JOURNAL OF VIROLOGY, July 2005, p. 8024–8031 Vol. 79, No. 13
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.13.8024–8031.2005

Enhanced Breadth of CD4 T-Cell Immunity by DNA Prime and Adenovirus Boost Immunization to Human Immunodeficiency Virus Env and Gag Immunogens

Lan Wu,[1,2]† Wing-pui Kong,[1]† and Gary J. Nabel[1]*

Vaccine Research Center, NIAID, National Institutes of Health, Room 4502, Bldg. 40, MSC-3005, 40 Convent Drive, Bethesda, MD 20892-3005,[1] and National Center for AIDS/STD Control and Prevention, China-CDC, 27 Nanwei Road, Xuan Wu District, Beijing, 100050, China[2]

Received 20 January 2005/Accepted 22 March 2005

A variety of gene-based vaccination approaches have been used to enhance the immune response to viral pathogens. Among them, the ability to perform heterologous immunization by priming with DNA and boosting with replication-defective adenoviral (ADV) vectors encoding foreign antigens has proven particularly effective in eliciting enhanced cellular and humoral immunity compared to either agent alone. Because adenoviral vector immunization alone can elicit substantial cellular and humoral immune responses in a shorter period of time, we asked whether the immune response induced by the prime-boost immunization was different from adenoviral vaccines with respect to the potency and breadth of T-cell recognition. While DNA/ADV immunization stimulated the CD8 response, it was directed to the same epitopes in Gag and Env immunogens of human immunodeficiency virus as DNA or ADV alone. In contrast, the CD4 response to these immunogens diversified after DNA/ADV immunization compared to each vector alone. These findings suggest that the diversity of the CD4 immune response is increased by DNA/ADV prime-boost vaccination and that these components work synergistically to enhance T-cell epitope recognition.

The ability of heterologous prime-boost vaccination to induce potent immunity to specific antigens has been documented for a variety of infectious disease pathogens. These responses have been observed with different viral vectors that boost plasmid DNA vaccines, including DNA/poxviruses (1, 6, 9, 24, 29, 36, 37), DNA/adenoviral (ADV) vectors (6, 16, 17, 20, 22, 38, 42, 47, 49, 50), and DNA/protein (2, 8, 13, 19, 21, 44) prime-boost vaccination combinations. In addition, different viral vector prime-boost combinations, such as alternative ADV serotypes or ADV/poxvirus vector combinations, have proven successful with different antigens in animal models (4–6, 15, 25, 28, 30, 31, 37).

Among these vaccine platforms, DNA/ADV vector boosting has conferred protective immunity to different infectious challenges, with promising results having been described for Ebola virus (42) and human immunodeficiency virus (HIV) (20, 38) in nonhuman primates. The success of this approach may be dependent on several factors. In some instances, immunity to the vector used for the primary immunization can neutralize it in repeated administrations, thus reducing the effective dose. The prime-boost vaccination may also allow for alternative modes of antigen presentation, depending on the gene delivery modality. Such differences in the cell specificity of gene expression and antigen processing can affect the cellular and humoral immune response.

At the same time, gene-based vectors, such as replication-defective adenoviruses, can induce significant immune responses when administered alone. For example, in the case of Ebola virus, adenoviral platforms have been used for viral pathogenesis studies (35, 39, 48) and vaccination (41, 42). Even though DNA priming and adenoviral boosting elicits more potent antibody and cellular immune responses (42), a single administration of adenoviral vector encoding Ebola virus gene products protects against a lethal challenge 1 month later, despite an immune response of a lower magnitude (41). When the threat of infection is immediate, this approach may be desirable; however, in cases where long-term protection might be preferred, the prime-boost combination may provide more durable immune protection (12, 22, 23, 34, 40). To understand the immunological basis of such protective immunity and determine whether the character of the immune response differs with heterologous vaccination modalities, we characterized the immune response to HIV Env and Gag antigens delivered by DNA, ADV, or DNA/ADV vectors according to the specificity of epitope recognition by T cells. These two antigens are representative of those observed by immunizing by using DNA and ADV with other antigens. Env typically elicits both CD4 and CD8 responses, while Gag induces a more prominent CD8 response. We find that the CD8 responses are boosted quantitatively by prime-boost immunization, but they remain fixed against the same epitopes. In contrast, the CD4 response to different epitopes increases following prime-boost vaccination.

MATERIALS AND METHODS

Mice. Female BALB/c and C57BL/6 mice, aged 6 to 8 weeks, were obtained from the Charles River Laboratory. Animal experiments were carried out in compliance with all federal and NIH policies.

Immunogens. The Env immunogen was a modified version of the BaL strain, a prototypic CCR5-tropic clade B virus. The gp145 form of this Env was used in the DNA vector, and gp140 was used for the ADV vector. Both versions contained mutations in the cleavage site, fusion domain, and interhelical region

* Corresponding author. Mailing address: Vaccine Research Center, NIAID, National Institutes of Health, Room 4502, Bldg. 40, MSC-3005, 40 Convent Drive, Bethesda, MD 20892-3005. Phone: (301) 496-1852. Fax: (301) 480-0274. E-mail: gnabel@nih.gov.

† L.W. and W.-P.K. contributed equally to this work.

(ΔCFI) to increase immunogenicity, as described previously (7). The clade B Gag immunogen represents a codon-modified form derived from the HXB2 strain (16) and was expressed under the regulation of a cytomegalovirus enhancer together with sequences from the HTLV-1 R region, which have been reported previously (51).

Vaccination schedule. Animals were injected with 50 μg of each plasmid DNA in the quadriceps muscle at 3-week intervals, for a total of three injections. The replication-defective adenoviral vector encoding each antigen was injected (10^{10} particles) intramuscularly. The DNA/ADV boost animals were injected with 50 μg DNA intramuscularly as described above, followed by the adenoviral boost 3 weeks after the third plasmid DNA injection. Intracellular cytokine staining was performed 10 days after the last injection for DNA and DNA/ADV, and ADV analyses were performed 3 weeks after a single ADV injection. In each case, the vaccination schedule has been determined from previous studies to represent the time at which the immune response can be maximally stimulated and optimally measured.

Immunologic analyses. The intracellular cytokine staining assay for tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) was performed with peptides from the respective immunogens as previously described (7, 17, 51). Each fraction in the figures includes two overlapping peptides. Pools were deconvoluted to test individual peptides at 2.5 μg/ml to determine the epitope specificity. Threshold levels were determined using control peptides directed to an irrelevant antigen from Ebola virus, as in past studies, and were set at threefold above the average background stimulation with the control peptides. No responses above this level were observed for the control peptides. Each analysis represents intracellular cytokine staining (ICS) of lymphocytes pooled from five animals and has been repeated independently in several instances to confirm the reproducibility of the results.

RESULTS

The response to DNA immunization with Env was examined initially for BALB/c mice by mapping epitopes that were recognized by T cells. Vaccination with a plasmid DNA encoding a modified clade B Env immunogen revealed a CD4 response to four peptide pairs that showed reactivity above the threshold level of 0.1%, a >5-fold increase above the background levels with control peptides, by ICS for IFN-γ and TNF-α (Fig. 1A, upper panel). In contrast, ADV vector immunization revealed a response to two epitope pairs, only one of which was the same as that stimulated by plasmid DNA vaccination (Fig. 1A, middle panel). In contrast, DNA/ADV prime-boost vaccination induced a response to at least 10 epitope pairs (Fig. 1A, lower panel). All the responses induced by DNA immunization were present within this pool, whereas only one of the adenoviral immunization epitope pairs was detected. The DNA/ADV immunization therefore elicited a stronger immune response to at least five epitopes that had not been detected with immunization by either DNA or ADV alone. In contrast, mapping of the CD8 epitopes revealed a different pattern of epitope responses. Two immunodominant peptide pairs in the V_3 region were identified, and these epitopes were recognized comparably by CD8 cells from animals immunized with DNA and ADV alone (Fig. 1B, upper and middle panels). While DNA/ADV immunization increased this response nearly fivefold, the same epitopes were recognized, suggesting a quantitative effect of the boost CD8 epitope recognition (Fig. 1C).

To evaluate whether this response was independent of the mouse strain and therefore generalizable, the CD4 and CD8 responses of C57BL/6 mice to the Env epitopes were mapped. In this strain, fewer CD4 epitopes were elicited by the ADV immunization than by the DNA alone (Fig. 2A, middle panel versus upper panel). Again, the DNA/ADV prime-boost immunization stimulated a response to several different epitope pairs compared to the DNA vaccine alone (Fig. 3A, lower panel versus upper panel). The CD8 response to Env in BALB/c mice was difficult to evaluate because of the absence of a substantial response to any epitope in this strain (Fig. 2B). Only two responses induced by ADV alone were observed, of marginal significance, and no substantial effects were seen using DNA or DNA/ADV vaccination (Fig. 2B, middle panel versus upper and lower panels), suggesting that this strain is a nonresponder for the Env antigen.

The immune responses to alternative immunogens were next analyzed to determine whether diversification of the CD4 response seen with DNA/ADV vaccination applied to an independent antigen, Gag. For BALB/c mice, the finding observed was similar to that for the Env response. Only two epitope pairs were recognized, slightly above background levels, after DNA immunization (Fig. 3, upper panel). A larger number of pairs (15) were observed after adenoviral immunization alone, but the magnitude and diversity of the response was greater with the DNA/ADV boost, where responses to 20 pairs were detected by ICS of CD4 cells (Fig. 3A, middle panel versus lower panel). Of these epitopes, the majority differed from those stimulated by either DNA or ADV boost alone. In contrast, six epitope pairs were identified with the CD8 response to Gag in BALB/c mice after DNA vaccination, and an additional one was seen after ADV priming alone (Fig. 3B, upper panel versus lower panel). Similar to the Env response, CD8 ICS increased in magnitude but retained the same specificity of epitope response as DNA or ADV vaccination alone (Fig. 3B, lower panel versus middle and upper panels).

Finally, the response to Gag in C57BL/6 mice was analyzed. While three responses were observed above background for DNA alone and six for ADV alone, eight were observed with DNA priming and ADV boosting, of which four had not been seen with either agent alone (Fig. 4A, lower panel versus middle and upper panels). In contrast, the CD8 response to Gag in C57BL/6 mice was focused on three epitope pairs, all of which were identical and boosted in magnitude with the DNA/ADV immunization (Fig. 4B, lower panel versus middle and upper panels).

DISCUSSION

In this study, we have examined the specificity of the immune response to DNA, ADV, and DNA/ADV prime-boost immunization in mice. The response was characterized with two different immunogens, Env and Gag, each of which was examined for two mouse strains. In each case, diversification of the $CD4^+$ T-cell immune response was observed following DNA/ADV immunization compared to vaccination with either modality alone. With the exception of a null response to Env in C57BL/6 mice for CD8 T cells. It is unsurprising that some inbred mouse strains do not respond to specific antigens given the homozygosity and limited major histocompatibility complex (MHC) class I diversity. This finding is unlikely to predict the success of this approach in human studies, where there is considerable polymorphism and diversification of MHC class I genes. Preliminary studies of humans suggest that such responses are readily elicited (data not shown). The response to two antigens in independent strains suggests also that these responses are likely to be representative and predictive of responses to other immunogens for various genetic backgrounds.

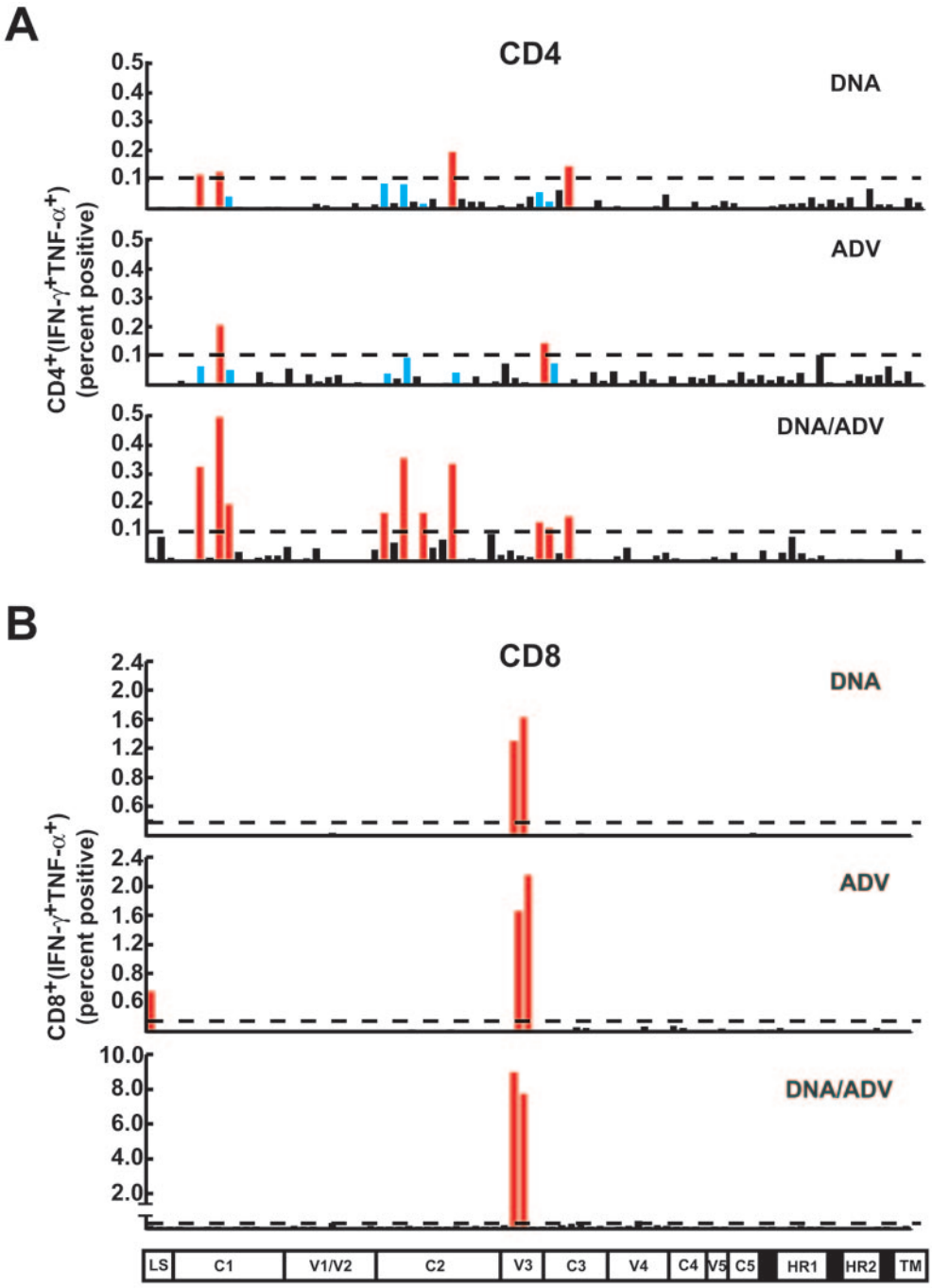

FIG. 1. Definition of CD4 and CD8 epitope responses to HIV Env in BALB/c mice. BALB/c mice were injected with DNA, ADV, or DNA/ADV vectors encoding Env, as indicated in Materials and Methods, and ICS to peptide pairs in the indicated positions was determined. The peptides to the HIV Env region were synthesized as 15-mers overlapping by 11, as previously described (7, 17). Responses to the (A) CD4 or (B) CD8 epitopes are shown. The minimal threshold response is indicated by the dashed line. Red bars indicate responses above the background, while blue bars indicate epitope responses below the background that are stimulated above the threshold using a second vaccination vector; however, the values indicated by the blue bars do not show significant stimulation. Black bars show the level of response to epitopes below the background level of detection. Any value below the threshold level indicated by the horizontal dashed line, whether blue or black, is below the background level and not statistically significant. LS indicates the leader sequence, and TM refers to the transmembrane domain.

It has been well recognized that heterologous prime-boost immunization confers a stronger response to immunogens and greater protection in several infectious disease models than immunization with either vector alone (10, 11, 14, 18, 25–27, 32, 33, 43, 45, 46); however, the immunologic basis for this effect has not been understood. Although the magnitudes of the immune responses elicited by prime-boost immunization have been greater than those elicited by either vector alone, it remained possible that the specificities of those responses were similar. In this study, we show that diversification of the CD4

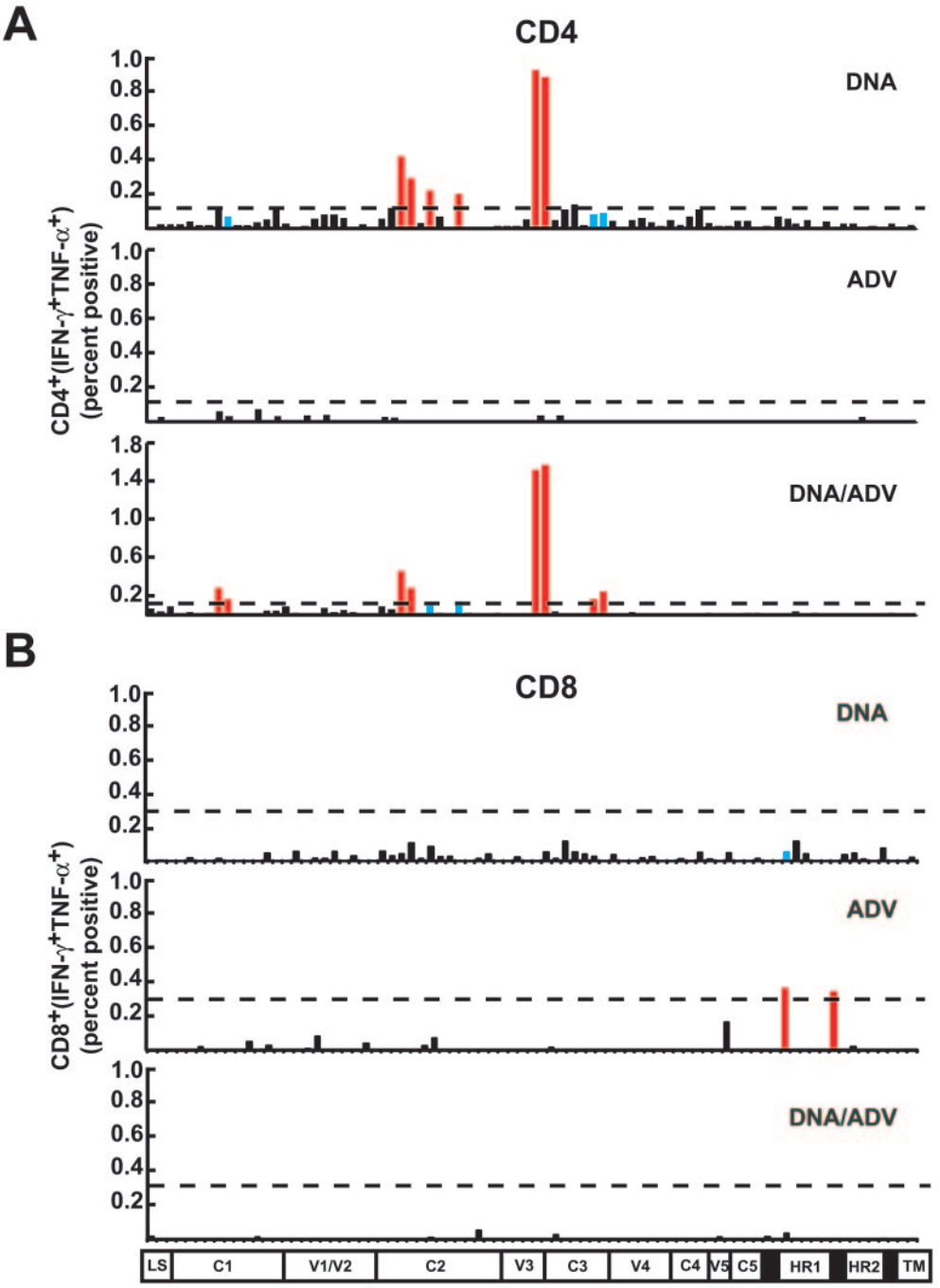

FIG. 2. Analysis of CD4 and CD8 epitope responses of C57BL/6 mice to HIV Env. C57BL/6 mice were injected with DNA, ADV, or DNA/ADV vaccines for Env, as indicated in Materials and Methods, and ICS to peptide pairs in the indicated positions was determined. The peptides to the HIV Env region were synthesized as 15-mers overlapping by 11. Responses to the (A) CD4 or (B) CD8 epitopes are shown. The minimal threshold response is indicated by the dashed line. Red, blue, and black bars are as defined in the legend to Fig. 1. LS indicates the leader sequence, and TM refers to the transmembrane domain.

T-cell response occurs following prime-boost immunization. In total, we have mapped the response to 159 Env peptides and 122 Gag peptides in two inbred mouse strains. When CD4 and CD8 responses were evaluated as positive responses to specific peptides above background levels, at least 20 new CD4 responses that were not seen with either modality alone appeared after DNA/ADV immunization; in contrast, no additional CD8 responses appeared. This association was highly significant ($P = 0.000001$, Fisher's exact test), indicating that the prime-boost immunization significantly increases the breadth of the CD4 responses while having only a quantitative effect on measurable CD8 responses. Although some responses with the heterologous prime-boost combination may be present at subthreshold amounts, the findings nonetheless indicate that this heterologous immunization allows for boosting that can enhance this effect and substantially

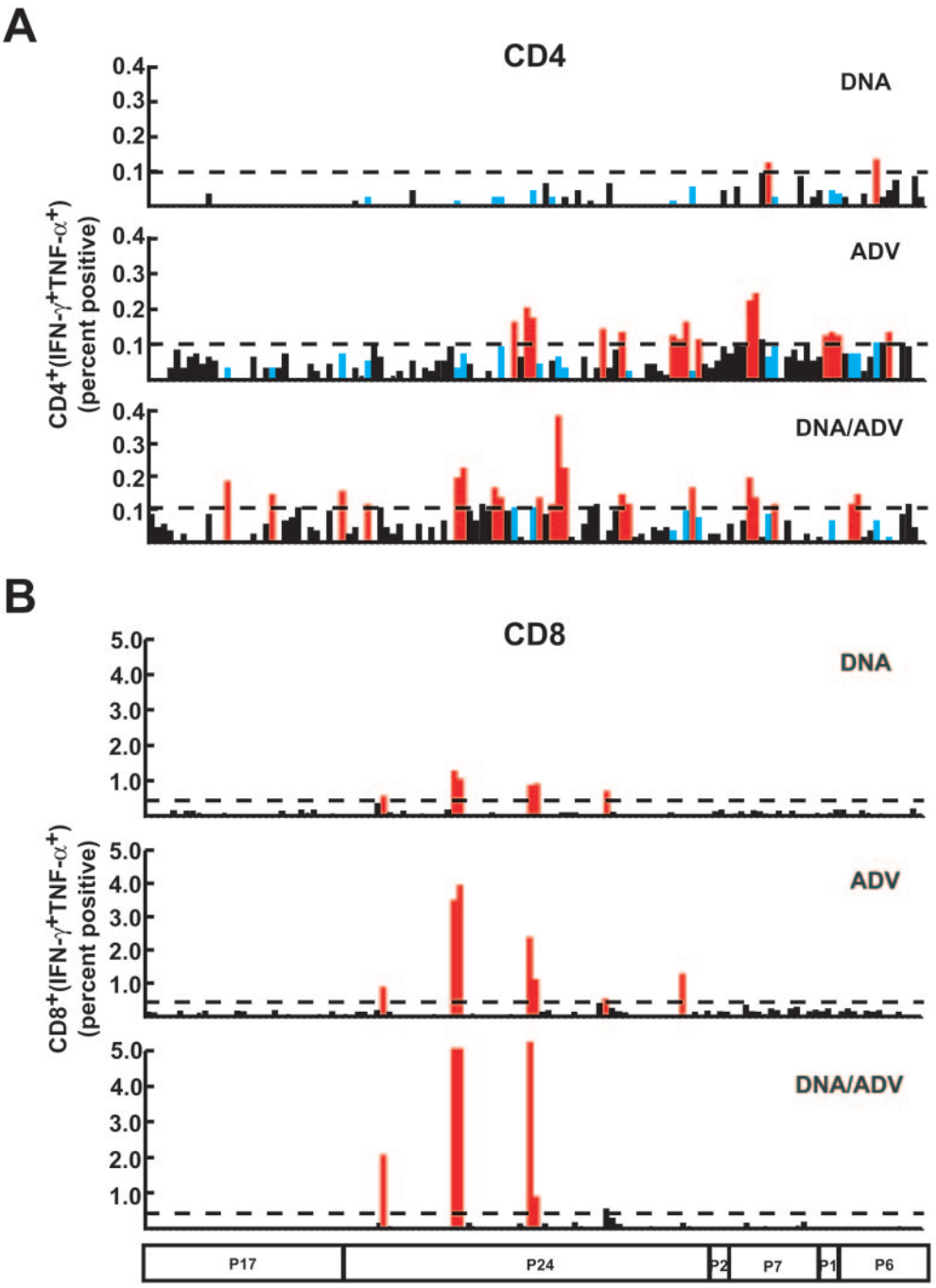

FIG. 3. Characterization of CD4 and CD8 epitope responses of BALB/c mice to HIV Gag. BALB/c mice were injected with the indicated vectors encoding Gag as described in Materials and Methods, and ICS to the indicated pairs of peptides was determined. The peptides to the HIV Gag region were synthesized as 15-mers overlapping by 11 (16, 17). Responses to the (A) CD4 or (B) CD8 epitopes are shown. The minimal threshold response is indicated by the dashed line. Red, blue, and black bars are as defined in the legend to Fig. 1.

increase the dominance of clones which recognize alternative epitopes in the antigen, specifically related to the CD4 response. In contrast, for CD8 cells, the pattern of immunodominance set by immunization with either agent alone results in nearly identical epitope recognition and a similar pattern of response following boosting.

Although the mechanisms that determine the breadth and potency of epitope recognition are unclear, it is likely that such responses are determined both by the T-cell repertoire and by the reactivity of T-cell receptors with a given antigen, as well as the mode of antigen processing and presentation. It has been shown that DNA vaccination can alter the hierarchy of immunodominance in T-cell responses (3), possibly through more efficient MHC loading after synthesis in antigen-presenting cells. While ADV appears to target early dendritic cells, which may differentiate to mature dendritic cells that more effectively

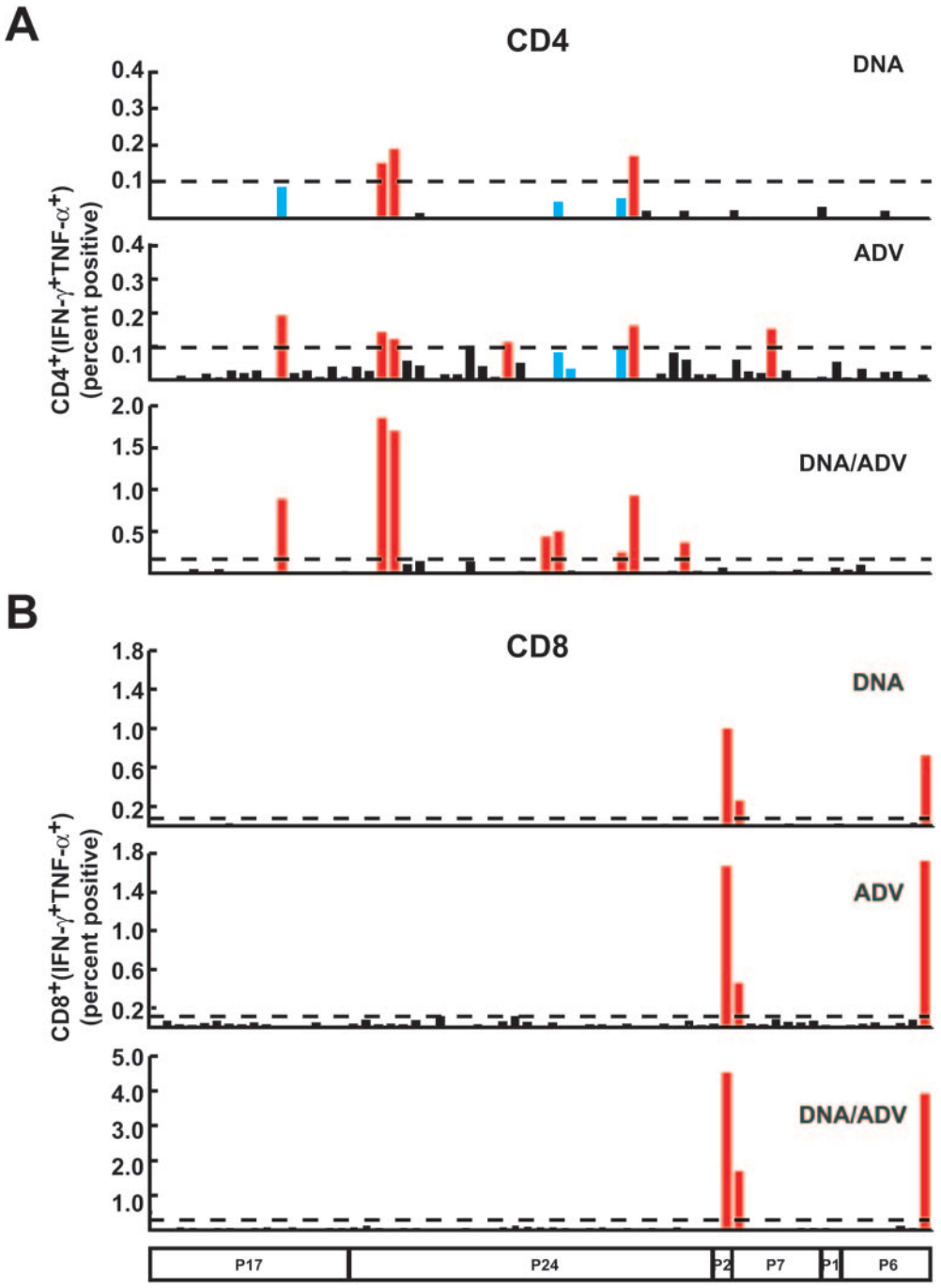

FIG. 4. Specificities of the CD4 and CD8 epitope responses of C57BL/6 mice to HIV Gag. C57BL/6 mice were immunized with the specified Gag immunogen vectors as described in Materials and Methods, and ICS to peptide pools from the HIV Gag region was determined. The peptides to the HIV Gag region were synthesized as 15-mers overlapping by 11 (16, 17). Responses to the (A) CD4 or (B) CD8 epitopes are shown. The minimal threshold response is indicated by the dashed line. Red, blue, and black bars are as defined in the legend to Fig. 1.

present antigens, they also synthesize larger quantities of protein, which may be taken up by endocytosis. Thus, the divergent cell targeting and antigen processing routes may complement one another, allowing a greater diversity of epitope recognition than with either agent alone.

These data have implications for the development and design of vaccines. In particular, because the immune response arises more rapidly after ADV immunization alone (41), it might be argued that this could be a preferable mode of immunization. If the specificities of the immune responses were identical, this approach would be reasonable; however, the data here suggest that alternative vectors, even when encoding the same antigen, stimulate different specificities in the CD4 immune response. Each of these vaccine modalities should therefore be considered independently for the evaluation of vaccine efficacy.

ACKNOWLEDGMENTS

We thank Ati Tislerics for assistance with manuscript preparation, Toni Garrison and Brenda Hartman for figure preparation, Richard Koup for comments on the manuscript, and Yiming Shao and members of the Nabel lab for helpful discussions and advice.

REFERENCES

1. **Amara, R. R., J. M. Smith, S. I. Staprans, D. C. Montefiori, F. Villinger, J. D. Altman, S. P. O'Neil, N. L. Kozyr, Y. Xu, L. S. Wyatt, P. L. Earl, J. G. Herndon, J. M. McNicholl, H. M. McClure, B. Moss, and H. L. Robinson.** 2002. Critical role for Env as well as Gag-Pol in control of a simian-human immunodeficiency virus 89.6P challenge by a DNA prime/recombinant modified vaccinia virus Ankara vaccine. J. Virol. **76:**6138–6146.
2. **Barnett, S. W., S. Rajasekar, H. Legg, B. Doe, D. H. Fuller, J. R. Haynes, C. M. Walker, and K. S. Steimer.** 1997. Vaccination with HIV-1 gp120 DNA induces immune responses that are boosted by a recombinant gp120 protein subunit. Vaccine **15:**869–873.
3. **Barouch, D. H., A. Craiu, S. Santra, M. A. Egan, J. E. Schmitz, M. J. Kuroda, T. M. Fu, J. H. Nam, L. S. Wyatt, M. A. Lifton, G. R. Krivulka, C. E. Nickerson, C. I. Lord, B. Moss, M. G. Lewis, V. M. Hirsch, J. W. Shiver, and N. L. Letvin.** 2001. Elicitation of high-frequency cytotoxic T-lymphocyte responses against both dominant and subdominant simian-human immunodeficiency virus epitopes by DNA vaccination of rhesus monkeys. J. Virol. **75:**2462–2467.
4. **Barouch, D. H., M. G. Pau, J. H. Custers, W. Koudstaal, S. Kostense, M. J. Havenga, D. M. Truitt, S. M. Sumida, M. G. Kishko, J. C. Arthur, B. Korioth-Schmitz, M. H. Newberg, D. A. Gorgone, M. A. Lifton, D. L. Panicali, G. J. Nabel, N. L. Letvin, and J. Goudsmit.** 2004. Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. J. Immunol. **172:**6290–6297.
5. **Casimiro, D. R., A. J. Bett, T. M. Fu, M. E. Davies, A. Tang, K. A. Wilson, M. Chen, R. Long, T. McKelvey, M. Chastain, S. Gurunathan, J. Tartaglia, E. A. Emini, and J. Shiver.** 2004. Heterologous human immunodeficiency virus type 1 priming-boosting immunization strategies involving replication-defective adenovirus and poxvirus vaccine vectors. J. Virol. **78:**11434–11438.
6. **Casimiro, D. R., L. Chen, T.-M. Fu, R. K. Evans, M. J. Caulfield, M.-E. Davies, A. Tang, M. Chen, L. Huang, V. Harris, D. C. Freed, K. A. Wilson, S. Dubey, D.-M. Zhu, D. Nawrocki, H. Mach, R. Troutman, L. Isopi, D. Williams, W. Hurni, Z. Xu, J. G. Smith, S. Wang, X. Liu, L. Guan, R. Long, W. Trigona, G. J. Heidecker, H. C. Perry, N. Persaud, T. J. Toner, Q. Su, X. Liang, R. Youil, M. Chastain, A. J. Bett, D. B. Volkin, E. A. Emini, and J. W. Shiver.** 2003. Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 *gag* gene. J. Virol. **77:**6305–6313.
7. **Chakrabarti, B. K., W. P. Kong, B.-Y. Wu, Z.-Y. Yang, J. Friborg, Jr., X. Ling, S. R. King, D. C. Montefiori, and G. J. Nabel.** 2002. Modifications of the human immunodeficiency virus envelope glycoprotein enhance immunogenicity for genetic immunization. J. Virol. **76:**5357–5368.
8. **Coban, C., M. T. Philipp, J. E. Purcell, D. B. Keister, M. Okulate, D. S. Martin, and N. Kumar.** 2004. Induction of *Plasmodium falciparum* transmission-blocking antibodies in nonhuman primates by a combination of DNA and protein immunizations. Infect. Immun. **72:**253–259.
9. **Dale, C. J., R. De Rose, I. Stratov, S. Chea, D. C. Montefiori, S. Thomson, I. A. Ramshaw, B. E. Coupar, D. B. Boyle, M. Law, and S. J. Kent.** 2004. Efficacy of DNA and fowlpox virus priming/boosting vaccines for simian/human immunodeficiency virus. J. Virol. **78:**13819–13828.
10. **Da Silva, D. M., J. T. Schiller, and W. M. Kast.** 2003. Heterologous boosting increases immunogenicity of chimeric papillomavirus virus-like particle vaccines. Vaccine **21:**3219–3227.
11. **Davidson, E. J., R. L. Faulkner, P. Sehr, M. Pawlita, L. J. Smyth, D. J. Burt, A. E. Tomlinson, J. Hickling, H. C. Kitchener, and P. L. Stern.** 2004. Effect of TA-CIN (HPV 16 L2E6E7) booster immunisation in vulval intraepithelial neoplasia patients previously vaccinated with TA-HPV (vaccinia virus encoding HPV 16/18 E6E7). Vaccine **22:**2722–2729.
12. **De Serres, G., J. Sciberras, M. Naus, N. Boulianne, B. Duval, and L. Rochette.** 1999. Protection after two doses of measles vaccine is independent of interval between doses. J. Infect. Dis. **180:**187–190.
13. **Epstein, J. E., Y. Charoenvit, K. E. Kester, R. Wang, R. Newcomer, S. Fitzpatrick, T. L. Richie, N. Tornieporth, D. G. Heppner, C. Ockenhouse, V. Majam, C. Holland, E. Abot, H. Ganeshan, M. Berzins, T. Jones, C. N. Freydberg, J. Ng, J. Norman, D. J. Carucci, J. Cohen, and S. L. Hoffman.** 2004. Safety, tolerability, and antibody responses in humans after sequential immunization with a PfCSP DNA vaccine followed by the recombinant protein vaccine RTS,S/AS02A. Vaccine **22:**1592–1603.
14. **Ferraz, J. C., E. Stavropoulos, M. Yang, S. Coade, C. Espitia, D. B. Lowrie, M. J. Colston, and R. E. Tascon.** 2004. A heterologous DNA priming-*Mycobacterium bovis* BCG boosting immunization strategy using mycobacterial Hsp70, Hsp65, and Apa antigens improves protection against tuberculosis in mice. Infect. Immun. **72:**6945–6950.
15. **Gilbert, S. C., J. Schneider, C. M. Hannan, J. T. Hu, M. Plebanski, R. Sinden, and A. V. Hill.** 2002. Enhanced CD8 T cell immunogenicity and protective efficacy in a mouse malaria model using a recombinant adenoviral vaccine in heterologous prime-boost immunisation regimes. Vaccine **20:**1039–1045.
16. **Huang, Y., W. Kong, and G. J. Nabel.** 2001. Human immunodeficiency virus type 1-specific immunity after genetic immunization is enhanced by modification of Gag and Pol expression. J. Virol. **75:**4947–4951.
17. **Kong, W. P., Y. Huang, Z. Y. Yang, B. K. Chakrabarti, Z. Moodie, and G. J. Nabel.** 2003. Immunogenicity of multiple gene and clade human immunodeficiency virus type 1 DNA vaccines. J. Virol. **77:**12764–12772.
18. **Kongkasuriyachai, D., L. Bartels-Andrews, A. Stowers, W. E. Collins, J. Sullivan, J. Sattabongkot, M. Torii, T. Tsuboi, and N. Kumar.** 2004. Potent immunogenicity of DNA vaccines encoding *Plasmodium vivax* transmission-blocking vaccine candidates Pvs25 and Pvs28-evaluation of homologous and heterologous antigen-delivery prime-boost strategy. Vaccine **22:**3205–3213.
19. **Konishi, E., A. Terazawa, and J. Imoto.** 2003. Simultaneous immunization with DNA and protein vaccines against Japanese encephalitis or dengue synergistically increases their own abilities to induce neutralizing antibody in mice. Vaccine **21:**1826–1832.
20. **Letvin, N. L., Y. Huang, B. K. Chakrabarti, L. Xu, M. S. Seaman, K. Beaudry, B. Korioth-Schmitz, F. Yu, D. Rohne, K. L. Martin, A. Miura, W. P. Kong, Z. Y. Yang, R. S. Gelman, O. G. Golubeva, D. C. Montefiori, J. R. Mascola, and G. J. Nabel.** 2004. Heterologous envelope immunogens contribute to AIDS vaccine protection in rhesus monkeys. J. Virol. **78:**7490–7497.
21. **Letvin, N. L., D. C. Montefiori, Y. Yasutomi, H. C. Perry, M. E. Davies, C. Lekutis, M. Alroy, D. C. Freed, C. I. Lord, L. K. Handt, M. A. Liu, and J. W. Shiver.** 1997. Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. Proc. Natl. Acad. Sci. USA **94:**9378–9383.
22. **Mascola, J. R., A. Sambor, K. Beaudry, S. Santra, B. Welcher, M. K. Louder, T. C. VanCott, Y. Huang, B. K. Chakrabarti, W. P. Kong, Z. Y. Yang, L. Xu, D. C. Montefiori, G. J. Nabel, and N. L. Letvin.** 2005. Neutralizing antibodies elicited by immunization of monkeys with DNA plasmids and recombinant adenoviral vectors expressing human immunodeficiency virus type 1 proteins. J. Virol. **79:**771–779.
23. **Matano, T., M. Kano, H. Nakamura, A. Takeda, and Y. Nagai.** 2001. Rapid appearance of secondary immune responses and protection from acute CD4 depletion after a highly pathogenic immunodeficiency virus challenge in macaques vaccinated with a DNA prime/Sendai virus vector boost regimen. J. Virol. **75:**11891–11896.
24. **McConkey, S. J., W. H. Reece, V. S. Moorthy, D. Webster, S. Dunachie, G. Butcher, J. M. Vuola, T. J. Blanchard, P. Gothard, K. Watkins, C. M. Hannan, S. Everaere, K. Brown, K. E. Kester, J. Cummings, J. Williams, D. G. Heppner, A. Pathan, K. Flanagan, N. Arulanantham, M. T. Roberts, M. Roy, G. L. Smith, J. Schneider, T. Peto, R. E. Sinden, S. C. Gilbert, and A. V. Hill.** 2003. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. Nat. Med. **9:**729–735.
25. **Moore, A. C., and A. V. Hill.** 2004. Progress in DNA-based heterologous prime-boost immunization strategies for malaria. Immunol. Rev. **199:**126–143.
26. **Moorthy, V. S., E. B. Imoukhuede, P. Milligan, K. Bojang, S. Keating, P. Kaye, M. Pinder, S. C. Gilbert, G. Walraven, B. M. Greenwood, and A. S. Hill.** 2004. A randomised, double-blind, controlled vaccine efficacy trial of DNA/MVA ME-TRAP against malaria infection in Gambian adults. PLoS Med. **1:**E33.
27. **Nyika, A., A. F. Barbet, M. J. Burridge, and S. M. Mahan.** 2002. DNA vaccination with *map1* gene followed by protein boost augments protection against challenge with *Cowdria ruminantium*, the agent of heartwater. Vaccine **20:**1215–1225.
28. **Ophorst, O. J., S. Kostense, J. Goudsmit, R. L. De Swart, S. Verhaagh, A. Zakhartchouk, M. Van Meijer, M. Sprangers, G. Van Amerongen, S. Yuksel, A. D. Osterhaus, and M. J. Havenga.** 2004. An adenoviral type 5 vector carrying a type 35 fiber as a vaccine vehicle: DC targeting, cross neutralization, and immunogenicity. Vaccine **22:**3035–3044.
29. **Pancholi, P., Q. Liu, N. Tricoche, P. Zhang, M. E. Perkus, and A. M. Prince.** 2000. DNA prime-canarypox boost with polycistronic hepatitis C virus (HCV) genes generates potent immune responses to HCV structural and nonstructural proteins. J. Infect. Dis. **182:**18–27.
30. **Patterson, L. J., N. Malkevitch, D. Venzon, J. Pinczewski, V. R. Gomez-Roman, L. Wang, V. S. Kalyanaraman, P. D. Markham, F. A. Robey, and M. Robert-Guroff.** 2004. Protection against mucosal simian immunodeficiency virus SIV_{mac251} challenge by using replicating adenovirus-SIV multigene vaccine priming and subunit boosting. J. Virol. **78:**2212–2221.
31. **Pinto, A. R., J. C. Fitzgerald, W. Giles-Davis, G. P. Gao, J. M. Wilson, and H. C. Ertl.** 2003. Induction of $CD8^+$ T cells to an HIV-1 antigen through a prime boost regimen with heterologous E1-deleted adenoviral vaccine carriers. J. Immunol. **171:**6774–6779.
32. **Putz, M. M., W. Ammerlaan, F. Schneider, G. Jung, and C. P. Muller.** 2004. Humoral immune responses to a protective peptide-conjugate against measles after different prime-boost regimens. Vaccine **22:**4173–4182.

33. **Ramiro, M. J., J. J. Zarate, T. Hanke, D. Rodriguez, J. R. Rodriguez, M. Esteban, J. Lucientes, J. A. Castillo, and V. Larraga.** 2003. Protection in dogs against visceral leishmaniasis caused by *Leishmania infantum* is achieved by immunization with a heterologous prime-boost regime using DNA and vaccinia recombinant vectors expressing LACK. Vaccine **21:**2474–2484.
34. **Ramsburg, E., N. F. Rose, P. A. Marx, M. Mefford, D. F. Nixon, W. J. Moretto, D. Montefiori, P. Earl, B. Moss, and J. K. Rose.** 2004. Highly effective control of an AIDS virus challenge in macaques by using vesicular stomatitis virus and modified vaccinia virus Ankara vaccine vectors in a single-boost protocol. J. Virol. **78:**3930–3940.
35. **Ray, R. B., A. Basu, R. Steele, A. Beyene, J. McHowat, K. Meyer, A. K. Ghosh, and R. Ray.** 2004. Ebola virus glycoprotein-mediated anoikis of primary human cardiac microvascular endothelial cells. Virology **321:**181–188.
36. **Robinson, H. L., D. C. Montefiori, R. P. Johnson, M. L. Kalish, S. L. Lydy, and H. M. McClure.** 2000. DNA priming and recombinant pox virus boosters for an AIDS vaccine. Dev. Biol. (Basel) **104:**93–100.
37. **Santra, S., D. H. Barouch, B. Korioth-Schmitz, C. I. Lord, G. R. Krivulka, F. Yu, M. H. Beddall, D. A. Gorgone, M. A. Lifton, A. Miura, V. Philippon, K. Manson, P. D. Markham, J. Parrish, M. J. Kuroda, J. E. Schmitz, R. S. Gelman, J. W. Shiver, D. C. Montefiori, D. Panicali, and N. L. Letvin.** 2004. Recombinant poxvirus boosting of DNA-primed rhesus monkeys augments peak but not memory T lymphocyte responses. Proc. Natl. Acad. Sci. USA **101:**11088–11093.
38. **Shiver, J. W., T. M. Fu, L. Chen, D. R. Casimiro, M. E. Davies, R. K. Evans, Z. Q. Zhang, A. J. Simon, W. L. Trigona, S. A. Dubey, L. Huang, V. A. Harris, R. S. Long, X. Liang, L. Handt, W. A. Schleif, L. Zhu, D. C. Freed, N. V. Persaud, L. Guan, K. S. Punt, A. Tang, M. Chen, K. A. Wilson, K. B. Collins, G. J. Heidecker, V. R. Fernandez, H. C. Perry, J. G. Joyce, K. M. Grimm, J. C. Cook, P. M. Keller, D. S. Kresock, H. Mach, R. D. Troutman, L. A. Isopi, D. M. Williams, Z. Xu, K. E. Bohannon, D. B. Volkin, D. C. Montefiori, A. Miura, G. R. Krivulka, M. A. Lifton, M. J. Kuroda, J. E. Schmitz, N. L. Letvin, M. J. Caulfield, A. J. Bett, R. Youil, D. C. Kaslow, and E. A. Emini.** 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature **415:**331–335.
39. **Simmons, G., R. J. Wool-Lewis, F. Baribaud, R. C. Netter, and P. Bates.** 2002. Ebola virus glycoproteins induce global surface protein down-modulation and loss of cell adherence. J. Virol. **76:**2518–2528.
40. **Skinner, M. A., B. M. Buddle, D. N. Wedlock, D. Keen, G. W. de Lisle, R. E. Tascon, J. C. Ferraz, D. B. Lowrie, P. J. Cockle, H. M. Vordermeier, and R. G. Hewinson.** 2003. A DNA prime-*Mycobacterium bovis* BCG boost vaccination strategy for cattle induces protection against bovine tuberculosis. Infect. Immun. **71:**4901–4907.
41. **Sullivan, N. J., T. W. Geisbert, J. B. Geisbert, L. Xu, Z. Y. Yang, M. Roederer, R. A. Koup, P. B. Jahrling, and G. J. Nabel.** 2003. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. Nature **424:**681–684.
42. **Sullivan, N. J., A. Sanchez, P. E. Rollin, Z.-Y. Yang, and G. J. Nabel.** 2000. Development of a preventive vaccine for Ebola virus infection in primates. Nature **408:**605–609.
43. **Tellier, M. C., R. Pu, D. Pollock, A. Vitsky, J. Tartaglia, E. Paoletti, and J. K. Yamamoto.** 1998. Efficacy evaluation of prime-boost protocol: canarypoxvirus-based feline immunodeficiency virus (FIV) vaccine and inactivated FIV-infected cell vaccine against heterologous FIV challenge in cats. AIDS **12:**11–18.
44. **Vajdy, M., M. Singh, J. Kazzaz, E. Soenawan, M. Ugozzoli, F. Zhou, I. Srivastava, Q. Bin, S. Barnett, J. Donnelly, P. Luciw, L. Adamson, D. Montefiori, and D. T. O'Hagan.** 2004. Mucosal and systemic anti-HIV responses in rhesus macaques following combinations of intranasal and parenteral immunizations. AIDS Res. Hum. Retrovir. **20:**1269–1281.
45. **Vindurampulle, C. J., L. F. Cuberos, E. M. Barry, M. F. Pasetti, and M. M. Levine.** 2004. Recombinant *Salmonella enterica* serovar Typhi in a prime-boost strategy. Vaccine **22:**3744–3750.
46. **Vordermeier, H. M., S. G. Rhodes, G. Dean, N. Goonetilleke, K. Huygen, A. V. Hill, R. G. Hewinson, and S. C. Gilbert.** 2004. Cellular immune responses induced in cattle by heterologous prime-boost vaccination using recombinant viruses and bacille Calmette-Guerin. Immunology **112:**461–470.
47. **Xiang, Z. Q., S. Pasquini, and H. C. Ertl.** 1999. Induction of genital immunity by DNA priming and intranasal booster immunization with a replication-defective adenoviral recombinant. J. Immunol. **162:**6716–6723.
48. **Yang, Z.-Y., H. J. Duckers, N. J. Sullivan, A. Sanchez, E. G. Nabel, and G. J. Nabel.** 2000. Identification of the Ebola virus glycoprotein as the main viral determinant of vascular cell cytotoxicity and injury. Nat. Med. **6:**886–889.
49. **Yang, Z.-Y., L. S. Wyatt, W. P. Kong, Z. Moodie, B. Moss, and G. J. Nabel.** 2003. Overcoming immunity to a viral vaccine by DNA priming before vector boosting. J. Virol. **77:**799–803.
50. **Yang, Z.-Y., B. K. Chakrabarti, L. Xu, B. Welcher, W.-P. Kong, K. Leung, A. Panet, J. R. Mascola, and G. J. Nabel.** 2004. Selective modification of variable loops alters tropism and enhances immunogenicity of human immunodeficiency virus type 1 envelope. J. Virol. **78:**4029–4036.
51. **Yang, Z.-Y., W.-P. Kong, Y. Huang, A. Roberts, B. Murphy, K. Subbarao, and G. J. Nabel.** 2004. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. Nature **428:**561–564.