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Immunogenicity of hybrid DNA vaccines expressing hepatitis B core particles carrying human and simian immunodeficiency virus epitopes in mice and rhesus macaques

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Abstract

An effective HIV vaccine will likely need to induce broad and potent CTL responses. Epitope-based vaccines offer significant potential for inducing multi-specific CTL, but often require conjugation to T helper epitopes or carrier moieties to induce significant responses. We tested hybrid DNA vaccines encoding one or more HIV or SIV CTL epitopes fused to a hepatitis B core antigen (*HBcAg*) carrier gene as a means to improve the immunogenicity of epitope-based DNA vaccines. Immunization of mice with a HBcAg-HIV epitope DNA vaccine induced $CD8^+$ T cell responses that significantly exceeded levels induced with DNA encoding either the whole HIV antigen or the epitope alone. In rhesus macaques, a multi-epitope hybrid HBcAg-SIV DNA vaccine induced CTL responses to 13 different epitopes, including 3 epitopes that were previously not detected in SIV-infected macaques. These data demonstrate that immunization with hybrid HBcAg-epitope DNA vaccines is an effective strategy to increase the magnitude and breadth of HIV-specific CTL responses.
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Keywords: Cytotoxic T lymphocyte response; Epitope; Hybrid vaccine; Hepatitis B core antigen; Human immunodeficiency virus; Simian immunodeficiency virus; nonhuman primate

Introduction

A vaccine capable of controlling or preventing HIV infection is needed to stem the AIDS epidemic. An effective vaccine will likely need to induce $CD8^+$ cytotoxic T lymphocytes (CTL) (McMichael and Rowland-Jones, 2001) and protective immunity against different viral variants may require induction of CTL responses against a broad range of epitopes, including subdominant and conserved sequences.

DNA immunization effectively induces T cell responses, including CTL (Donnelly et al., 1997), making this strategy an attractive approach for vaccination against HIV. Studies in nonhuman primates have shown that DNA vaccines afford various levels of protection against challenge with avirulent or pathogenic AIDS viruses (Amara et al., 2001; Barouch et al., 2000; Boyer et al., 1997; Kent et al., 1998; Robinson et al., 1999; Rosati et al., 2005; Singh et al., 2005). However, in most studies, boosting with a viral vaccine vector, numerous booster immunizations, or high doses of DNA was required to induce strong CTL responses and suppress infection, suggesting that new strategies are needed to improve DNA vaccine potency.

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Epitope-based vaccines offer advantages for induction of CTL. Unlike whole antigens, epitope vaccines can be designed to include the relevant epitopes while excluding sequences outside epitope domains that can negatively influence immunogenicity (Collins et al., 1998; Gratton et al., 1998; Maggi et al., 1994; Romagnani and Maggi, 1994). Epitope-based vaccines can also induce more potent immune responses than whole antigen vaccines (Ishioka et al., 1999; Restifo et al., 1995). The combination of an epitope-based strategy in the context of a DNA vaccine may, therefore, provide a highly effective strategy to elicit HIV-specific CTL.

Epitopes often require conjugation to a longer, immunogenic T helper peptide (Restifo et al., 1995; Shirai et al., 1994; Vitiello et al., 1995) or a carrier moiety (Griffiths et al., 1993; Layton et al., 1993; Michel et al., 1988; Schlienger et al., 1992; Schodel et al., 1994a) to achieve significant immunogenicity. The hepatitis B virus nucleocapsid antigen (HBcAg) has been used as an efficient carrier moiety for peptide or protein vaccines (Milich et al., 1995; Schodel et al., 1994a; Ulrich et al., 1998), and immunization with purified hybrid HBcAg-B cell epitope particles enhances antibody responses against the carried epitope (Milich et al., 1995; Schodel et al., 1994a; Tindle et al., 1994; Ulrich et al., 1998). This prompted us to investigate HBcAg as a carrier for CTL epitopes in the context of a DNA vaccine as a method to augment CTL responses. Here, we demonstrate hybrid DNA vaccines encoding HIV or SIV epitopes fused to a *HBcAg* carrier gene increase CTL epitope immunogenicity in mice and can be used to induce broad CTL responses against multiple epitopes in nonhuman primates.

Results

Generation of HBcAg-epitope DNA vaccines

Heterologous epitopes can be inserted into either the immunodominant antibody binding or the C-terminus regions of the *HBcAg* gene without disrupting the core particle (Borisova et al., 1996). To test this concept as a DNA vaccine, an H-2D^d-restricted HIV CTL epitope, RGPGRAFVTI (Takeshita et al., 1995) recognized in Balb/c mice, was inserted into the immunodominant region of a plasmid encoding the *HBcAg* carrier gene (*pHBc*) (Fig. 1), resulting in the hybrid DNA vaccine, pHBc-V3-10. To determine if the hybrid vaccine expressed core particles, Vero cells were transfected with pHBc-V3-10 and the supernatants were subjected to sedimentation in a 20% glycerol cushion to pellet the core particles. For comparison, supernatants from Vero cells transfected with either the parent HBcAg vector (positive control) or DNA encoding irrelevant antigen (negative control) were also analyzed. Core particles were measured by ELISA detection of a hepatitis B e antigen (HBeAg) epitope that is exposed on particles expressed by the HBc plasmids due to deletion of the C-terminal arginine-rich region of the *HBcAg* gene. HBeAg was readily detected in the fraction from the parent HBcAg carrier vector (pHBc) and the hybrid pHBc-V3-10 vector, but not from DNA encoding the HIV envelope gene

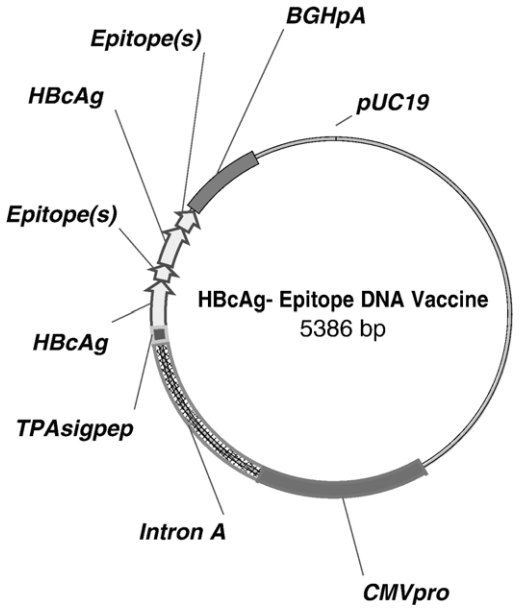

Fig. 1. Schematic of the chimeric hepatitis B core antigen-epitope plasmid design. Vector elements include the human cytomegalovirus immediate early promoter (CMVpro) with intron A sequences, the tissue plasminogen activation signal peptide (TPAsigpep), the coding sequence for hepatitis B core antigen (HBcAg), the bovine growth hormone polyadenylation signal (BGHpA), and the pUC19 origin of replication. One to three CTL epitopes were inserted into either the immunodominant loop or at the C-terminus of the *HBcAg* gene.

(*pHIVgp120*) (Fig. 2), confirming that the hybrid vector expressed hepatitis B core particles.

A multi-epitope SIV DNA vaccine encoding19 SIV_{mac239}-specific CTL epitopes that bind the Mamu-A*01 MHC class I molecule (Allen et al., 2001) was also generated. The epitopes were inserted into either the internal region or C-terminus of the *HBcAg* gene. Separation of epitopes onto different plasmids reduces competition between epitopes (Rodriguez et al., 2002). Therefore, the two immunodominant CTL epitopes, $Gag_{181–189}$ CM9 and $Tat_{28–35}$ SL8 (Allen et al., 2001), were inserted into 2 separate HBcAg vectors, and the remaining 16 epitopes were separated among 7 additional vectors resulting in a cocktail of 9 hybrid HBc-SIV vectors, each carrying 1–3 epitopes (Table 1) separated by two alanines. All 9 HBc-SIV vectors expressed variable, but comparable, levels of core particles *in vitro* (data not shown).

Hybrid HBcAg-epitope DNA vaccines increase epitope immunogenicity

We investigated HBcAg for the ability to enhance the immunogenicity of the HIV epitope. Groups of 8 Balb/c mice were immunized with three 1 μg DNA doses of either the hybrid vector (pHBc-V3-10) or a control vector encoding the same epitope without carrier (pV3-10) using a gene gun to deliver the DNA directly into the cells of the epidermis. A 3rd group of mice was immunized with a co-delivery of the parent HBcAg carrier and the epitope (pHBc+pV3-10) to determine if expression of the *HBcAg* carrier gene on a separate plasmid is sufficient to enhance epitope immunogenicity, and a 4th group

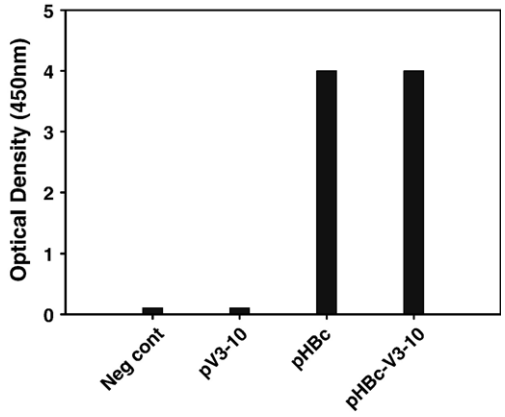

Fig. 2. Detection of hepatitis B core particles following plasmid expression *in vitro*. Core particles in cell supernatants from transfected Vero cells were pelleted through a glycerol cushion, resuspended in sample buffer, and detected by a commercial ELISA kit that measures expression of a HBeAg epitope revealed on truncated core particle. Pelleted supernatant from untransfected Vero cells was used as a negative control. The plasmid pV3-10 expresses the HIV V3 epitope without carrier; pHBc expresses the HBcAg carrier; and pHBc-V3-10 expresses HBcAg containing the HIV V3 epitope inserted into the immunodominant loop.

was immunized with DNA encoding the whole HIVgp120 antigen (pHIVgp120) so that the epitope-based approaches could be compared to a whole gene approach. The pHIVgp120 plasmid encodes the same epitope in the context of its natural sequences and was previously shown to induce significant responses against this epitope in mice (Fuller and Haynes, 1994). After the 2nd and 3rd DNA doses, HIV epitope-specific IFN-γ and CTL responses were measured by ELISPOT (Fig. 3A) and ^{51}Cr-release (Fig. 3B) assays, respectively. Results from both assays were consistent and show that the hybrid HBc-V3-10 DNA vaccine induced significantly higher CD8$^+$ T cell responses than either the epitope alone (pV3-10, $P=0.0015$) or the whole *HIVgp120* gene (pHIVenv, $P<0.0001$). The low immune responses induced by the pHIVgp120 plasmid after 3 doses may be due to poor expression of the glycosylated native protein in mouse cells (Grundner et al., 2004) and is consistent with our previous findings where 4–5 doses were required to induce a significant response (Fuller and Haynes, 1994). CD8$^+$ T cell responses in mice immunized with pHBc-V3-10 increased after the 2nd boost despite the presence of high antibody titers against the carrier HBcAg (>1:100,000) after the 1st boost (not shown), indicating that pre-existing responses against HBcAg did not prevent boosting of the HIV-specific response. Co-delivery of the HBcAg plasmid with the CTL

Table 1
Mamu-A*01-restricted, SIV-specific CTL epitopes inserted into chimeric HBcAg-SIV DNA vaccines

Vaccine vector	CTL epitopes	Sequence	Insert position
1. pHBc-SIV-CM9	$Gag_{181-189}$CM9	CTPYDINQM	Internal
2. pHBc-SIV-SL8	Tat_{28-35}SL8 [a]	STPESANL	Internal
3. pHBc-SIV-SI9	$Env_{763-771}$SI9	SWPWQIEYI	C-terminus
4. pHBc-SIV-A	$Vif_{144-152}$QA9	QVPSLQYLA	C-terminus
	$Pol_{143-152}$LV10	LGPHYTPKIV	
	$Env_{729-738}$ST10	SPPSYFQTHT	
5. pHBc-SIV-B	$Env_{235-243}$CL9	CAPPGYALL	C-terminus
	$Pol_{147-155}$YI9	YTPKIVGGI	
	Pol_{51-61}EA11	EAPQFPHGSSA	
6. pHBc-SIV-C	$Gag_{340-349}$VT10	VNPTLEEMLT	Internal
	$Pol_{621-629}$SV9	STPPLVRLV	
7. pHBc-SIV-D	Pol_{34-43}QF10	QMPRQTGGFF	Internal
	$Vif_{100-107}$VI8	VTPDYADI	
	Tat_{28-35}TL8 [a]	TTPESANL	
8. pHBc-SIV-E	$Pol_{474-483}$IL10	IYPGIKTKHL	C-terminus
	$Env_{622-630}$TL9	TVPWPNASL	
	$Pol_{957-964}$MI8	MTPAERLI	
9. pHBc-SIV-F	$Pol_{588-596}$QV9	QVPKFHLPV	C-terminus
	$Gag_{372-380}$LA9	LAPVPIPFA	
	$Pol_{359-368}$GM10	GSPAIFQYTM	

CTL epitopes were inserted into either an internal position replacing an immunodominant antibody-binding region or the C-terminus of the *HBcAg* gene. Multiple CTL epitopes in a single vaccine were separated by two alanines and inserted in the order indicated.

[a] Since recognition of Tat_SL8 and Tat_TL8 by PBMC from infected macaques is indistinguishable, they are considered the same epitope.

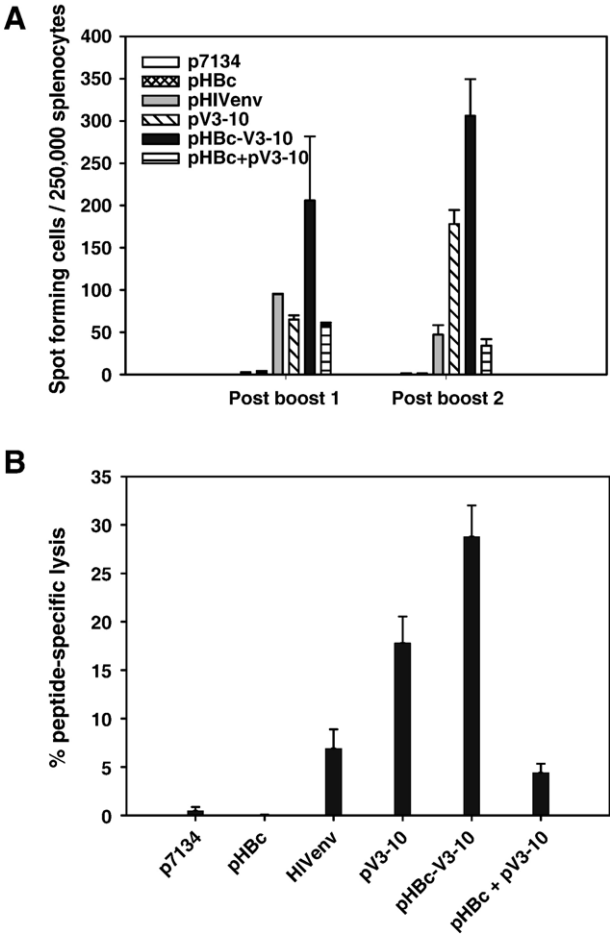

Fig. 3. (A) Frequencies of HIV epitope-specific CD8$^+$ T cell responses as measured by IFN-γ ELISPOT 2 weeks after the first or second booster immunization. (B) HIV epitope-specific cytotoxic T lymphocyte responses measured 2 weeks after the second boost. DNA vaccine vectors: p7134=empty vector backbone, pHBc=parent vector encoding hepatitis B core antigen, pHIVgp120=whole HIVgp120, pV3-10=HIV CTL epitope, HBc-V3-10=HIV CTL epitope inserted into HBcAg, HBcAg+V3-10=co-delivery of pHBc and pV3-10.

epitope plasmid (pHBc+pV3-10) did not enhance $CD8^+$ T cell responses, demonstrating the necessity of linking the epitope to the *HBcAg* gene. Immune responses were reduced in the group receiving the co-delivery of separate pHBcAg and pV3-10 plasmids when compared to mice immunized with the pV3-10 alone (Fig. 3), an outcome that is likely due to competition for transcription factors between the co-delivered plasmids. For gene gun delivery formulation of two or more plasmids onto the same gold beads results in the plasmids being delivered into the same cell (Arrington et al., 2002) and competition between the vectors for transcription factors within the same cell reduces plasmid expression and immune responses (Arrington et al., 2002; Hooper et al., 2003).

The amount of antigen expressed can influence the potency of a DNA vaccine. To determine if differences in antigen expression influenced the magnitude of the HIV-specific $CD8^+$ T cell response induced by each DNA vaccine, mRNA expression was analyzed by Northern blot. B16 cells were transfected with pHBc, pHIVgp120, pHBc-V3-10, or pV3-10; and Northern hybridization analysis was performed on total RNA extracted from cell lysates using a synthetic probe specific for the 5′ untranslated region in each plasmid. As shown in Fig. 4, mRNA from the pHIVgp120 (lane C) plasmid was undetectable by Northern blot, although HIVgp120 could be detected in cell supernatants by sandwich ELISA (not shown). This result indicates that the higher immunogenicity observed with the epitope-based vaccines when compared to the whole gene vaccine was due, in part, to higher levels of antigen expression. The hybrid vector, pHBc-V3-10 (lane D), expressed lower levels of mRNA than its parent HBcAg carrier vector (lane A). However, comparable levels of mRNA expression were seen in pHBc-V3-10 (lane D) and its control vector, pV3-10 (plasmid expressing the CTL epitope without HBcAg, lane E) indicating that pHBc-V3-10 was able to induce higher $CD8^+$ T cell responses than pV3-10 by conferring enhanced immunogenicity, not expression, to the inserted epitope.

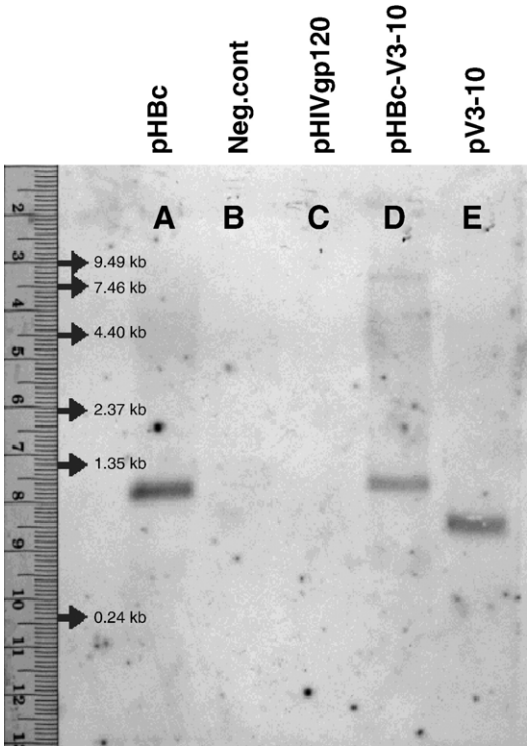

Fig. 4. mRNA expression by DNA vaccine plasmids. Total RNA was extracted from cell lysates of transfected B16 cells and analyzed by Northern blot hybridization using a cDNA probe specific for the 5′ untranslated region of each plasmid. Lane A: pHBc; lane B: negative control (cell lysate from untransfected cells); lane C: pHIVgp120; lane D: pHBc-V3-10; lane E: pV3-10.

Table 2
Tetramer-positive $CD3^+/CD8^+$ T cells induced in mice following immunization with HIV epitope DNA vaccines

DNA vaccine	Antigens	%Tetramer-positive $CD3/CD8^+$ T lymphocytes
p7134	None, control empty plasmid	0.50
pHBc	HBcAg	0.42
pV3-10	HIV CTL epitope	3.84
pHBc-V3-10	HIV CTL epitope fused to HBcAg	5.84
pV3-15	HIV CTL and Th epitopes	6.59
pHBc-V3-15	HIV CTL and Th epitopes fused to HBcAg	10.7

Pools of splenocytes from 4 mice per group were analyzed following a prime and two booster immunizations.

Linkage of CTL and T helper epitopes significantly improves the ability of epitope-based vaccines to induce $CD8^+$ T cell responses (Ishioka et al., 1999; Shirai et al., 1994; Vitiello et al., 1995). We therefore tested the effects of inserting a linked HIV-specific CTL-T helper epitope into the immunodominant region of HBcAg. A second hybrid DNA vaccine, pHBc-V3-15, was generated that expresses a 15-mer HIV-specific CTL-T helper epitope (V3-15) fused to the *HBcAg* carrier gene. V3-15 is recognized by $CD4^+$ T cells with the class II molecule $I\text{-}A^d$ and includes 10 overlapping amino acids corresponding to the minimal $H\text{-}2D^d$-restricted V3-10 CTL epitope (Takeshita et al., 1995). The immunogenicity of pHBc-V3-15 was compared to a control DNA vaccine encoding the linked CTL-T helper epitope in the absence of the HBcAg carrier (pV3-15). For comparison, additional groups of mice were immunized with the pHBc-V3-10 or pV3-10 vectors encoding only the minimal CTL epitope, or negative control vectors encoding either hepatitis core antigen (pHBc) or the empty expression vector cassette, p7134. Following the 3rd dose, $CD8^+$ T cell responses were measured using $H\text{-}2D^d$ MHC class I tetramers complexed to the HIV V3-10 CTL epitope.

Consistent with our previous results, HBcAg enhanced the immunogenicity of the inserted epitopes, resulting in a marked elevation of the $CD8^+$ T cell response (Table 2). Frequencies of tetramer-positive cells increased from 3.84% (V3-10) to 5.84% (pHBc-V3-10) when the epitope was expressed in the context of HBcAg. Similarly, insertion of the longer CTL-T helper epitope (V3-15) into HBcAg increased the frequency of tetramer-positive cells from 6.59% (pV3-15) to 10.7% (pHBc-V3-15) (Table 2). Interestingly, linking the CTL epitope fused to the HIV-specific T helper epitope (pV3-15) or the HBc carrier (HBc-V3-10) resulted in comparable frequencies of HIV-specific $CD8^+$ T cells (6.59% vs. 5.84%, respectively)

demonstrating that the T helper epitope and HBcAg carrier were similarly effective in enhancing $CD8^+$ T cell responses. However, pHBc-V3-15 DNA vaccine induced the highest $CD8^+$ T cell response demonstrating that combining the HBcAg carrier and T helper epitope afforded a synergistic effect on the resulting $CD8^+$ T cell response.

Hybrid HBcAg-SIV epitope DNA vaccine induces CTL in rhesus macaques

Vaccine strategies that work well in mice can fail in larger species, including humans and nonhuman primates. Therefore, we tested a hybrid HBcAg-SIV epitope DNA vaccine in rhesus macaques. Six macaques were immunized with a hybrid HBc-SIV DNA vaccine encoding a SIV gag-specific Mamu A*01-restricted CTL epitope ($Gag_{181-189}$CM9: CTPYDINQM) (Allen et al., 1998) inserted into the immunodominant core region. PBMC were isolated after the 3rd dose and assayed for the presence of CM9-specific $CD8^+$ T cells by cytolytic assay and tetramer staining. Significant levels of CTL and tetramer-positive cells were detected in 5 of the 6 monkeys (Table 3). Levels of CTL and tetramer-positive cells correlated with the levels of HBcAg-specific antibody and T cell proliferative responses in each monkey, providing further evidence for an adjuvant role of HBcAg (Table 3).

HBcAg-SIV CTL epitope DNA vaccines induce multi-specific SIV-specific CTL responses in monkeys

An effective HIV vaccine may need to induce CTL responses against multiple epitopes. We therefore tested the hybrid HBcAg-epitope DNA vaccine strategy for the ability to induce multi-specific CTL responses in rhesus macaques. Five Mamu-A*01-positive rhesus macaques were immunized with 3 doses (32 μg/dose) of an HBcAg-multi-epitope SIV DNA vaccine encoding19 Mamu-A*01-restricted epitopes (Table 1), along with a DNA vaccine expressing whole SIV tat to provide SIV-specific T cell help. Macaques received 3 DNA doses spaced 12 weeks apart. PBMC were isolated 8 days after the third immunization and assayed by ELISPOT for peptide-specific $CD8^+$ T cell responses against 8 representative epitopes encoded by the vaccine. As shown in Fig. 5A, IFN-γ $CD8^+$ T cell responses against 3–6 of the 8 epitopes tested were detected in each macaque. The breadth of the response differed between each animal with only the immunodominant Gag_CM9 (Allen et al., 1998) detected in all 5 macaques.

$CD8^+$ T cells that have cytolytic effector function may be critical for control of HIV/SIV replication (Calarota et al., 2006; Goulder et al., 2000; Yang et al., 1997), but IFN-γ-producing $CD8^+$ T cell responses detected by the ELISPOT assay do not always correlate with the cytolytic effector response (Calarota et al., 2006). To determine if the HBcAg-multi-epitope DNA vaccine induced $CD8^+$ T cells with cytolytic activity, a second group of macaques was immunized with 3 doses of HBc-multi-epitope DNA vaccine without the plasmid expressing whole tat, and cytolytic effector responses were assayed by ^{51}Cr-release assay. As shown in Fig. 5B, the vaccine induced significant CTL responses (≥10% lysis) to 5–7 epitopes in each monkey, and the repertoire of the CTL response was distinct in each monkey with only a single epitope ($Env_{235-243}$ CL9) recognized by all 3 macaques.

Among all 8 macaques immunized with the HBc-multi-epitope vaccine, SIV-specific $CD8^+$ T cell responses were detected by either ELISPOT or CTL assay against a total of 13 of the 19 epitopes. Our inability to detect CTL responses against 6 of the epitopes is not due to defective vector expression because all 9 vectors resulted in comparable levels of HBcAg expression (not shown). Analysis of the responses induced by each vector (Table 1 and Fig. 5) shows that CTL were detected against at least 1 epitope encoded by each vector containing either a single epitope or multiple epitopes inserted at the C-terminus of the *HBcAg* gene (Table 1, vectors 1–5, 8, and 9) but not those vectors containing multiple epitopes inserted into the internal position of HBcAg (Table 1, vectors 6 and 7). This suggests that epitope presentation may be compromised when more than one epitope is expressed within the immunodominant region of the *HBcAg* gene. CTL responses were detected against 3 epitopes (Pol_{51-61}EA11, $Env_{622-630}$TL9, and $Env_{763-771}$SI9) that were previously not observed in SIV-infected macaques (Allen et al., 2001) suggesting that the DNA vaccine was able to induce responses against epitopes that may be subdominant in the context of a natural infection.

Table 3
Immunogenicity of a hybrid HBcAg-Mamu-A*01 SIV $Gag_{181-189}$ CM9 CTL epitope DNA vaccine in Rhesus macaques following a prime and two boosts

Monkey	%SIV $Gag_{181-189}$ CM9-specific lysis (restimulated PBMC[a])	%Tetramer-positive $CD3/CD8^+$ T lymphocytes (restimulated PBMC[a])	Anti-HBcAg titer	HBcAg-specific proliferation (stimulation index)
M95045	6	0.2	100	1.6
M95058	19	6.7	25,600	16.1
M96031	34	23.7	102,400	33.7
M96118	35	21.6	51,200	nd
M96123	38	26.6	409,600	nd
M94004	14	17.5	102,400	nd

[a] PBMC were restimulated in vitro with peptide for 2 weeks. nd=Not done.

Discussion

Presentation of epitopes in the context of immunogenic particles is an effective strategy to increase epitope immunogenicity in protein-based vaccines (Delpeyroux et al., 1986; Michel et al., 1988; Schlienger et al., 1992; Schodel et al., 1994b). We investigated this effect in the context of a DNA vaccine encoding HIV and SIV CTL epitopes fused to the gene encoding hepatitis B core antigen. Our results demonstrate that hybrid HBc-CTL epitope DNA vaccines express core particles *in vitro*, are more immunogenic than DNA vaccines encoding either whole antigen or the epitope in the absence of carrier, and can be used to induce responses against multiple CTL epitopes in nonhuman primates.

The results reported here are consistent with previous studies using, instead, hepatitis B surface antigen (HBsAg) as a carrier

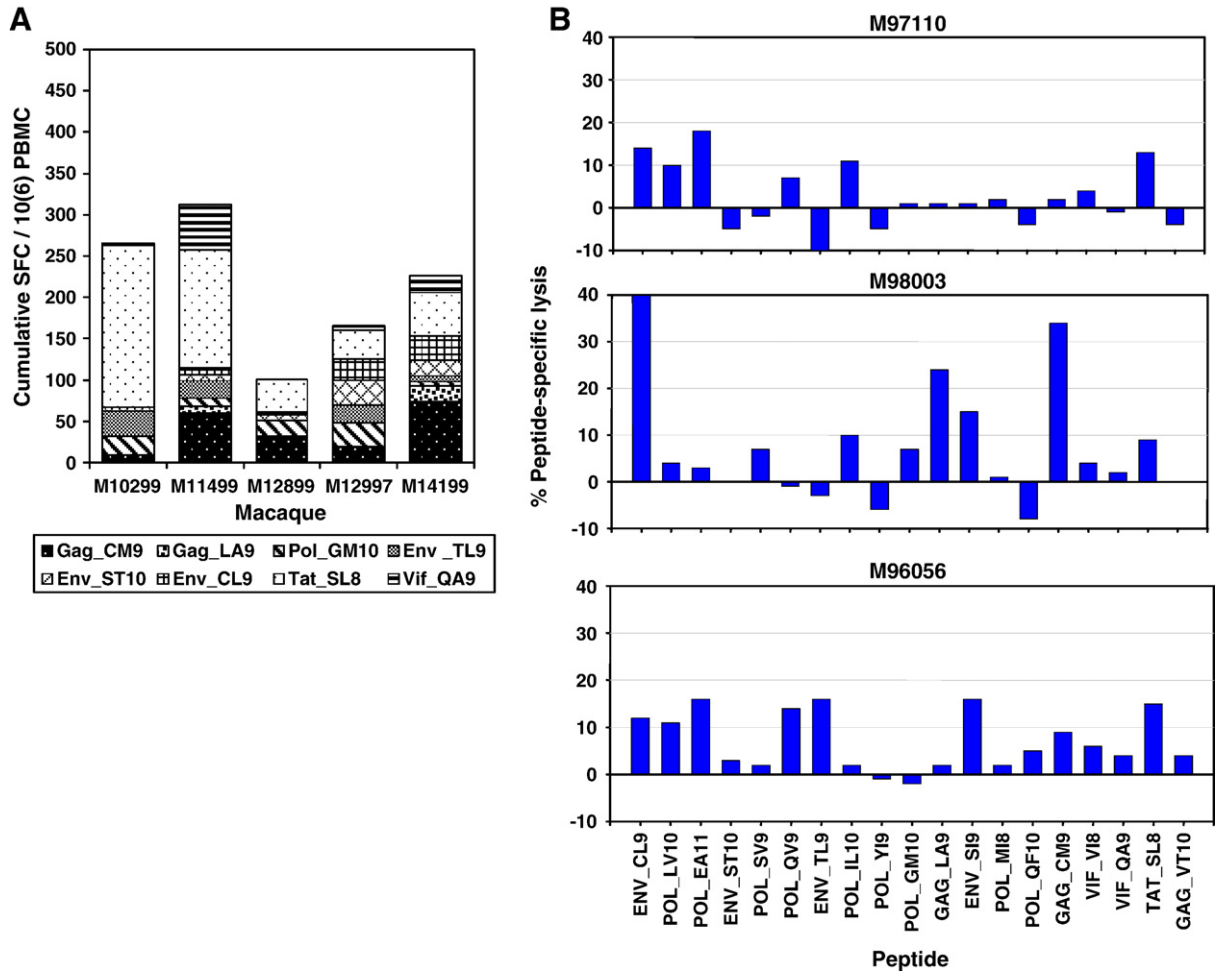

Fig. 5. SIV-specific T cell responses in 5 Mamu-A*01-positive rhesus macaques immunized with 3 doses of a cocktail of 9 HBcAg-epitope DNA vaccines encoding 19 Mamu-A*01 CTL epitopes (Table 1) with (panel A) or without (panel B) a plasmid expressing the *SIV tat* gene. (A) $CD8^+$ T cell responses measured by ELISPOT in Mamu-A*01-positive macaques following the 3rd dose. Immunizations were spaced 4–8 weeks apart. Responses were measured 8 days after the final dose. In all cases, background levels were less than 10 spot forming cells (SFC)/10^6 PBMC. Responses significantly higher than background levels (twice the SFCs from untreated PBMC plus 20 spots) were considered positive. (B) Cytolytic T cell responses in 3 Mamu-A*01-positive rhesus macaques following the 3rd dose. Immunizations were spaced 12 weeks apart. PBMC were isolated 2 weeks after the 3rd dose and stimulated with peptide in a 14-day culture. CTL activity was measured at an effector:target ratio of 20:1 in a ^{51}Cr-release assay using peptide-pulsed B-LCLs from a Mamu-A*01+ rhesus macaque as targets. Data are expressed as net lysis after subtraction of background lysis measured using irrelevant peptides (see Materials and methods) that bound the Mamu-A*01 molecule but were not encoded in the vaccine cocktail. Background lysis in all cases was 1–3%.

moiety to enhance B and T cell epitope immunogenicity in DNA vaccines (Fomsgaard et al., 1998; Le Borgne et al., 1998; Major et al., 1995; Marsac et al., 2005; Puaux et al., 2004; Wunderlich et al., 2000) and studies using HBcAg as a carrier in DNA vaccines to enhance antibody responses against B cell epitopes (Lesinski et al., 2001; Wu et al., 2005). Here, we extend these findings to show that the *HBcAg* carrier gene can be used to increase the immunogenicity of DNA vaccines encoding $CD8^+$ T cell epitopes.

The increased epitope immunogenicity was likely due, in part, to the induction of potent T helper responses against the HBcAg carrier (Milich et al., 1997b) but was dependent on the linkage of the CTL epitope to HBcAg. Linking CTL epitopes to T helper epitopes in peptide vaccines similarly increases CTL responses (Restifo et al., 1995; Shirai et al., 1994; Vitiello et al., 1995). Linked sequences may be more immunogenic because epitopes longer than the 8- to10-mers that define most minimal CTL epitopes could be more effectively processed by antigen presenting cells. In addition, linking the CTL epitope to HBcAg or a T helper epitope may bring the CTL and Th cells closer together allowing for more effective transmission of stimulatory lymphokines secreted by T helper cells.

The HBc-epitope carrier likely increases epitope immunogenicity via its ability to self-assemble into a highly immunogenic particle and present the epitope in the context of the particle (Milich, 1988). Phagocytic macrophages can process particulate antigens via the exogenous class I pathway (Kovacsovics-Bankowski et al., 1993; Pfeifer et al., 1993), and particulate protein-based vaccines induce $CD8^+$ T cell responses via this mechanism (Griffiths et al., 1993; Layton et al., 1993; Schirmbeck et al., 1995). Similarly, *in vivo* expression of the hybrid HBcAg-epitope DNA vaccines may afford dual presentation of T cell epitopes in the context of both the endogenous (via intracellular expression of the epitopes) and

exogenous (via *in vivo* expression and secretion of HBc-epitope particles) class I pathways.

Another factor that likely contributes to the adjuvant effects of HBcAg is its ability to directly activate B cells to serve as primary antigen presenting cells *in vivo* (Kratz et al., 1999; Milich et al., 1997a). The high density of repetitive structural features of HBcAg allows it to cross-link mIg receptors on naïve B cells, leading to mIg-receptor-mediated uptake of HBcAg and induction of the costimulatory molecules B7.1 and B7.2 (Milich et al., 1997a). As a result, HBcAg can enhance naïve B cell APC function as much as 10^5-fold as compared with non-B-cell APC (Kratz et al., 1999). This feature is unique to HBcAg and likely contributes to HBcAg being more immunogenic than HBsAg (Hoofnagle et al., 1974). In this respect, HBcAg may be a more effective carrier of foreign epitopes than HBsAg.

We previously showed gene gun DNA immunization induced $CD8^+$ T cell responses in rhesus macaques and protected against challenge with a heterologous primary isolate of SIV (Fuller et al., 2002). In that study, 7 DNA doses were required before significant CTL responses were detected in all animals. In contrast, the HBc-SIV epitope DNA vaccines used here induced SIV-specific $CD8^+$ CTL responses in all 14 immunized rhesus macaques after only 3 doses. In a separate study, this HBc-epitope DNA vaccine was effectively used to prime for $CD8^+$ T cell responses in rhesus macaques that were increased to unprecedented levels when boosted with a single dose of recombinant modified vaccinia Ankara (MVA) encoding the same CTL epitope (Allen et al., 2000b). In that study, tetramer-positive $CD8^+$ T cell frequencies of 1.2–20.0% detected following a single MVA boost significantly exceeded levels of 0.4–3.0% reported in a previous study where macaques were boosted with the same MVA vaccine following priming with a DNA vaccine encoding the same epitope but in the absence of HBcAg carrier (Hanke et al., 1999). These results suggest that immunization with hybrid HBc-epitope DNA vaccines should reduce the number of doses required for priming the immune response and improve the ability of combined DNA prime-viral vector boost strategies to induce $CD8^+$ T cell responses.

The hybrid HBc-epitope DNA vaccines encoding multiple epitopes induced multi-specific CTL responses in rhesus macaques. Among 8 animals, responses were detected against a total of 13 of the 19 epitopes encoded by the vaccine. Interestingly, CTL responses against 3 epitopes previously not recognized in macaques chronically infected with SIV (Allen et al., 2001) were also detected, suggesting that the HBcAg-multi-epitope vaccine induced responses against subdominant epitopes. These observations support the potential for using this approach to increase the breadth of CTL responses against HIV.

In each macaque, the multi-epitope DNA vaccine induced responses to different repertoire of epitopes. Inherent variability in immune responsiveness to vaccination in the outbred macaque species is a factor that likely contributed to this result. Similarly, although 14 of the 19 CTL epitopes used in this vaccine have been recognized in SIV_{mac239}-infected Mamu-A*01 macaques, each infected animal responded to a different subset of these epitopes, even at the same time-point post-infection (Allen et al., 2001). Thus, an effective HIV multi-epitope vaccine may need to include a broad range of epitopes to ensure that vaccinated individuals will respond to a minimum repertoire of epitopes needed for protection.

An important goal in the development of vaccine strategies against HIV is to identify vaccines that enhance both the potency and the breadth of the virus-specific $CD8^+$ T cell response. Here, we report that a hybrid HBc-epitope DNA vaccine strategy can be used to increase epitope immunogenicity and induce broad CTL responses. The HBc-multi-epitope DNA vaccine described here was recently tested for immunotherapy in combination with an antiretroviral drug in SIV-infected macaques (Fuller et al., 2006) and shown to increase the magnitude and breadth of the virus-specific $CD8^+$ T cell response, and this effect was associated with reduced viral load and prevention of disease progression (Fuller et al., 2006). These studies demonstrate that the hybrid HBc-epitope DNA vaccine strategy may be a promising approach for treating or preventing HIV infection as well as other diseases where inducing CTL is a goal.

Materials and methods

HIV epitope DNA vaccines

Expression vector PJV7134 (PowderJect Vaccines Inc., Madison, WI) contains the cytomegalovirus (CMV) immediate early promoter/intron A sequences that transcribe the coding sequence for the signal peptide of the human tissue plasminogen activator (hTPA), followed by the bovine growth hormone polyadenylation signal, pUC19 origin of replication, and ampicillin resistance gene. A unique *Bsp*120I site at the end of the hTPA coding region allows for translational fusions of CTL epitopes to the signal peptide. To construct expression vectors pV3-10 and pV3-15, complementary oligonucleotides respectively encoding either the minimal H-$2D^d$-restricted HIV CTL epitope, RGPGRAFVTI (HIV IIIB $Env_{311–320}$ RI10) (Takeshita et al., 1995) or the epitope embedded in the longer HIV-specific T helper epitope, RIQRGPGRAFVTIGK (HIV IIIB $Env_{308–322}$ RK15) (Shirai et al., 1994), and appropriate overhangs for insertion into the *Bsp*120I site were synthesized (Biosource, Camarillo, CA) with appropriate *Bsp*120I overhangs. PJV7134 was digested with *Bsp*120I (New England Biolabs, Beverly, MA) and the oligonucleotides were annealed and ligated into the *Bsp*120I site to generate the epitope expression plasmids pV3-10 (CTL epitope) pV3-15 (CTL and Th epitopes).

Hybrid HBcAg-epitope DNA vaccines

The HBcAg carrier expression vector pHBc expresses HBcAg under the control of the CMV immediate early/intron A promoter (PJV7198, PowderJect Vaccines, Inc., Madison, WI). HBcAg was truncated at amino acid 144 at the C-terminus and fused to the human TPA signal peptide sequence at the N-terminus. A unique *Bsp*120I restriction was added within the immunodominant c/e1 region between amino acids 80 and 81,

and a unique *Not*I restriction site at the C-terminus to facilitate insertion of epitopes at either site (Lesinski et al., 2001). To construct hybrid HBcAg-epitope DNA vaccines, pHBc was digested with either *Bsp*120I or *Not*I. Oligonucleotides encoding *Bsp*120I or *Not*I-flanked, codon-optimized HIV or SIV CTL epitopes flanked with *Bsp*120I overhangs were synthesized, annealed, and ligated into pHBc either at the immunodominant region or the C-terminus of the *HBcAg* gene (Fig. 1). For inserts containing multiple CTL epitopes (Table 1), each epitope was separated and flanked by 2 alanines. Clones containing inserts were identified by PCR as described (Lesinski et al., 2001) and sequenced to confirm insertion of the correct coding sequences and orientation.

HIVgp120 DNA vaccine

The expression plasmid pHIVgp120 encodes the glycoprotein 120 gene from HIVBru *Env* under the control of the CMV immediate early promoter and was generated as described (Fuller and Haynes, 1994).

SIV tat DNA vaccine

The SIV tat DNA vaccine encodes the entire *SIV tat* gene from SIV17E-Fr under the control of the CMV immediate early promoter and was generated as described (Vogel et al., 2003).

Detection of HBcAg particles

Vero cells were transfected with pHBc, pHBc-V3-10, or pHIVgp120 using Polyfect Transfection Reagent (Qiagen, Valencia, CA). Negative controls were untransfected cells. Twenty-four hours after transfection, cell culture supernatants were overlaid on a 20% glycerol cushion (20% glycerol in TE buffer; 10 mM Tris, pH8 and 1 mM EDTA) and pelleted by centrifugation for 3 h at 100,000×*g*. The partially purified particles were resuspended in 200 μl TE buffer, and 75 μl was analyzed for the presence of an HBeAg epitope exposed in truncated HBcAg particles (Schodel et al., 1993) by Murex HBeAg/anti-HBe kit (Dartford, UK) according to the manufacturer's instructions.

Rhesus macaques

Rhesus macaques were identified as Mamu-A*01-positive by PCR-SSP and by direct sequencing as previously described (Knapp et al., 1997). Macaques were maintained in accordance with the NIH Guide to the Care and Use of Laboratory Animals and under the approval of the University of Wisconsin Research Animal Resource Center (RARC) review committee.

DNA immunizations

Plasmid DNA was precipitated onto 1- to 3-μm gold particles as previously described (Roy et al., 2000) at a rate of 2.0 μg DNA/mg of gold. The plasmids pV3-10 and pHBc were co-delivered into the same cells by coating 1.0 μg of each plasmid on the same gold beads. The 9 plasmids in the HBc-SIV epitope cocktail vaccine were co-delivered by coating each plasmid on separate gold particles that were mixed just prior to immunization. Abdominal fur was clipped from 5- to 6-week-old Balb/c mice (H-2D^d), and both abdominal and inner leg fur were clipped from rhesus macaques just prior to DNA delivery. DNA-coated gold particles were accelerated into the abdominal skin of mice and into the skin of both the abdominal and inguinal lymph node regions of rhesus macaques by particle mediated epidermal delivery (PMED) using an XR-1 research delivery device (PowderJect Vaccines, Inc., Madison, WI). DNA/gold was delivered at a helium pressure of 400 lb/in.^{2} (psi) for mice and 500–600 psi for monkeys. Each delivery consisted of either 0.5 mg (mice) or 1.0 mg (macaques) of gold and 1.0 μg (mice) or 2.0 μg (monkeys) of DNA. A dose of 2 μg (mice) or 32 μg (macaques) of DNA per immunization was achieved by administering DNA into 2 or 16 sites in mice and monkeys, respectively. Consecutive DNA immunizations were spaced 4 (mice) or 8–12 (macaques) weeks apart.

IFN-γ ELISPOT assays

ELISPOT assays were performed on mouse splenocytes and PBMC from rhesus macaques as previously described (Arrington et al., 2002; Fuller et al., 2006).

Northern blot

Plasmid vectors pHBc-V3-10, pV3-10, or the parent pHBc vector were transfected into B16 cells using Polyfect Transfection Reagent (Qiagen, Valencia, CA). B16 cells were used because we have found they consistently express plasmid-encoded genes well. After 40 h, cell lysates were collected and total RNA extracted using the SV Total RNA Isolation kit (Promega, Madison, WI). The Northern blot analysis was standardized and performed as described (Gilman, 1997). Specimens from different groups were assayed twice, in parallel, and the results were consistent in terms of yield and reproducibility. 10 μg of RNA was subjected to gel electrophoresis, transferred to nitrocellulose, and hybridized with a [^{32}P]dATP-labeled cDNA probe specific for the 5′ untranslated region in each plasmid. A 0.24–9.5 kb RNA Ladder (Life Technologies, Rockville, MD) was used to confirm correct sizes of mRNA expressed by each plasmid.

HIV cytotoxicity assays

HIV cytotoxicity assays in mice were performed using peptide-pulsed P815 cells labeled with 100 μCi of sodium ^{51}chromate (NEN Life Sciences, Boston, MA) as previously described (Fuller and Haynes, 1994).

Tetramer staining

Soluble tetrameric Mamu-A*01 MHC class I/SIV Gag$_{181–189}$ CM9 (CTPYDINQM) or H-2D^d MHC class I/HIV Env$_{311–320}$ RI10 (RGPGRAFVTI) peptide complexes were generated, and

tetramer staining was performed as described (Allen et al., 2000a). For macaques, 1×10^6 PBMC from 2-week CTL cultures were washed in FACS buffer consisting of phosphate-buffered saline (Gibco, Rockville, MD) with 2% fetal calf serum (BioCell, Rancho Dominguez, CA). Cells were stained in the dark in 100 μl FACS buffer with 0.1 μg Mamu-A*01-PE tetramer, 10 μl anti-rhesus CD3 FITC-conjugated antibody (BioSource, Camarillo, CA), and 1 μl anti-$CD8^+$-PECy5 (Coulter, Fullerton, CA) for 30 min at room temperature. The cells were then washed with FACS buffer and fixed with 450 μl of 2% paraformaldehyde. Data were acquired on a Becton Dickinson FACSCalibur instrument and analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Background tetramer staining of PBMC was less than 0.02% (data not shown).

For mice, frozen pooled splenocytes were thawed, washed, and stained in the dark with 1 μg H-$2D^d$-PE tetramer in 70 μl FACS buffer for 2 1/4 h followed by staining with 2 μl of anti-mouse CD3-PerCP and 1 μl anti-mouse CD8-APC for an additional 40 min. Pooled splenocytes from mice within the same group were used to obtain a sufficient number of cells for the analysis. The cells were then washed with FACS buffer and fixed with 450 μl of 2% paraformaldehyde. Data were acquired on a Becton Dickinson FACSCalibur instrument and analyzed using FloJo software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Populations of tetramer-positive cells were readily distinguished as a distinct subset from background $CD3^+$/$CD8^+$ T cells. Background tetramer staining of *in vitro* stimulated cultures from naive mice was $<0.08\%$ (data not shown).

SIV cytotoxicity assays

The CTL activity of *in vitro* stimulated PBMC from rhesus macaques was determined by a standard ^{51}Cr-release using γ-irradiated, peptide-pulsed B-LCLs from a Mamu-A*01+ rhesus macaque as targets as previously described (Allen et al., 2000a). Data reported for each CTL epitope are based on CTL assays tested at 20:1 effector:target ratios and reflect %net-specific lysis after background subtraction. Background lysis for each macaque was 1–3% (data not shown) and was determined by averaging the gross %lysis from each of 3 CTL assays using irrelevant peptides (AAPTSAPV, LAPVPIPF, and STPPLVRLV) that bound the Mamu-A*01 molecule (Allen et al., 2001), but was not included in the DNA vaccine. A response was considered positive if the net-specific lysis was $\geq10\%$.

HBcAg proliferation assay

Rhesus macaque PBMC were isolated by Ficoll density gradient centrifugation and washed 3 times with RPMI 1640 supplemented with Human AB+ serum (R10AB). PBMC (100,000/well) were aliquoted into 96-well round-bottomed plates in quadruplicate in 150 μl R10AB. Purified HBcAg protein was added at 1 μg/well in 50 μl R10AB. Medium containing no antigen was added to negative control wells and 5 μg/ml ConA was added to positive control wells. Plates were incubated for 4 days before adding 1 μCi tritiated thymidine to each well. After 16- to 18-h incubation in the presence of radionucleotide, cells were harvested onto glass fiber mats and counted via scintillation. Counts per minute (CPM) were averaged for quadruplicate wells and stimulation indices were calculated by dividing the average CPM obtained from test wells by the average CPM from the negative control wells.

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