell vaccine approaches may involve certain risks and limitations; this paradigm appears to have reached an impasse.

In September 2009, there was ground-breaking news that the RV144 large-scale efficacy trial in Thailand demonstrated a partial effect of reducing HIV-1 infection rate in the recipients of ALVAC (canarypox)/gp120 prime-boost vaccine [46]. Although the results demonstrated limited effects, they demonstrated the possibility of preventing HIV infection with the active immunization for the first time. Furthermore, although there was no apparent correlation between protection and virus-specific cellular immune response or neutralizing antibody levels in the vaccinees, more detailed analyses of the total host responses are expected in the future. Taking the vaccine formulation with the gp120 protein boost into account, some antibody-mediated reactions may be involved in this partial protection. On the other hand, a new T-cell-targeted vaccine also demonstrated protective efficacy in a macaque study in the same year. A rhesus cytomegalovirus-vectored vaccine expressing SIV Gag, Rev-Tat-Nef, and Env persistently infected rhesus macaques, primed, and maintained robust SIV-specific $CD4^+$ and $CD8^+$ effector memory T-cell responses in the absence of neutralizing antibodies [47]. The report suggests that T cell vaccines may have greater potential than previously estimated. Although the importance of broadly neutralizing antibody production would not change despite tremendous difficulties, cellular immunity-targeted candidate vaccines should be also clinically tested for proofs of concept.

4.3. BCG-Vectored HIV Vaccine. The most practical advantage of the BCG vector is its high safety. In addition to being effective at inducing protective immunity, an HIV-1 vaccine regimen must be shown to be safe, affordable, and compatible with other vaccines before it can be considered promising [39]. In this respect, vectors that have already been used in humans without serious complications and with low cost should be utilized for HIV vaccines. BCG is a unique live vaccine vector because of its easy antigen delivery to the professional APC to be presented to T cells. Therefore, this bacterium is expected to be an important vector for HIV vaccine development.

At the early stage of rBCG research in the 1990s, Aldovini and Young [48] demonstrated immunogenicity of rBCG against genetically engineered HIV-1 antigens in mice. We independently worked on an rBCG-vectored anti-HIV vaccine simultaneously. First, we demonstrated effective cellular immune induction against SIV Gag antigen by the rBCG vector in rhesus macaques [49, 50]. Furthermore, we cloned an extracellular α antigen (antigen 85B) gene from both BCG [51] and *Mycobacterium kansasii* [52], and established a foreign antigen secretion system in mycobacteria [53]. Based on this system, we extensively evaluated several rBCG constructs for candidate HIV vaccines and reported that an rBCG-HIV vaccine could induce protective humoral immune responses in guinea pigs [54]. These studies suggest that rBCG-based vaccines are feasible as AIDS vaccines. However, the CTL activity did not reach protective levels with a single injection of rBCG-HIV vaccine in the macaque model. To overcome the low immunogenicity of the rBCG vaccine in CTL induction, we utilized various strategies for enhancing the immune potential of the BCG vector.

4.4. Prime-Boost Regimen for Enhancing Immune Responses. The first strategy by which we tried to improve the potential of the rBCG-HIV vaccine was the use of a safe recombinant viral vector for a booster vaccine. With respect to safety, traditional live vaccines, which have been administered safely to both the healthy and the HIV-infected individuals, may be the vectors of choice for HIV-1 vaccines. To fully take advantage of the benefits of such traditional vaccines in the development of anti-HIV vaccines, we studied BCG Tokyo 172 strain and the replication-deficient vaccinia vaccine strain DIs [55, 56] both of which have been shown to be nonpathogenic when inoculated into immune-deficient animals as live recombinant vaccine vehicles [57]. The vaccinia virus DIs have been tested clinically as a smallpox vaccine in Japanese infants and proved to be quite safe. We chose this highly attenuated virus as a booster vaccine vector and constructed recombinant DIs (rDIs) expressing the HIV *gag* [58] or SIV *gag-pol* gene [59]. Both rDIs constructs were found to be effective in eliciting HIV- or SIV-Gag-specific immunity in mice. When they were administered as a booster antigen after priming with an SIV-DNA vaccine, the cellular immunity to SIV Gag was greatly enhanced [59]. In brief, we tested a new combination regimen: priming with rBCG-SIV Gag followed by boosting with rDIs-SIV Gag.

In the macaque study, we found that BCG/DIs vaccination induced a long-lasting and effective cellular immunity that was able to control a highly pathogenic virus SHIV C2/1 [60], after mucosal challenge [61]. A possible mechanism of effective Gag-specific cell-mediated immunity is shown in Figure 2. The strong Th1 response induced by the BCG vector may contribute to eliciting the Gag-specific CTL response. How these immune inductions are correlated with protective efficacy requires further investigation. In this study, the BCG/DIs vaccination developed high levels of cellular immunity in the macaques that were protected against the loss of $CD4^+$ T lymphocytes with reduced viral RNA levels after virus challenge. Furthermore, the BCG/DIs group showed no evidence of clinical diseases or mortality

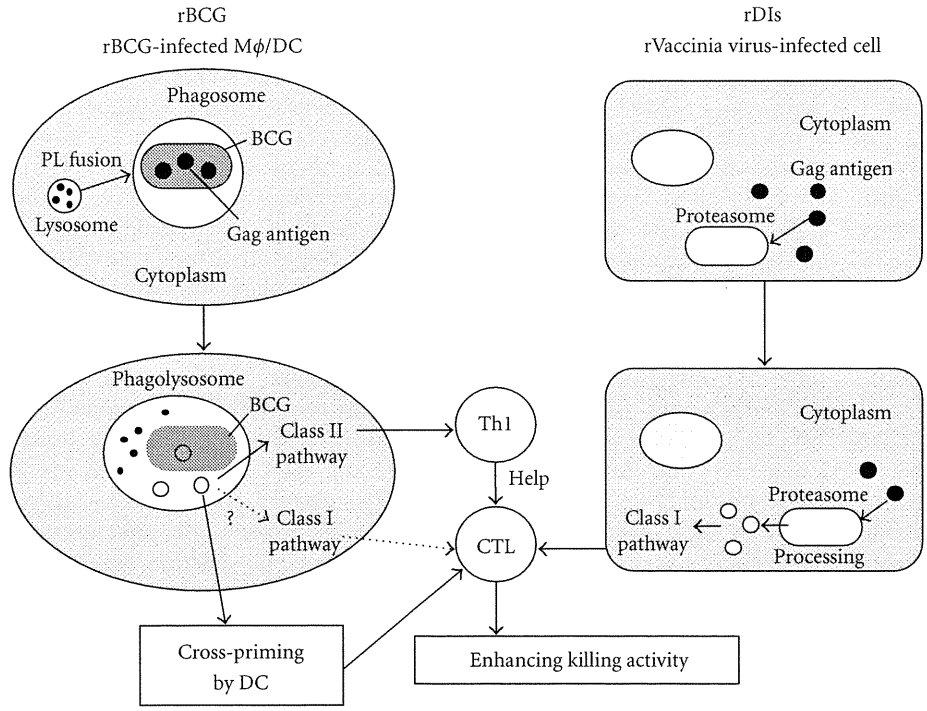

FIGURE 2: A possible mechanism of effective Gag-specific cell-mediated immunity induction with the rBCG/rDIs prime-boost vaccine. Abbreviations: DC, dendritic cell; Mϕ, macrophage; PL, phagosome-lysozome; Th1, type 1 helper T cell; CTL, cytotoxic T lymphocyte.

after viral challenge during the 1-year observation period [61]. These results suggest that the BCG/DIs prime-boost regimen might be a potential candidate for an effective and safe anti-HIV vaccine. Recent studies in macaques subjected to BCG/Ad5 [62] and BCG/MVA [63] regimens strongly support the effectiveness of the BCG vector. In the latter study, a hemolysin-expressing BCG strain, which was devised for more efficient antigen presentation to the CTL precursor, elicited a robust and broad range of HIV-1 specific T-cell responses along with recruitment of multiple T-cell clonotypes into the memory pool.

4.5. Codon Optimization Strategy. The major issue with BCG vehicle vaccines is the low expression level of the foreign antigen gene in BCG cells. In general, sufficient levels of foreign antigen-specific immune responses are obtained with high doses of rBCG between 10- and 100-fold greater than that needed for a practical dose against TB in humans [54]. This is considered the main limitation for the clinical use of rBCG-based vaccines. To address this substantial issue, we applied a codon optimization strategy for foreign genes in the rBCG system to increase its expression level. The aims of the study were to increase the immunogenicity of the foreign antigen, decrease inoculation dosages as small as the conventional BCG vaccine against TB, avoid adverse reactions, prevent possible association with Th2-type immune responses, and ward off the exacerbation of retroviral infections.

First, we determined the in vitro effects of codon optimization of the HIV gene in rBCG. Although the effect of codon optimization in mammalian cells is well documented [64–66], its effect in rBCG vehicle had never been fully elucidated. We targeted the HIV-1 *gag p24* gene as a model antigen to clarify the effect of codon optimization in the rBCG system. A specially designed synthetic p24 gene consisting of mycobacterial-preferred codons resulted in an increase in their GC content from 43.4% to 67.4%. Furthermore, codon-optimized rBCG was generated without any detectable changes in its characters including the growth rate. This rBCG exhibited a dramatic increase in Gag p24 antigen production approximately 40-fold greater than the non-optimized rBCG. Moreover, we successfully obtained data regarding the enhancement of immune responses in codon-optimized rBCG-immunized mice [67]. Inoculation of mice with a single low dose of the codon-optimized bacteria elicited effective cellular immunity. In the ELISPOT assay, the number of Gag-specific IFN-γ spot-forming cells elicited by codon-optimized rBCG was significantly greater than that elicited by non-optimized recombinants [67]. These cellular immune responses would decrease if the $CD8^+$ T cells were depleted. The results also suggest that effective MHC-class I-restricted CTL responses are inducible by vaccination with codon-optimized rBCG. Furthermore, Gag-specific lymphocyte proliferative responses were also detected in the codon-optimized rBCG-immunized mice [67].

We also applied this strategy to an SIV Gag construct and successfully generated an rBCG harboring the codon-optimized SIV *gag* gene with an expression 10-fold greater than that of the native *gag* gene. In the macaque study, compared with a native *gag* gene construct, a low-dose (10^6 bacilli) injection of this construct induced optimal priming of Gag-specific $CD4^+$ and $CD8^+$ T cells and prolonged the maintenance of memory T-cell response after vaccinia DIs

boost [68]. These results imply that the quality of the priming vaccine is a critical factor for inducing a desirable immune response against immunodeficiency viruses. Thus, the codon optimization strategy should generally be applied to other foreign genes in rBCG-based vaccine development.

5. Vaccine for Other Infectious Diseases

There were various candidate rBCG vaccines targeting infectious diseases other than TB or HIV. Stover et al. [69] reported that the rBCG system would be useful in Lyme disease vaccine development; the vaccine incorporated with the surface protein of *Borrelia burgdorferi* first reached clinical phase I trials. However, the vaccine was rejected due to its low antibody production response [70]. Two groups [71, 72] applied rBCG in malaria vaccine development and demonstrated efficacy in a mouse model. Malaria is recognized as one of the three major infectious diseases as well as TB and AIDS. Although there is a long history of malaria vaccine development, we have not seen any licensed vaccine. The strategy to induce cellular immunity against conserved antigens using BCG vector could be effective to overcome substantial difficulties in producing vaccine due to antigenic diversity and unique life cycle of this parasite. In addition, BCG vector was tested for vaccine discovery against some viral diseases. A rBCG expressing the measles virus nucleoprotein demonstrated protection against measles virus pneumonia in macaques [73]. Furthermore, we demonstrated that a rBCG with a single hepatitis C virus (HCV) NS5 CTL epitope into antigen 85B induced HCV-specific CTL response in mice [74]. HCV is recognized as one of the major infectious pathogens of which the global infection rate is ~3%. Although the priority for preventive HCV vaccine development has become lower because of the remarkable progress in the treatment, BCG vector of targeting CTL induction may have implication for therapeutic vaccine against this disease. All these candidates at the early stage of rBCG study could not proceed to further development stages at those times. The rBCG-based vaccine development for these diseases should be reconsidered because the advanced technology that enhances the potential of BCG vectors has become currently available.

6. Conclusion and Future Perspective

As described in Section 3, several rBCG-based candidate vaccines are currently being evaluated for the development of TB vaccines. Such human trials would provide a greater insight into the paradigm of immune correlation in *M. tuberculosis* infection. In addition, the application of the codon optimization strategy enables us to utilize this bacterial vector as a primer of a heterologous prime-boost regimen for a preventive HIV vaccine. These results could suggest that the BCG vector is possible divalent vaccine controlling both TB and HIV/AIDS with a single construct; such study may help resolve the serious public health problem in the sub-Saharan African countries in which both diseases are highly prevalent [14].

Another potential outcome is the utility of the BCG vector for infant vaccines. One of the largest advantages of rBCG vaccines is their applicability to newborns. Because BCG as a TB vaccine is integrated into the expanded program on immunization in many countries, we have the earliest chance to immunize newborns with BCG within 3 months of birth before they are exposed to a variety of infectious pathogens. Substituting the current BCG with a novel rBCG vaccine possessing protective antigens against pathogens that cause serious diseases in infants, such as severe diarrhea and respiratory diseases, could be effective in developing countries. Such vaccine concepts should be also tested in appropriate animal models before they are tested in humans. Thus, after much trial and error in the last 2 decades, rBCG-based vaccines may contribute to the control of global infectious diseases in the near future.

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Paradigm change in immune correlation: cellular or humoral?

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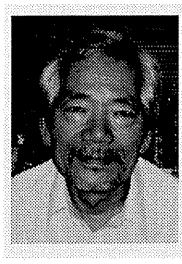

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"Although the importance of the induction of broadly neutralizing antibodies by vaccines cannot be denied, cellular immunity-targeted candidate vaccines ... should also be clinically tested."

Current status of HIV/AIDS vaccine development

Recently, considerable progress has been achieved in the field of HIV vaccine development [1,2], and it has been suggested that the immune correlates of protection against viral infection comprise a complicated combination of viral and host immunological and genetic factors [3]. It is well known that some HIV-1-infected individuals do not exhibit disease progression as long as HIV-1 replication is well controlled by host immunity [4]. Despite great efforts to understand the immune status of such long-term nonprogressors and macaques immunized with live-attenuated simian immunodeficiency virus (SIV) vaccine, we still suffer from lack of sufficient knowledge on potential immune correlates of protection. In addition, the structural features and variability of the envelope (Env) glycoprotein (gp) of HIV-1 are responsible for our inability to elicit potent broadly neutralizing antibodies against HIV-1 by active immunization.

History of HIV vaccine efficacy trials

The history of efficacy trials for preventive HIV vaccines is marked by a series of paradigm shifts in immune correlation. First, Vaxgen Co. (CA, USA) tested a genetically engineered surface Env gp120 vaccine in humans. Despite induction of effective virus-neutralizing antibodies during the initial phases of the trial, a large-scale Phase III trial revealed the ineffectiveness of the vaccine [5]. This failure changed the strategy of HIV vaccine research from antibodytargeted to cell-mediated immunity-targeted. Considering the cell-associated features of HIV-1, cell-mediated immunity, especially that conferred by virus-specific cytotoxic T lymphocytes (CTLs), should be an important arm of the host immune system in regards to HIV infections. Indeed, it has been suggested that immunodeficiency virus-specific cellular immunity effectively controls viral replication during the natural course of infection [6,7]. Based on these considerations, various vaccine modalities, including live viral vectors and DNA, have been tested to elicit strong CTL and type 1 T-helper cell responses in nonhuman primate models. Although the DNA vaccine was not sufficiently immunogenic in macaques, boosting in DNA-primed individuals with viral vector vaccines, such as vaccinia virus [7], Sendaivirus [8] and adenovirus [9], amplified CTL responses and resulted in effective control of immunodeficiency virus replication. Among such viral vectors, vaccination with adenovirus type 5 (Ad5) elicited the strongest CTL induction. However, in 2007, the Merck (NJ, USA) STEP trial testing the efficacy of a recombinant (r) Ad5 vaccine was discontinued at Phase IIb because the vaccine failed to provide protective immunity [10]. Instead, the vaccinated group showed a significantly higher HIV-1 infection rate than the placebo group, which indicated that rAd5 immunization

EXPERT REVIEWS

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may have some unknown enhancing effect on HIV-1 infection. This trial provided key findings that T-cell vaccine approaches may involve risks and limitations, and that the chimeric simian human immunodeficiency virus macaque AIDS model is not suitable for evaluation of the protective immunity of candidate vaccines [11]. The results do not necessarily mean that T-cell vaccines have no potential applications, but rather that this paradigm seemed to have reached a deadlock.

In September 2009, RV144, a large-scale efficacy trial conducted in Thailand, reported a partial reduction in the HIV-1 infection rate among the vaccinees who received the canarypox/gp120 prime–boost vaccine [12]. The results indicated only a limited effect but first demonstrated the possibility of prevention of HIV infection through active immunization. Although there was no clear correlation of protection with a virus-specific, cell-mediated immune response and neutralizing antibody levels in the vaccinees, detailed analysis of the host responses is expected. Taking into account the vaccine formulation of the gp120 boost, some types of antibody-mediated reaction other than neutralization may be involved in this partial protection. This might generate a novel paradigm of immune correlates of protection against HIV-1 infection.

Bacillus Calmette–Guérin vector for HIV vaccine development

Mycobacterium bovis bacillus Calmette–Guérin (BCG) is a unique vector in the field of vaccine research because of its safety record, affordability and easy antigen delivery to the professional antigen-presenting cells, and thereby to the T cells. Most of the current candidate T-cell vaccines utilized DNA vaccines for priming HIV-specific cellular responses. However, because of its low immunogenicity in humans and product cost issues, an alternative vector is needed for global use. Therefore, BCG has been extensively studied as a primer of heterologous vaccination regimens.

> **"...it has been suggested that immunodeficiency virus-specific cellular immunity effectively controls viral replication during the natural course of infection."**

The characteristic responses to BCG involve the induction of innate immunity by cellular components via Toll-like receptors, a strong and long-lasting type 1 T-helper cell response and triggering of the CTL priming. As we reported in 2005, a prime–boost regimen combining rBCG–SIVgag with a nonreplicating vaccinia virus Dairen I strain (DIs), which was similar to modified vaccinia Ankara (MVA) and expressed SIV Gag, induced a long-lasting and effective cellular immunity that was able to control a highly pathogenic simian human immunodeficiency virus after mucosal challenge in macaques [13]. This indicated that the BCG/DIs prime–boost regimen may have the potential to serve as an effective and safe anti-HIV vaccine. Recent studies in macaques subjected to BCG/Ad5 [14] and BCG/MVA [15] regimens strongly support the effectiveness of the BCG vector. In the latter study, a hemolysin-expressing BCG strain, which was devised for more efficient antigen presentation to CTL precursors, elicited robust and broad-range HIV-1-specific T-cell responses along with recruitment of multiple T-cell clonotypes into the memory pool.

Another strategy for enhancing the potential of the BCG vector is codon optimization. Owing to the limitation on the dose that can be delivered in humans, the rBCG construct should be capable of optimal foreign antigen expression. To address this issue, we applied a codon-optimization strategy to the rBCG system and successfully generated a rBCG harboring the codon-optimized SIV *gag* gene with a tenfold higher expression than the native *gag* gene [16]. In the macaque study, a low-dose (10^6 bacilli) injection of this construct, in comparison with that of the native *gag* gene construct, induced optimal priming of Gag-specific $CD4^+$ and $CD8^+$ T cells, and prolonged the maintenance of memory T-cell response after vaccinia DIs boost [17]. These results imply that the quality of the priming vaccine is a critical factor for inducing a desirable immune response against immunodeficiency viruses.

> **"A DNA-prime and recombinant adenovirus type 5-boost regimen induced broad simian immunodeficiency virus-specific cellular responses without neutralizing antibodies and protected rhesus macaques from heterologous mucosal simian immunodeficiency virus challenge."**

These rBCG-/viral vector-based combination regimens are expected to progress to human clinical study because, as McShane *et al.* reported, the BCG/MVA-antigen 85A prime–boost vaccine, which is quite similar to the rBCG-based HIV vaccines, efficiently induced anti-TB T-cell responses in humans and proceeded to a Phase IIb trial in July 2009, indicating the advantage of BCG over other T-cell vaccine vectors with regard to human immunogenicity [18].

Future prospects

Recent studies in macaques have confirmed the effectiveness of T-cell vaccines. A DNA-prime and rAd5-boost regimen induced broad SIV-specific cellular responses without neutralizing antibodies and protected rhesus macaques from heterologous mucosal SIV challenge [19]. Furthermore, a rhesus cytomegalovirus vector expressing SIV Gag, Rev-Tat-Nef and Env persistently infected rhesus macaques, and primed and maintained robust SIV-specific $CD4^+$ and $CD8^+$ effector memory T-cell responses in the absence of neutralizing antibodies [20]. These reports suggest that T-cell vaccines may have a greater potential than previously estimated. Although the importance of the induction of broadly neutralizing antibodies by vaccines cannot be denied, cellular immunity-targeted candidate vaccines, whose efficacy has been tested in a macaque-SIV study, should also be clinically tested. Such human trials would provide a greater insight into the paradigm of immune correlation, and help achieve the ultimate goal of establishing a dual humoral and cellular immune barrier against HIV infection at the site of viral entry, especially at mucosal sites.

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Different Vaccine Vectors Delivering the Same Antigen Elicit CD8$^+$ T Cell Responses with Distinct Clonotype and Epitope Specificity[1]

Mitsuo Honda,*† Rui Wang,‡ Wing-Pui Kong,* Masaru Kanekiyo,* Wataru Akahata,* Ling Xu,* Kazuhiro Matsuo,† Kannan Natarajan,‡ Howard Robinson,§ Tedi E. Asher,* David A. Price,*¶ Daniel C. Douek,* David H. Margulies,‡ and Gary J. Nabel[2]*

Prime-boost immunization with gene-based vectors has been developed to generate more effective vaccines for AIDS, malaria, and tuberculosis. Although these vectors elicit potent T cell responses, the mechanisms by which they stimulate immunity are not well understood. In this study, we show that immunization by a single gene product, HIV-1 envelope, with alternative vector combinations elicits CD8$^+$ cells with different fine specificities and kinetics of mobilization. Vaccine-induced CD8$^+$ T cells recognized overlapping third V region loop peptides. Unexpectedly, two anchor variants bound H-2D^d better than the native sequences, and clones with distinct specificities were elicited by alternative vectors. X-ray crystallography revealed major differences in solvent exposure of MHC-bound peptide epitopes, suggesting that processed HIV-1 envelope gave rise to MHC-I/peptide conformations recognized by distinct CD8$^+$ T cell populations. These findings suggest that different gene-based vectors generate peptides with alternative conformations within MHC-I that elicit distinct T cell responses after vaccination. ***The Journal of Immunology,*** **2009, 183: 2425–2434.**

Whereas protective immune responses against viral infections have been achieved by vaccination, HIV-1 has proven recalcitrant to preventive vaccination. Although humoral and cellular immunity contribute to the control of HIV-1 (1–3), it has not been possible to elicit the broadly neutralizing Abs required to prevent infection by diverse strains, prompting the development of T cell vaccine approaches. Recently, gene-based vaccines, including naked DNA and replication-defective viral vectors, have been used to stimulate antiviral T cell immunity in both nonhuman primates (4, 5) and humans (6, 7). Furthermore, in nonhuman primates, vaccine-induced T cells directed against processed immunodominant peptides can provide a degree of protection against acute and chronic immunodeficiency virus infections (8–11). However, immunodominance profiles and variation in the T cell responses elicited by vaccination are not yet well understood (9, 10, 12), and the differences in immunogenicity of alternative prime-boost vaccine regimens are ill defined. Further explorations of vaccine efficacy are required in human clinical studies. At the same time, murine models allow better analysis of genetic and immunological factors that regulate vaccine responses and are more amenable to mechanistic studies.

Virus-specific CD8$^+$ CTL recognize peptide epitopes that are generated by an intracellular processing pathway and are presented at the cell surface bound to MHC class I molecules (MHC-I). Because recognition of MHC-I/peptide complexes by the $\alpha\beta$ TCR is based on both MHC and peptide specificity, strategies for improving CTL immune responses have included the identification of immunodominant viral peptides, improvement of immunogenic peptide affinity for MHC-I, and efforts to maximize the functional affinity of the TCR for MHC-I/peptide complexes (13–15).

The immunogenicity of HIV-1 envelope (Env)[3] has been analyzed in animals (16) and humans (6). The third V region (V3) of the HIV-1 gp120 envelope glycoprotein is essential for coreceptor binding upon HIV entry (2, 17), and thus, Env has been a focus for the study of immunogenicity in experimental animals and humans. In mice, this region also serves as an immunodominant epitope, recognized both by CTL and by neutralizing Abs (17, 18). The V3 loop peptide in the CXCR4-tropic HIV-1 strain IIIB is an immunodominant CTL antigenic determinant in mice of several different H-2 haplotypes (19), and MHC-I tetramers have been used to analyze specific immunity to HIV-1_{IIIB} (20). However, because HIV-1_{IIIB} is not commonly found among natural isolates, it may have limited value as a target sequence in Env-directed HIV vaccines. To explore a potentially more clinically relevant virus (9, 10), we have selected HIV-1_{BaL} as a vaccine candidate that represents a CCR5-tropic virus and is more closely related to the strains responsible

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[3] Abbreviations used in this paper: Env, HIV-1 envelope; Ad, adenovirus; β_2m, β_2-microglobulin; BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; CM, central memory; EM, effector memory; V3, third V region.

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for HIV-1 transmission, in contrast to the CXCR4-tropic HIV-1_{IIIB} (9, 16). We have used Env as the substrate for recombinant vector-based vaccines and have studied prime-boost combinations with DNA or recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (rBCG) priming, followed by recombinant adenovirus (rAd) boosting.

In this study, we first identified functional peptides related to the immunodominant V3 loop peptide of HIV-1_{BaL} that bind well to the H-2D^d restriction element. These peptides were used to make a set of H-2D^d/peptide tetramers that enabled the detection and characterization of disparate subpopulations of HIV-specific CD8$^+$ T cells induced by DNA or rBCG priming before rAd boosting compared with rAd Env vector immunization alone. Structural analysis and TCR sequencing were used to examine the molecular basis for differential recognition of specific H-2D^d/peptide complexes by distinct populations of CD8$^+$ T cells.

Materials and Methods

Cell culture and peptide induction of surface MHC-I expression

A TAP-defective cell line, LKD8, expressing H-2D^d (21), was propagated in DMEM supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 50 μM 2-ME. Cell cultures were incubated with indicated peptides overnight either with or without the addition of human β_2-microglobulin (β_2m). Cells for flow cytometric analysis in all studies were incubated with the viability dye ViVid (Molecular Probes) (22). Subsequently, cells were stained with mAb 34-5-8S, which binds a peptide-dependent, but not peptide-specific H-2D^d epitope, or with the conformation-independent anti-H-2D^d mAb 34-2-12S, followed by anti-mouse IgG-PE (Sigma-Aldrich) (23). Stained cells were analyzed using a modified BD LSR II flow cytometer with FlowJo software (Tree Star), and the results are shown as Δ mean fluorescence intensity.

Production and preparation of rBCG, rAd, and plasmid DNA-expressing modified HIV-1 Env

We used a previously characterized vector encoding gp140ΔCFIΔV1V2 and prepared a rBCG vaccine expressing this modified Env gene.

Immunization

BALB/c mice purchased from The Jackson Laboratory were maintained in the Vaccine Research Center Animal Care Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, under pathogen-free conditions. The animal studies were approved by the Vaccine Research Center Animal Care and Use Committee and conducted in accordance with all federal and National Institutes of Health policies and regulations. Mice were immunized with 10^8 viral particles of rAd in saline i.m., 50 μg of DNA in saline i.m., or 0.5 mg of rBCG in saline intradermally (supplemental Table S-II).[4]

Flow cytometric analysis of tetramer staining and intracellular cytokine production

PBMC and spleen cells (10^6) were simultaneously and sequentially incubated with PE- and/or allophycocyanin-conjugated H-2D^d tetramers containing human β_2m for 15 min at room temperature; cells were then stained for CD3 (BD Pharmingen), CD8 (BD Pharmingen), CD16/32 (Beckman Coulter), CD44 (BD Pharmingen), CD62L (eBioscience), CD127 (eBioscience), KLRG-1 (Southern Biotechnology Associates), and CCR7 (Biolegend). The following synthetic peptides were used for tetramer production: Env-modified PA9 (IGPGRAFYA), Env-modified PI10 (IGPGRAFYTI), native PT10 (IGPGRAFYTT), native PT9 (IGPGRAFYT), P18I10 (RGPGRAFVTT), and motif control (AGPARAAAL) (National Institute of Allergy and Infectious Diseases Tetramer Core Facility). In some experiments, immune spleen cells were incubated with the peptide (2 μg/ml), anti-CD28 (2 μg/ml; BD Pharmingen), anti-CD49d (2 μg/ml; BD Pharmingen), anti-CD107a (10 μl; BD Biosciences), and anti-CD107b (5 μl; BD Biosciences) for 6 h and stained with H-2D^d/V3 tetramers, as described previously (24). Optimal concentrations of all Abs and tetramers used in this study were determined in pilot titration experiments. Intracellular cytokine production was quantified, as described previously (22).

[4] The online version of this article contains supplemental material.

TCR clonotype analysis

Small, live CD16$^-$/CD19$^-$/CD32$^-$CD8α^+ H-2D^d tetramer-positive spleen cells (10,000 per condition) were sorted to greater than 98% purity using a modified FACS DIVA (BD Biosciences) (25). Unbiased analysis of *TCR* gene expression was conducted, as described previously, using a strand-switch anchored RT-PCR with *TCRA* and *TCRB* C region primers (26). All sequences were analyzed with reference to the international ImMunoGeneTics information system website V-align (http://imgt.cines.fr).

Protein expression, structure determination, and crystallographic refinement

The soluble extracellular segment (aa 1–275) of H-2D^d was expressed in *Escherichia coli* as inclusion bodies, solubilized, and refolded in vitro with similarly expressed murine β_2m and either the PA9 or PI10 peptide, essentially as described previously for the H-2D^d/β_2m/P18I10 complex (27). Crystals were frozen in liquid nitrogen after dipping in paratone oil and examined by synchrotron radiation at beamline X29A at the National Synchrotron Light Source at Brookhaven National Laboratory. Data were collected from single crystals in a nitrogen stream at 100 K, and were indexed, scaled, and merged using HKL2000. The PA9-containing complex crystallized in space group $P2_12_12$ with one complex (H chain, β_2m, and peptide) in the asymmetric unit, and a Matthews coefficient of 2.50. The PI10 complex in space group $P2_12_12_1$ also had one complex per asymmetric unit, and a Matthews coefficient of 3.14. Data collection and refinement statistics are reported in Table S-III. The structures were readily solved by molecular replacement with MOLREP of the CCP4 suite, using the H-2D^d/β_2m complex from 1QO3 from which both peptide and Ly49A had been removed. Refinement was conducted in CNS 1.2, manual fitting of each of the peptides was accomplished with Coot (28), and molecular graphics figures were prepared with PyMOL (http://pymol.sourceforge.net/). The PA9 and PI10 complexes were determined to 2.4 and 2.1 Å, respectively, with corresponding R_{work}/R_{free} of 22.8/27.5 and 21.9/25.1. Coordinates of the refined models and structure factors have been deposited in the protein data bank (D^d-PA9, 3E6F and D^d-PI10, 3E6H). Side chain accessibility was calculated with AREAIMOL of the CCP4 suite.

Data analysis and statistics

All comparisons between recombinant and control groups and between immunization groups were conducted using ANOVA tests assuming variances with the JMP program (SAS Institute). Data are expressed as the mean $\pm$ SD.

Results

Identification of variant V3 peptide epitopes elicited by HIV-1_{BaL} Env immunization

Because previous studies of the immune response in BALB/c mice to HIV$_{IIIB}$ Env revealed that a decamer peptide spanning the V3 loop, RGPGRAFVTI (P18I10), was immunodominant for the H-2D^d-restricted CD8$^+$ T cell response (20), we asked whether the HIV$_{BaL}$ Env (29) was cross-reactive, and whether the two responses were of comparable magnitude (Fig. 1*A* and Table S-I). Mice primed with HIV-1_{BaL} expressed in a DNA plasmid vector and boosted with rAd showed a recall response to the pool of overlapping 15-mer peptides representing the entire BaL Env protein and to the specific BaL-derived peptides PT9 and PT10, but did not respond to the P18I10 peptide. Mice immunized with the corresponding HIV-1_{IIIB} vaccine responded to the cognate peptide pool as well as to P18I10, but failed to respond to either PT9 or PT10. However, the recall response (as measured by the percentage of CD8$^+$ T cells producing intracellular IFN-γ) to either native PT9 or PT10 in HIV-1_{BaL}-immunized mice was consistently weaker than the response to the P18I10 peptide following HIV-1_{IIIB} immunization (5.6 $\pm$ 2.8% for PT9 and 4.6 $\pm$ 2.5% for PT10 as compared with 15.3 $\pm$ 1.5% for P18I10). Thus, responses to each of the vaccines were specific for the delivered peptide epitopes.

Inspection of the amino acid sequence of the HIV-1_{BaL} V3 loop suggested that processed peptides derived from this region might not bind to the H-2D^d-presenting element to the same degree as the immunodominant peptide derived from the HIV-1_{IIIB} isolate.

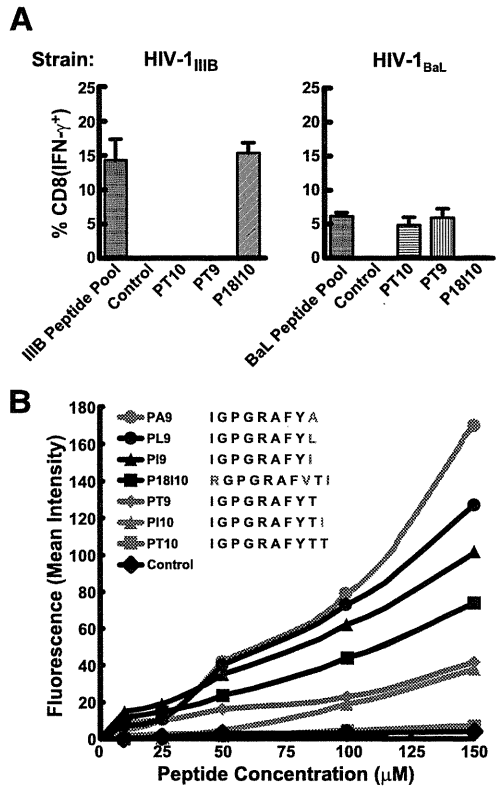

FIGURE 1. $CD8^+$ T cells from HIV Env-immunized mice show specificity for individual virus isolates, exhibit distinct potencies, and recognize variant antigenic peptides that associate differently with H-2D^d. *A*, Mice were primed with DNA encoding either HIV-1_{BaL}- or HIV-1_{IIIB}-modified Env and boosted with rAd expressing either HIV-1_{BaL} or HIV-1_{IIIB} Env, as described in *Materials and Methods* and Table S-II. Splenocytes were harvested 14 days after boosting, stimulated in vitro for 6 h with the V3 epitope peptides (2.5 μM) P18I10 (RGPGRAFVTI), PT10 (IGPGRAFYTT), or PT9 (IGPGRAFYT); pools of overlapping 15-mer peptides spanning HIV-1_{BaL} Env (BaL peptide pool) or HIV-1_{IIIB} Env (IIIB peptide pool); or an irrelevant Ebola Env peptide as a control (29); and then stained for intracellular IFN-γ production, as described previously (22). Functional profiles of $CD8^+$ T cell responses to native and variant peptides in HIV-1_{BaL} Env-vaccinated mice are shown in Fig. S1. *B*, Peptide-induced surface expression of H-2D^d with V3 loop-related peptides. TAP-negative LKD8 cells were incubated with the indicated concentrations of each peptide and stained with mAb 34-5-8, as described in *Materials and Methods*. Control peptide is WKEATTTLLCASDAK. Results are shown as the mean fluorescence intensity over background (Δ mean fluorescence intensity). Red lines indicate peptides used for tetramer construction.

Therefore, we examined whether peptides from this region of the BaL V3 loop would bind to H-2D^d in an epitope stabilization assay. The H-2D^d peptide-binding motif, determined first by analysis of peptides that copurify with H-2D^d (30, 31) and further characterized in x-ray structures of H-2D^d complexed with the IIIB-derived peptide P18I10 (27), consists of G at position 2, P at position 3, R at position 5, and a C-terminal hydrophobic residue at position 9, 10, or 11. Because H-2D^d is known to bind well to both nonamer and decamer peptides, and because the C-terminal anchor residue strongly influences peptide binding, we evaluated a set of synthetic 9-mer and 10-mer peptide variants for their ability to bind to H-2D^d (Fig. 1*B*). Using LKD8, a TAP-deficient H-2D^d-positive cell line, as an indicator (21), we observed a hierarchy of binding, as follows: PA9 > PL9 > PI9 > P18I10 > PT9 > PI10 > PT10. Thus, several nonamer and decamer variants of the PT9 and PT10 sequences found in the BaL Env immunogen bind H-2D^d with higher apparent affinity than either the native 9-mer or 10-mer. Because PA9 binds H-2D^d better than the putative endogenously generated PT9, and because PI10 binds H-2D^d better than the putative endogenously generated PT10, we expected that tetramers prepared with these variant peptides would have greater stability and would be more effective reagents with which to monitor specific T cells. However, it remained possible that subtle differences in either the proportion of molecules bound by the higher affinity peptides or the conformations of the epitopic residues of these peptides when bound to H-2D^d might influence either the specificity of the T cells elicited or the ability of such T cells to be detected with specific tetramers.

We analyzed the fine specificity of the HIV-1_{BaL} response to PA9, PT9, PT10, and PI10 using intracellular cytokine staining for IFN-γ, IL-2, and TNF-α (Fig. S1). In all vaccine vectors, the amino acid sequence encoded in the functional epitope was IG PGRAFYTT, which includes both PT9 and PT10. The native PT9 and PT10 peptides, which have apparently lower affinities for H-2D^d, elicited no triple cytokine-positive $CD8^+$ T cells in HIV-1_{BaL} Env-immunized mice. However, the higher affinity, anchor-variant peptides, PA9 and PI10, elicited a significant proportion of triple-positive cells (46 and 47%, respectively). All the V3 peptides specifically stimulated immune $CD8^+$ T cells and not $CD4^+$ T cells (data not shown), whereas a pool of HIV-1_{BaL} Env gp120 peptides stimulated both $CD4^+$ and $CD8^+$ T cells.

Diversity of $CD8^+$ T cells reactive with H-2D^d tetramers elicited by different prime-boost combinations

Having established that the two native and two variant peptides could stimulate cytokine production in DNA-primed/rAd-boosted $CD8^+$ T cells, we proceeded to explore the responses elicited by various vaccines and their prime-boost regimens with respect to T cell specificity using H-2D^d tetramers prepared with PA9, PT9, PI10, and PT10. To determine whether these different tetramers reacted with distinct T cell subsets or the same subsets bearing cross-reactive TCRs, double-staining experiments were performed. As controls, vaccine-elicited cells were double stained with the same tetramers labeled with PE or allophycocyanin (Fig. 2*A*). As expected, in each case, most of the positive cells clearly stained simultaneously with both the PE and allophycocyanin tetramers. Other controls, using P18I10 and a motif peptide known to bind H-2D^d, but lacking epitopic side chains (Table S-I), showed no reactivity (Fig. 2*A*, *right two panels*). For some tetramer combinations, D^d-PA9 and D^d-PI10, D^d-PT10 and D^d-PI10, and D^d-PT9 and D^d-PI10, minimal double staining was observed (colored panels in Fig. 2*B*). Furthermore, the differential staining patterns were not affected by performing the staining sequentially in either direction (Fig. 2*C*, paired with matching colored panels in Fig. 2*B*). These results indicate that these MHC-I/peptide complexes were recognized by distinct T cell subsets. In contrast, D^d-PT9/D^d-PA9, D^d-PT10/D^d-PT9, and D^d-PT10/D^d-PA9 double staining revealed that 82, 65, and 6% of the positive cells bound both tetramers, respectively (nonhighlighted panels in Fig. 2*B*). $CD8^+$ T cells from unimmunized mice did not react with any of the tetramers (data not shown). Thus, the Env V3 tetramers were specific for immune $CD8^+$ T cells of mice immunized with HIV-1_{BaL} Env and also could detect distinct populations of immune T cells. Several MHC tetramer pairs reacted with the same T cells, whereas D^d-PI10 tetramers detected a distinct T cell population.

Differential fine specificity of BaL Env V3-specific $CD8^+$ T cells after immunization with rAd alone, priming with DNA, or priming with rBCG

Because of the high degree of cross-reactivity between D^d-PA9 and D^d-PT9 tetramers (Fig. 2*B*), their distinct reactivity from that

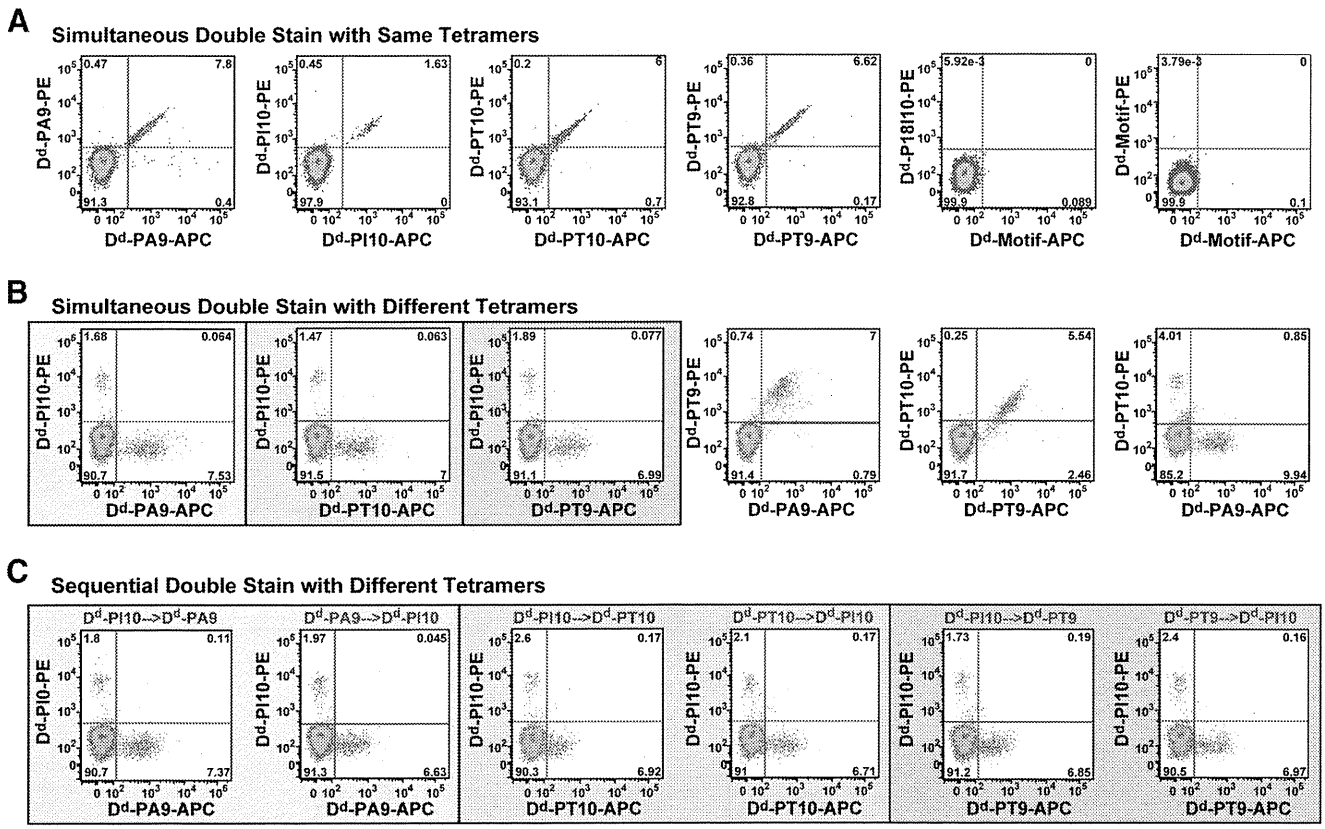

FIGURE 2. Reactivity of H-2D^d/V3 peptide tetramers in immune cell populations showing double-staining profiles for D^d-PA9, D^d-PI10, D^d-PT9, and D^d-PT10 tetramers. Spleen cells from BALB/c mice immunized with rBCG/rAd were analyzed for reactivity with the H-2D^d/peptide tetramers conjugated to PE or allophycocyanin. *A*, Simultaneous double staining of immune spleen cells with each of the four tetramers. D^d-P18I10 and D^d-motif tetramers were used as controls that bear the P18I10 and the binding motif peptide AGPARAAAL, respectively (Table S-I). Percentage of positive cells is indicated in each quadrant. Plots are gated on live CD16$^-$/CD19$^-$/CD32$^-$/CD3$^+$/CD8$^+$ T cells, as described in *Materials and Methods*. *B*, Simultaneous double staining with different tetramers. *C*, Pairs of tetramers highlighted in *B* were also used in sequential double-staining experiments. The differential staining profiles of CD8$^+$ T cells between D^d-PI10 vs D^d-PA9, D^d-PI10 vs D^d-PT10, and D^d-PI10 vs D^d-PT9 were similarly detected in either DNA/rAd or rAd immunizations (data not shown).

of the D^d-PI10 tetramer (Fig. 2, *B* and *C*), and their ability to produce functional responses after immunization with HIV-1$_{BaL}$ vector vaccines (Fig. S1), we further analyzed immune responses with the two tetramers that were not cross-reactive, D^d-PA9 and D^d-PI10. Responses were measured following different immunization schemes with rAd, DNA, and rBCG vectors, either alone or in DNA/rBCG prime-rAd boost combinations (Fig. 3). rAd elicited higher frequency responses than either DNA ($p < 0.001$) or rBCG ($p < 0.0001$) as detected by the percentage of D^d-PA9 tetramer-positive cells (Fig. 3*A*). A similar result was observed, at lower magnitude, with the D^d-PI10 (Fig. 3*B*) and D^d-PT10 tetramers (data not shown).

To study the effects of prime-boost immunization, we next monitored the CD8$^+$ T cell responses after DNA/rAd and rBCG/rAd (Fig. 3*C*). Boosting of DNA- or rBCG-primed mice with rAd (Table S-II) resulted in differential timing of the peak response, as detected with either the D^d-PA9 or D^d-PI10 tetramers. At 4 days after the boost, the D^d-PI10-responsive CD8$^+$ T cell subset in the rBCG/rAd group was preferentially elicited (mean value, 5.8 ± 1.5%; blue lines in *left panel* in Fig. 3*C*), and lower levels were achieved with DNA/rAd or rAd alone without priming (red and green lines in *left panel* in Fig. 3*C*, respectively; both $p < 0.001$). This D^d-PI10 tetramer-binding CD8$^+$ T cell population decreased by day 8 postboost and remained stable until day 90. A switch in the dominance of CD8$^+$ T cell populations from D^d-PI10 to D^d-PA9 specificity in mice immunized with rBCG/rAd was observed 14 days after rAd vector boosting. Although the peak D^d-PI10 response occurred earlier than the peak D^d-PA9 response (day 4 as compared with day 14), the magnitude of the D^d-PA9 response was significantly greater than the maximal D^d-PI10 response (24.6 ± 10.5% as compared with the D^d-PI10 response described above). The rBCG-vector control/rAd group showed results very similar to those for the rAd-alone vector group (data not shown).

The maturation and differentiation status of D^d-PA9 and D^d-PI10-specific CD8$^+$ T cells was compared between DNA/rAd and rBCG/rAd regimens at the peak of the immune response 14 days after rAd boosting (Fig. 4). The majority of the D^d-PA9-specific CD8$^+$ T cells in the spleen showed an effector cell CD127^{low} CD62L^{low}CD44^{high} phenotype in both the DNA/rAd and the rBCG/rAd immunization protocols (57.6 and 73.5% of gated cells, respectively; Fig. 4*A*). The remaining D^d-PA9-specific CD8$^+$ T cells were CD127^{high}CD62L^{low}CD44^{high} effector memory (EM; 33.7 and 16.4% in DNA/rAd and rBCG/rAd, respectively) and CD127^{high}CD62L^{high}CD44^{high} central memory (CM; 0.76 and 3.98% in DNA/rAd and rBCG/rAd, respectively). In contrast, of the D^d-PI10-specific CD8$^+$ T cells analyzed at the same time, the majority were EM (66.7 and 31.6% in DNA/rAd and rBCG/rAd, respectively) and CM (11.6 and 28.9% in DNA/rAd and rBCG/rAd, respectively). Thus, at the peak of the immune response, D^d-PA9-specific CD8$^+$ T cells were substantially skewed toward more differentiated effector phenotypes relative to the contemporaneous D^d-PI10-specific CD8$^+$ T cell populations (Figs. 4*C* and

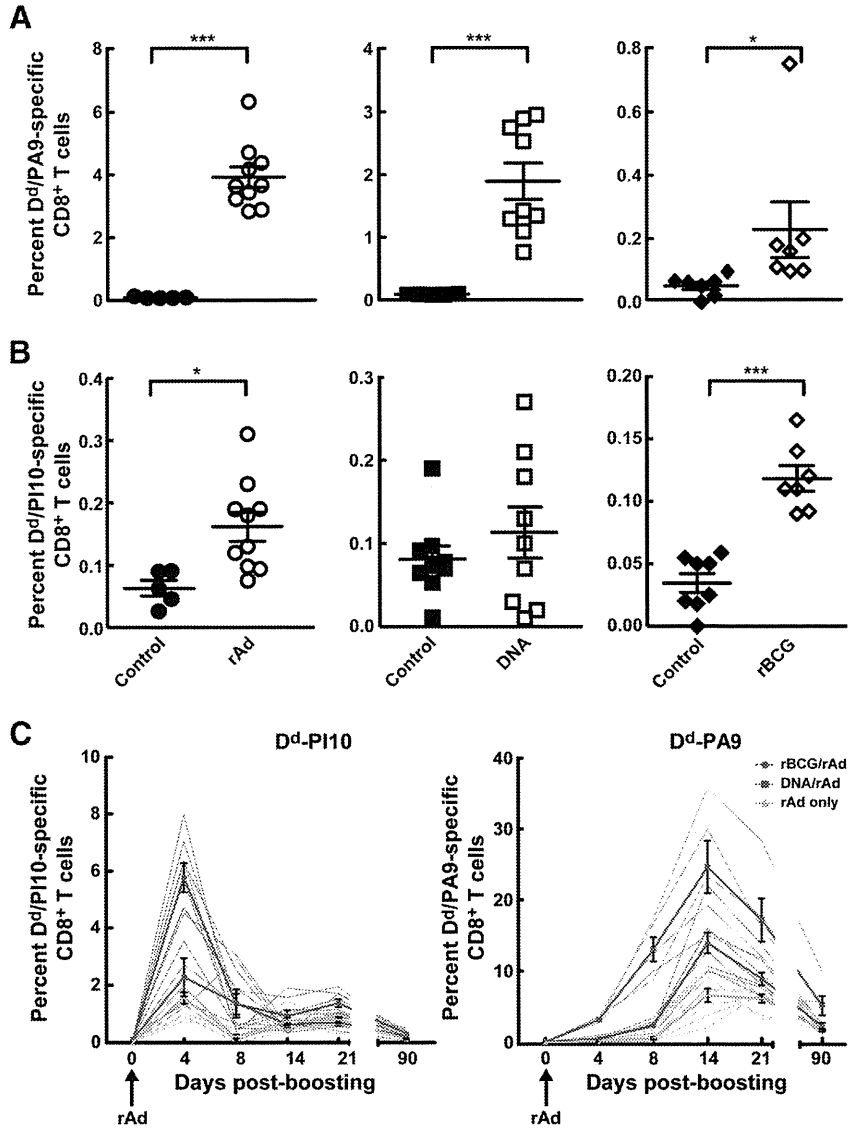

FIGURE 3. H-2D^d/V3 peptide tetramer-positive CD8$^+$ T cell responses in mice immunized with rAd, DNA, rBCG, DNA/rAd, or rBCG/rAd. *A*, Mice were immunized once with rAd (*left*), three times with DNA (*middle*), or once with rBCG (*right*), as described in Table S-II, and Ag-specific CD8$^+$ T cells were detected with D^d-PA9 (*A*) and D^d-PI10 (*B*) tetramers. Control refers to immunization with vector alone. Response patterns similar to those observed with D^d-PA9 were also observed with the D^d-PT10 tetramer (data not shown). *C*, After DNA priming (Table S-II), animals were boosted with rAd (DNA/rAd, red dotted lines). Animals were also immunized with rBCG/rAd (blue dotted lines) or rAd without priming (green dotted lines), as shown in Table S-II, and analyzed for the generation of CD8$^+$ T cells specific for D^d-PI10 (*left panel*) and D^d-PA9 (*right panel*). Dotted lines show data for each animal, and solid lines show mean values of five animals in each group. D^d-PT10 responses were lower, but showed a similar pattern to those specific for D^d-PA9 (data not shown).

S2), indicating clear differences between the two distinct CD8$^+$ T cell subsets according to MHC-I/peptide specificity.

Analysis of TCR *gene expression in Env V3-specific CD8$^+$ T cell populations*

To characterize the *TCR* gene usage of these tetramer-positive cells at 14 days postboost, we analyzed *TCRA* and *TCRB* gene expression at the clonotypic level. In rBCG/rAd-immunized mice, the tetramer-positive populations were clonotypically distinct (Fig. 5). Remarkably, D^d-PA9-specific CD8$^+$ T cells sorted from two different mice immunized with rBCG/rAd contained dominant *TCRB* sequences that were identical at the nucleotide level, representing 100 and 80% of the sequences; the corresponding *TCRA* sequences were distinct. In one mouse, the *TCRA* sequences were very restricted, whereas in the other five different sequences were observed. In contrast, the CD8$^+$ T cell population specific for D^d-PI10 was more diverse, although the distinct tetramer-positive CD8$^+$ T cells exhibited similar effector potential, as determined by CD107 expression (data not shown). Thus, D^d-PA9-specific and D^d-PI10-specific CD8$^+$ T cells, in addition to representing discrete tetramer-staining populations, also exhibit distinct TCR usage.

MHC-peptide structures suggest a basis for recognition by different T cell populations

To gain further insight into the nature of the MHC-I/peptide epitopes that constituted these different H-2D^d/peptide tetramers, we determined the high resolution x-ray crystal structures of H-2D^d complexed with PA9 and with PI10, and compared these with the previously published structure of H-2D^d bound to the related HIV-1_{IIIB} envelope peptide P18I10 (Figs. 6 and S3). Details of the structure determination and crystallographic refinement are provided in *Materials and Methods* and in Table S-II. The structures of PA9 and PI10, each complexed with H-2D^d and murine β_2m, were determined to a resolution of 2.4 and 2.1 Å, respectively. P18I10 bound to H-2D^d has been structurally characterized as the trimeric H chain/β_2m/peptide complex (27) and also with the same peptide in complex with the murine NK cell receptor Ly49A (32). We compared the two newly determined D^d-PA9 and D^d-PI10 structures with D^d-P18I10. The comparisons are focused on the $\alpha1\alpha2$ domain and bound peptide to illustrate the conformational differences of the three different bound peptides. For all three structures, the N-terminal five residues of the peptides superpose precisely (root mean square deviation of 0.053 to 0.127 Å for the three pairwise superpositions), but there is considerable

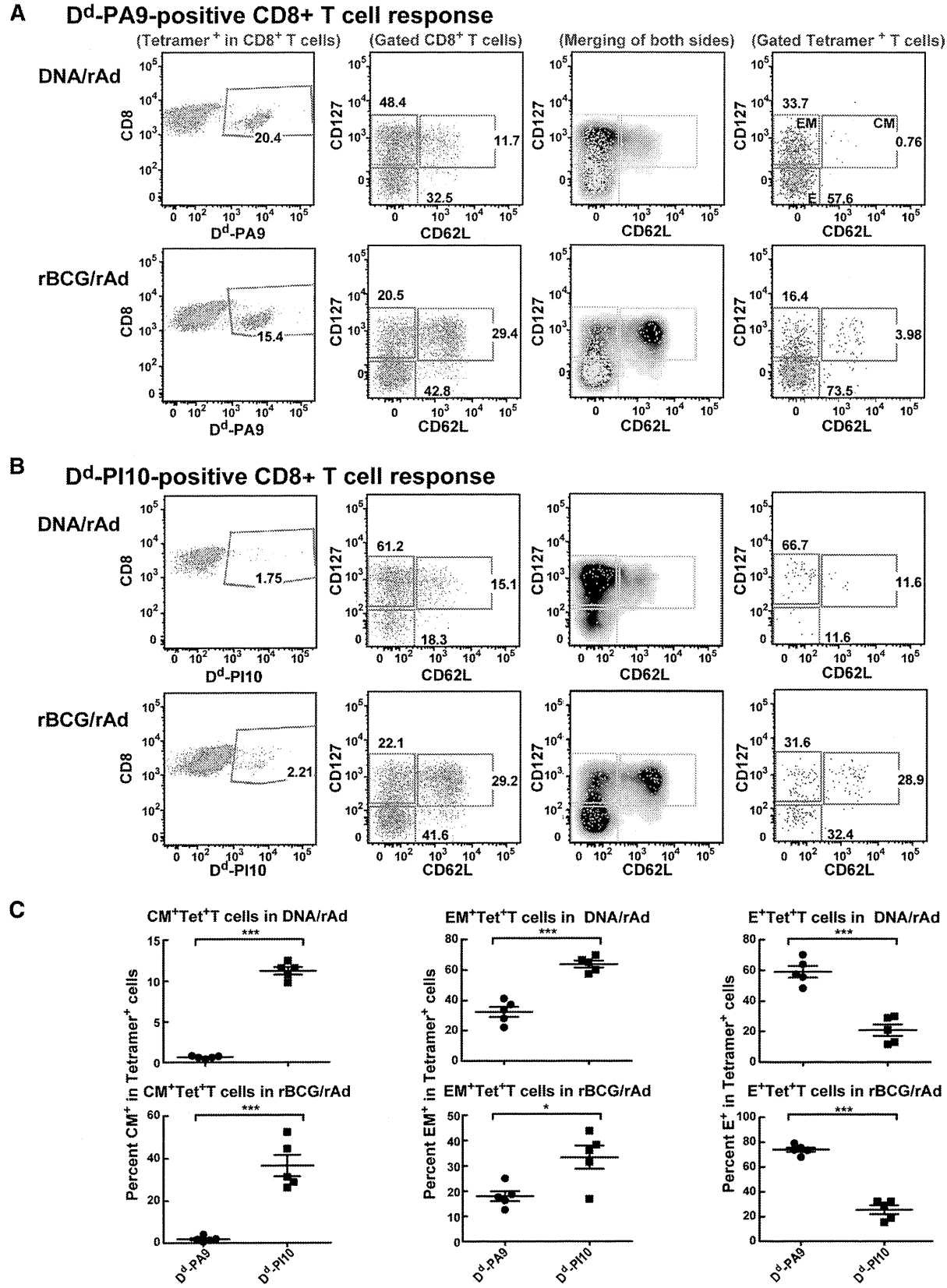

FIGURE 4. Phenotypes of D^d-PA9-specific and D^d-PI10-specific $CD8^+$ T cells elicited by immunization with DNA/rAd and rBCG/rAd. The differentiation phenotypes of tetramer-binding $CD8^+$ T cells specific for D^d-PA9 (*A*) and D^d-PI10 (*B*), harvested from the spleens of immunized mice at day 14 postboost (Fig. 3*C*), were analyzed by flow cytometry. Representative data are shown in *A* and *B*. The overall phenotypic distribution of the $CD8^+$ T cell population is shown in the *second column*; the *fourth column* shows the same analysis for the tetramer-positive events. Both sets of data are combined in the *third column*, which shows the tetramer-positive events (yellow) superimposed on the total $CD8^+$ T cell population (gray density cloud) in a bivariate plot of CD127 vs CD62L. *C*, Shows the phenotypic subset distribution of D^d-PA9-specific and D^d-PI10-specific $CD8^+$ T cells elicited by DNA/rAd (*upper panels*) and rBCG/rAd (*lower panels*) in each animal gated according to standard definitions. CM, $CD44^{high}CD127^{high}CD62L^{high}$; EM, $CD44^{high}CD127^{high}CD62L^{low}$; E (effector), $CD44^{high}CD127^{low}CD62L^{low}$. The CM cells were further defined to be $CCR7^+KLRG\text{-}1^{int}$ (data not shown).

D^d/PA9-#1

TCRBV	CDR3	TCRBJ	%	n
4	CASSTRQNAEQFF	2.1	100	81
TCRAV	**CDR3**	**TCRAJ**	**%**	**n**
16	CAMRGANYGNEKITF	48.01	84	21
16	CALRGANYGNEKITF	48.01	16	4

D^d/PA9-#2

TCRBV	CDR3	TCRBJ	%	n
4	CASSTRQNAEQFF	2.1	80	16
13	CASGDGGYTEVFF	1.1	15	3
13	CASSDLGRNYAEQFF	2.1	5	1
TCRAV	**CDR3**	**TCRAJ**	**%**	**n**
12	CALSDWPGANTGKLTF	52.01	47	9
14	CAENSGGSNYKLTF	53.01	16	3
13	CAIDPGTGSKLSF	58.01	16	3
14	CAAPPGFASALTF	35.02	10	2
6	CALVEGTGSKLSF	58.01	10	2

D^d/PI10-#1

TCRBV	CDR3	TCRBJ	%	n
1	CTCSADWGDTQYF	2.5	76	47
5	CASSQDNAGEVFF	1.1	13	8
13	CASGDAGVEQYF	2.7	6	4
29	CASSLSNAEQFF	2.1	5	3
TCRAV	**CDR3**	**TCRAJ**	**%**	**n**
4	CAADMDYANKMIF	47.01	46	21
13	CAMGNSGGSNYKLTF	53.01	24	11
16	CAMRESNNNAPRF	43.1	20	9
16	CAMREGGGNEKITF	48.01	4	2
16	CAMRESNNNAPKF	43.01	4	2
4	CVADMDYANKMIF	47.01	2	1

D^d/PI10-#2

TCRBV	CDR3	TCRBJ	%	n
13	CASIGQGAETLYF	2.3	28	9
13	CASKGLGDTGQLYF	2.2	13	4
13	CASAGGEGNTEVFF	1.1	9	3
4	CASSFGTGGNTLYF	1.3	6	2
13	CASGDGADQDTQYF	2.5	6	2
14	CASSSGTDQAPLF	1.5	6	2
13	CASSESGGPQDTQYF	2.5	6	2
13	CASRGQGMGQNTLYF	2.4	3	1
13	CASSDRPQDTQYF	2.5	3	1
29	CASSYAETLYF	2.3	3	1
29	CASSPPGQGYEQYF	2.7	3	1
13	CASMGGYAEQFF	2.1	3	1
14	CASSLHPRDWGGALEQYF	2.7	3	1
1	CTCSADWGDTQYF	2.5	3	1
17	CASSRGGNQDTQYF	2.5	3	1
TCRAV	**CDR3**	**TCRAJ**	**%**	**n**
9	CAVIEGGRALIF	15.01	17	2
13	CAMGNSGGSNYKLTF	53.01	8	1
12	CALSDQNTGYQNFYF	49.01	8	1
12	CALSCLPGTGSNRLTF	28.01	8	1
6	CALGALYGGSGNKLIF	32.02	8	1
12	CILRGTGGNNKLTF	56.01	8	1
6	CALGDHRIFF	31.01	8	1
7	CAASDLNNNAPRF	43.02	8	1
14	CAARGYQNFYF	49.01	8	1
6	CVLGEPNTGKLTF	27.01	8	1
7	CASDSGYNKLTF	11.01	8	1

FIGURE 5. Clonotypic analysis of splenic $CD8^+$ T cells specific for D^d-PA9 and D^d-PI10. D^d/V3 peptide-specific tetramer-positive splenic $CD8^+$ T cells were sorted by flow cytometry 14 days after booster immunization with rAd in rBCG-primed animals; *TCR* gene expression was analyzed, as described in *Materials and Methods*. Clonotypes are shown in order of frequency for each sorted population with TCRBV/TCRAV usage, CDR3 sequences, and their TCRBJ/TCRAJ usage. Identical CDR3 amino acid sequences are color coded.

variation in the remaining residues. The complex with PA9 (Fig. 6, *A*, *D*, *G*, and *J*) shows exposure of peptide residues F7 and Y8, with the C-terminal A9 buried in the F pocket. With the additional residue found in the 10-mers, PI10 (Fig. 6, *B*, *E*, *H*, and *K*) and P18I10 (Fig. 6, *C*, *F*, and *I*), residues 6, 7, 8, and 9 buckle out. PI10 forms an aromatic stacking interaction between F7 and Y8, hiding much of Y8 from exposure to the TCR. P18I10, with the added flexibility of the additional residue, but lacking the potential for the aromatic stacking found in PI10, thrusts F7 back toward the α1 helix. Calculations of solvent-exposed surface area per peptide residue (Fig. S3) are consistent with the visual impression: PA9 exposes residues G4, A6, and Y8; PI10 exposes G4, A6, F7, and T9; and P18I10 exposes G4, A6, F7, and T9. These conformational differences may explain the difference in the binding of D^d-PI10,

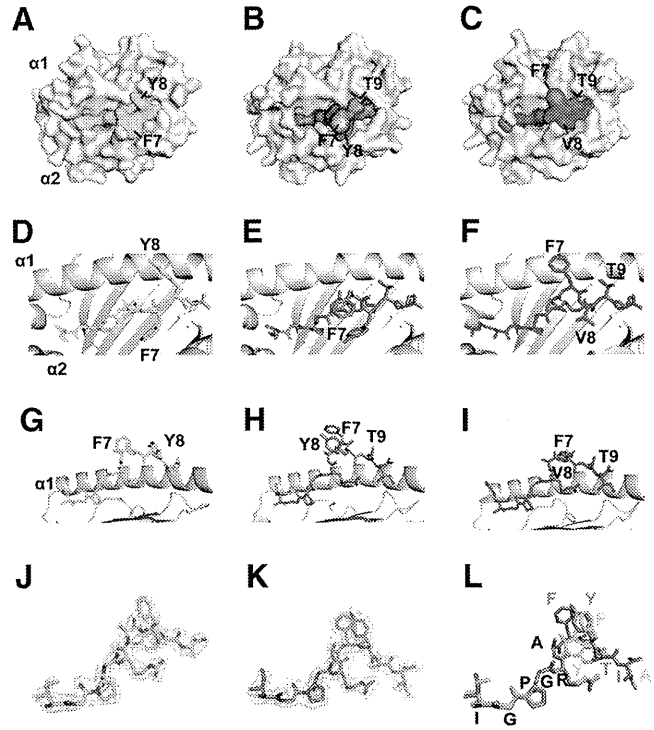

FIGURE 6. H-$2D^d$/peptide structures reveal distinct conformations of different bound peptides. Representations of x-ray structures of D^d-PA9 (*A*, *D*, and *G*), D^d-PI10 (*B*, *E*, and *H*), and D^d-P18I10 (*C*, *F*, and *I*) are shown in surface (*A–C*) and ribbon (*D–I*) representations. Peptides are colored cyan (PA9), orange (PI10), and magenta (P18I10). Viewpoint of *A–F* is from above, and of *G–L* from the side. Peptides PA9 and PI10, with accompanying electron density maps (contoured at 1σ), are shown in *J* and *K*. *L*, Shows the superposition of PA9 and PI10.

D^d-PA9, and D^d-P18I10 tetramers to specific $CD8^+$ T cells, and the differences in exposure of peptide residues 7, 8, and 9 between PA9 and PI10 offer a conceptual framework for possible differences in priming between PT9 and PT10 (see *Discussion*).

Discussion

Virus-specific $CD8^+$ T cells that arise after MHC-I-restricted presentation of peptides derived from the Env play a key role in the recognition of HIV-1-infected cells and in the control of virus replication. Although most viral proteins contain a large number of potential MHC-binding peptide epitopes, in general only a few of these epitopes evoke significant $CD8^+$ T cell responses. In a number of mouse strains, and also among human $CD8^+$ T cell responses, epitopes derived from the V3 loop of the HIV envelope are overrepresented (19, 33). In this study, we examined $CD8^+$ T cells elicited by different HIV-1_{BaL} vector vaccines using different H-$2D^d$/peptide tetramers. We suspected that the predicted immunodominant peptide of this isolate, PT10 (IGPGRAFYTT), or possibly the nonamer contained within, PT9 (IGPGRAFYT), might bind H-$2D^d$ with low affinity. Therefore, we explored the endogenous peptides as well as a set of related peptide variants for their ability to bind H-$2D^d$ in a MHC-I stabilization assay in TAP-deficient cells. Two additional peptides, PA9 and PI10, were selected for further comparative studies based on their ability to bind H-$2D^d$ (Fig. 1*B*) and elicit potent functional responses after immunization with HIV-1_{BaL} vector vaccines (Fig. S1).

What is the difference between these tetramer-specific $CD8^+$ T cell subsets? First, differences among these MHC-I/peptide complexes in the size of the exposed peptide loop as well as in the conformation of the exposed peptide present alternative targets for

TCR engagement. The difference in the reactivity of CD8$^+$ T cells specific for D^d-PI10 and D^d-PT10 is more subtle, but the finding that PT10 binds H-2D^d with significantly lower affinity supports the view that it binds differently than PI10, and leads to the conclusion that the PT10 peptide is presented in a distinct conformation from PI10. Second, clear differences were apparent in the kinetics with which CD8$^+$ T cells specific for D^d-PI10 appeared relative to D^d-PA9-reactive CD8$^+$ T cells; these distinct mobilization kinetics confirm differential Ag recognition by D^d-PI10-specific and D^d-PA9-specific CD8$^+$ T cell populations. Several explanations are possible for these differential kinetics. One is that the proportion of D^d-PI10-reactive cells after priming is greater and that these expand more prominently following the boost. Another is that the D^d-PA9-reactive cells are of higher avidity for their cognate MHC/peptide complex, and, as shown in another model system (34–36), may eventually outcompete their lower avidity counterparts (37, 38). It is also possible that both mechanisms play a role in our immunization scheme, but additional experiments will be needed to assess both the relative proportion of reactive T cells as well as the relative avidity of these different CD8$^+$ T cell populations for their respective Ags.

The maturation and differentiation status (39, 40) of the two V3-specific tetramer-positive CD8$^+$ T cell populations was clearly different at the peak of the immune responses. D^d-PI10-specific CD8$^+$ splenocytes were CM rich, whereas the corresponding D^d-PA9-specific cells behaved much more like a primary population with higher peak effector cell numbers, thereby suggesting a greater degree of maturation and expansion. Together with the tetramer-specific double-staining profiles discussed above, it is apparent that HIV-1_{BaL} Env-specific immunodominant CD8$^+$ T cell responses are mediated by distinct V3 epitope-specific subsets, each with a different phenotype. It is noteworthy in this context that the ability of rAd vectors to generate CM seems to be important. In this mouse study, modeling the events that might be occurring in vaccinated humans, boosting DNA-primed animals with rAd induced more notable maturation of dominant D^d-PA9-specific CD8$^+$ T cells into effector cells. In contrast to this effector cell differentiation, generation of CM was significantly decreased in this immunization regimen. Lately, EM has been reported to be responsible for protection of animals from SIV challenge (36). Because low-dose rAd boosting increased the proportion of CM (data not shown) and because CM have been shown to expand in vivo and mediate protective immunity against pathogenic virus (39, 41), both low- and high-dose immunizations with rAd might be worth testing to determine whether one regimen will be preferable in generating more effective protective T cell immunity. In general, further studies in nonhuman primate challenge models will be needed to permit evaluation of the character of the T cell response that contributes to protection, and such studies have been initiated.

Of particular interest is the observation that the different Env vaccine/immunization regimens elicited CD8$^+$ T cell populations with different fine specificities and clonotype usage. The x-ray crystallographic analysis of the D^d-PA9 and D^d-PI10 complexes revealed a structural basis for these differences in peptide specificity, suggesting that alternative vaccination regimens lead to differences in Ag processing and presentation that in turn elicit distinct populations of Ag-specific T cells. Structural differences in the size of the exposed peptide loop (nonamer vs decamer) and in the conformation of the exposed peptide were apparent. Although we have been unable to obtain crystals of either D^d-PT9 or D^d-PT10, we speculate that D^d-PT9 has a stable peptide conformation very similar to that of D^d-PA9. D^d-PT10, however, lacking the strong p10 isoleucine anchor residue, may have at least two distinct conformations of the bound peptide, one with the position 9T down in the F pocket and the position 10T up exposed to solvent extending beyond the peptide-binding groove, and a second with the position 10T down in the F pocket. Such conformational dimorphism of MHC-I-bound peptides has been suggested by two different high resolution structures of HLA-B*2705, complexed with either pVIPR (RRKWRRWHL) (42) or pGR (RRRWHR WRL) (43). These two peptides have been reported to induce cross-reactive CD8$^+$ T cells.

The observation that distinct populations of T cells that recognize D^d-PA9, D^d-PT9, D^d-PT10, and D^d-PI10 are differently elicited by a single protein immunogen is reminiscent of the two different classes (types A and B) of hen egg lysozyme-specific CD4$^+$ T cells observed by Unanue and colleagues (44). Type A conventional T cells recognize a distinct conformation of the peptide/I-A^k complex generated in the presence of H2-DM in late endocytic vesicles, whereas type B cells identify a distinct conformation of the same peptide/I-A^k complex produced by peptide exchange in the absence of H2-DM. There clearly are differences in the MHC-II processing and presentation systems observed for hen egg lysozyme as compared with the Env vaccine systems that we have studied. However, the distinct T cell populations that we observe may be indicative of different conformations of peptide/MHC-I complexes generated by processing and presentation from different types of APC, by a cellular mechanism similar to that observed for hen egg lysozyme/I-A^k. Complex mechanisms of Ag processing and presentation, as well as variations in the T cell repertoire, play important roles in the variability and specificity of the Ag-specific T cell response (45, 46). The differences in Ag-specific CD8$^+$ T cell induction between the two prime-boost regimens are most likely due to differences in the priming Ags. The i.m. injection of plasmid DNA-encoding Env results in cellular gene expression, which typically leads to Ag processing through a proteasomal pathway (47). In contrast, the injected rBCG must enter an endosomal/lysosomal pathway to generate peptides for presentation by MHC-I (48). Thus, endogenously generated PT9 and PT10 may be presented differently in the context of H-2D^d in different APC, and moreover, PT10 may be displayed in two distinct conformations (Fig. 7). Furthermore, this mechanistic explanation enables an understanding of the relative immunodominance profiles between D^d-PI10-specific and D^d-PA9-specific CD8$^+$ T cell populations. Differences in epitope abundance and compartmentalization (49), both temporally and between the different vaccination regimens, could explain the observed differential induction of CD8$^+$ T cells specific for D^d-PI10 and D^d-PA9.

Immunization to elicit protective T cell responses offers a reasonable strategy for design of vaccines against a variety of infectious diseases (50, 51), and MHC-I/peptide tetramers can be effective for evaluating not only the extent, but also the specificity of T cell immunity. The general approach to evaluating CD8$^+$ T cell-inducing vaccines in this study relied on several experimental strategies. First, we focused on the region of the immunizing envelope Ag known to elicit the immunodominant response. Second, we empirically tested a set of clade-specific and synthetic variant peptides for their ability to bind the known MHC-I-presenting molecule. Using both the optimal binding peptides and those that were putatively endogenously generated to produce H-2D^d tetramers, we detected different CD8$^+$ T cell subsets specific for PI10 and PA9. Structural understanding of the MHC-I/peptide complex may facilitate our ability to identify Ag-specific CD8$^+$ T cell activation in vivo in response to vector-based vaccines. Such an approach toward exploring variant peptides for MHC-I binding and for tetramer production may allow broader detection of T cell responses to HIV-1.