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Mamu-A*01/K^b transgenic and MHC Class I knockout mice as a tool for HIV vaccine development

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ABSTRACT

We have developed a murine model expressing the rhesus macaque (RM) Mamu-A*01 MHC allele to characterize immune responses and vaccines based on antigens of importance to human disease processes. Towards that goal, transgenic (Tg) mice expressing chimeric RM (α1 and α2 Mamu-A*01 domains) and murine (α3, transmembrane, and cytoplasmic H-2K^b domains) MHC Class I molecules were derived by transgenesis of the H-2K^{b}D^b double MHC Class I knockout strain. After immunization of Mamu-A*01/K^b Tg mice with rVV-SIVGag–Pol, the mice generated CD8$^+$ T-cell IFN-γ responses to several known Mamu-A*01 restricted epitopes from the SIV Gag and Pol antigen sequence. Fusion peptides of highly recognized CTL epitopes from SIV Pol and Gag and a strong T-help epitope were shown to be immunogenic and capable of limiting an rVV-SIVGag–Pol challenge. Mamu-A*01/K^b Tg mice provide a model system to study the Mamu-A*01 restricted T-cell response for various infectious diseases which are applicable to a study in RM.

Introduction

Homologues of the human major histocompatibility complex (MHC) Class I (Bontrop et al., 1995), Class II (Slierendregt et al., 1992) and TCR genes (Levinson et al., 1992) can be identified in rhesus macaque (RM) and are remarkably similar to human counterparts. The close evolutionary similarity between RM and humans makes them suitable animal models to study the immunology of human immunodeficiency virus (HIV) infection using the RM infectious homologue simian immunodeficiency virus (SIV) (Letvin et al., 1983, 1984) and other examples of prominent infectious disease models with clinical applications (Barry et al., 2006; Belshe et al., 1977; Desrosiers et al., 1989; Felsenfeld and Schmidt, 1975; Polozov et al., 1978; Raengsakulrach et al., 1999; Rivailler et al., 2004; Sun et al., 2006; Wang, 2001; Yang et al., 1999; Yue et al., 2007; Zhu et al., 1997). Specifically, both SIV and HIV are primate lentiviruses which use the CD4 protein as a primary receptor and chemokine receptors as coreceptors for cell entry (Boyson et al., 1996; Klatzmann et al., 1984). After infection with SIV, most RM develop a disease similar to HIV-1-induced AIDS (King et al., 1990), consequently SIV infection of the RM is currently considered the best animal model for HIV infection of humans. RM are considered the pre-clinical standard for HIV/AIDS vaccine development utilizing SIV challenge.

Several vaccine studies in RM have suggested that strong immune responses to SIV protect against the development of AIDS in some monkeys (Daniel et al., 1985; Day et al., 2006; Lehner et al., 1996; Mossman et al., 1996). CD8$^+$ T cells contribute to this protective immunity based on accumulated evidence implicating T cell responses in the control of HIV/SIV replication (Brodie et al., 1999; Charini et al., 2001; Hirsch et al., 1994; Matano et al., 1998; Schmitz et al., 1999). The Mamu-A*01 Class I MHC allele is present in about 25% of RM of Indian origin (Knapp et al., 1997; Vogel et al., 1995). Tetramers for the single Mamu-A*01-restricted cytotoxic T lymphocyte (CTL) epitope Gag-CM9 (CTPYDINQM) have been developed to analyze Gag-specific CTL in the peripheral blood of SIV infected RM (Allen et al., 1998; Charini et al., 2001; Kuroda et al., 1998).

SIV disease progression was significantly delayed in Mamu-A*01 positive RM infected with SIV compared to monkeys whom did not express this allele (Evans et al., 1999, 2000; Muhl et al., 2002; Pal et al., 2002; Zhang et al., 2002). The studies also showed that RM that express the Class I Mamu-A*01 molecule control viremia after infection by a chimeric lentivirus composed of elements from both HIV and SIV,

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referred to as SHIV, more effectively than Mamu-A*01 negative animals (Mothe et al., 2003). This improved control of viral replication correlated with the development of a high-frequency dominant epitope Gag-specific CTL response following infection (Seaman et al., 2005). Similarly, the Mamu B*17 alleles also has been associated with slower disease progression in SIV-infected RM (Allen et al., 1998; Evans et al., 2000; Loffredo et al., 2007, 2008). A comparable set of observations was more recently made for RM with the B*08 allele (Loffredo et al., 2007, 2008). The term elite controllers (EC) has been coined to distinguish RM which durably control SIV viral load, and several different Mamu alleles (Mamu-A*02, B*08, B*17) have been implicated (Friedrich et al., 2007; Loffredo et al., 2007). It is an important question whether the mechanisms for control of SIV replication are synonymous with HIV-infected humans also designated as EC and who are characterized by unique HLA alleles.

To begin to address some of these questions in an easily manipulatable model, we developed a transgenic mouse expressing the

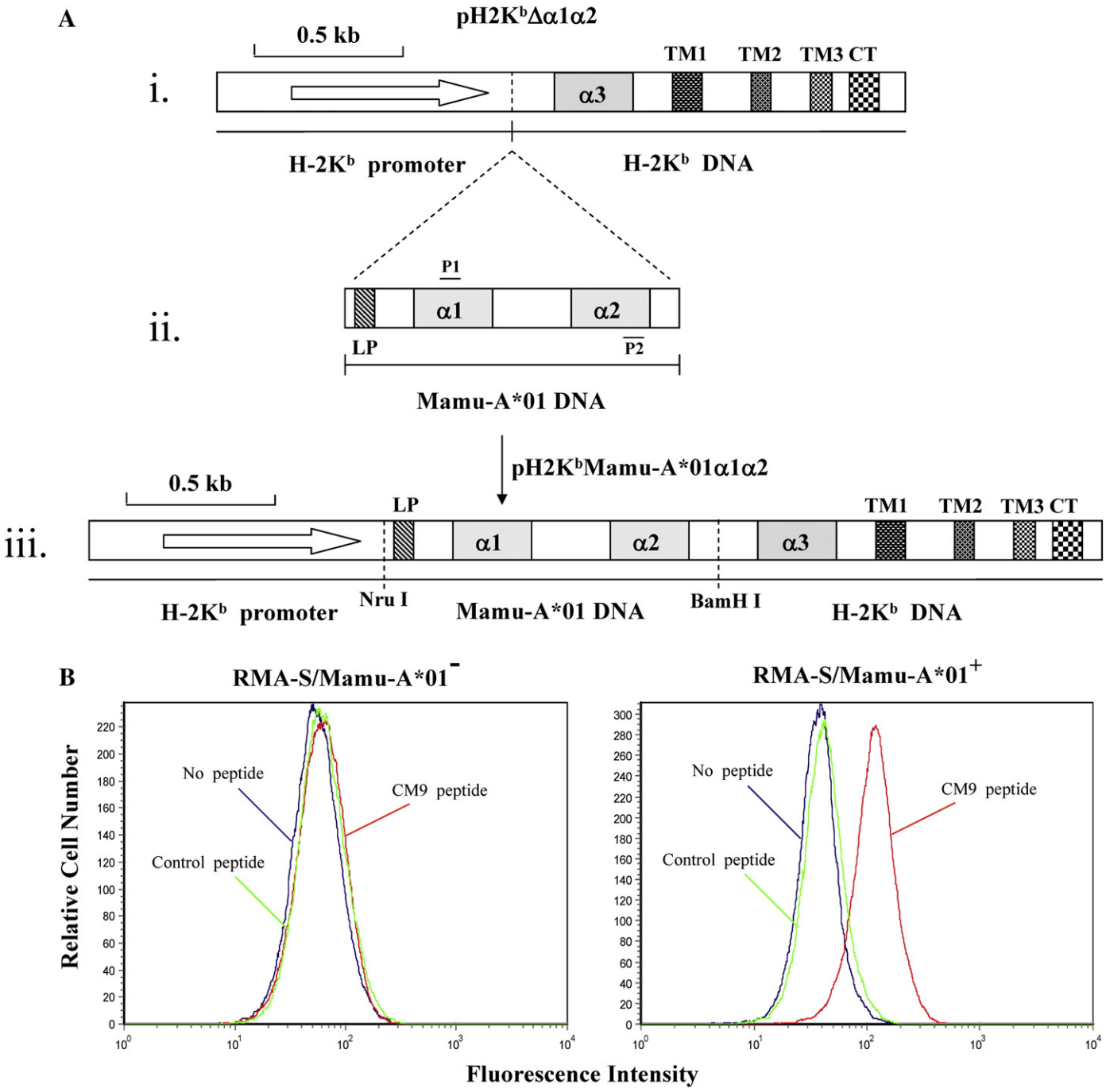

Fig. 1. (A) Scheme for generating H-2K^b promoter Mamu-A*01/K^b transgene construct. P1 and P2 are sites of Mamu-A*01 specific primers. See Materials and methods for details of the construction of the chimeric gene. The stippled boxes correspond to exons which encode the TM and cytoplasmic (CT) domains of the H-2K^b gene (i). Clear boxes represent introns and the endogenous promoter segment is designated by a box containing an arrow indicating the direction of transcription (i). The leader peptide (LP), α1 and α2 domains of the cloned MamuA*01 MHC gene are shown as represented in the genome (ii), and their position between Nru I and Bam HI sites of the modified H-2K^b gene that was introduced by transgenesis into the DKO mice (iii). (B) Stabilization of cell surface expression of Mamu-A*01/K^b in RMA-S cells with CM9 peptide or (C) four known Mamu-A*01 restricted SIV peptides LA9, LV10, GM10 and MI8. See Table 1 for amino acid sequences. After overnight culture of Mamu-A*01/K^b-RMA-S cells at 26 °C, 10^{-5} M of the indicated peptides were added or no peptide for the control, with subsequent incubation for 4 h at 37 °C. Analysis was initiated by staining with MAb W6/32 conjugated to FITC, followed by flow cytometry. The GYKDGNEYI peptide represents an irrelevant peptide (control peptide).

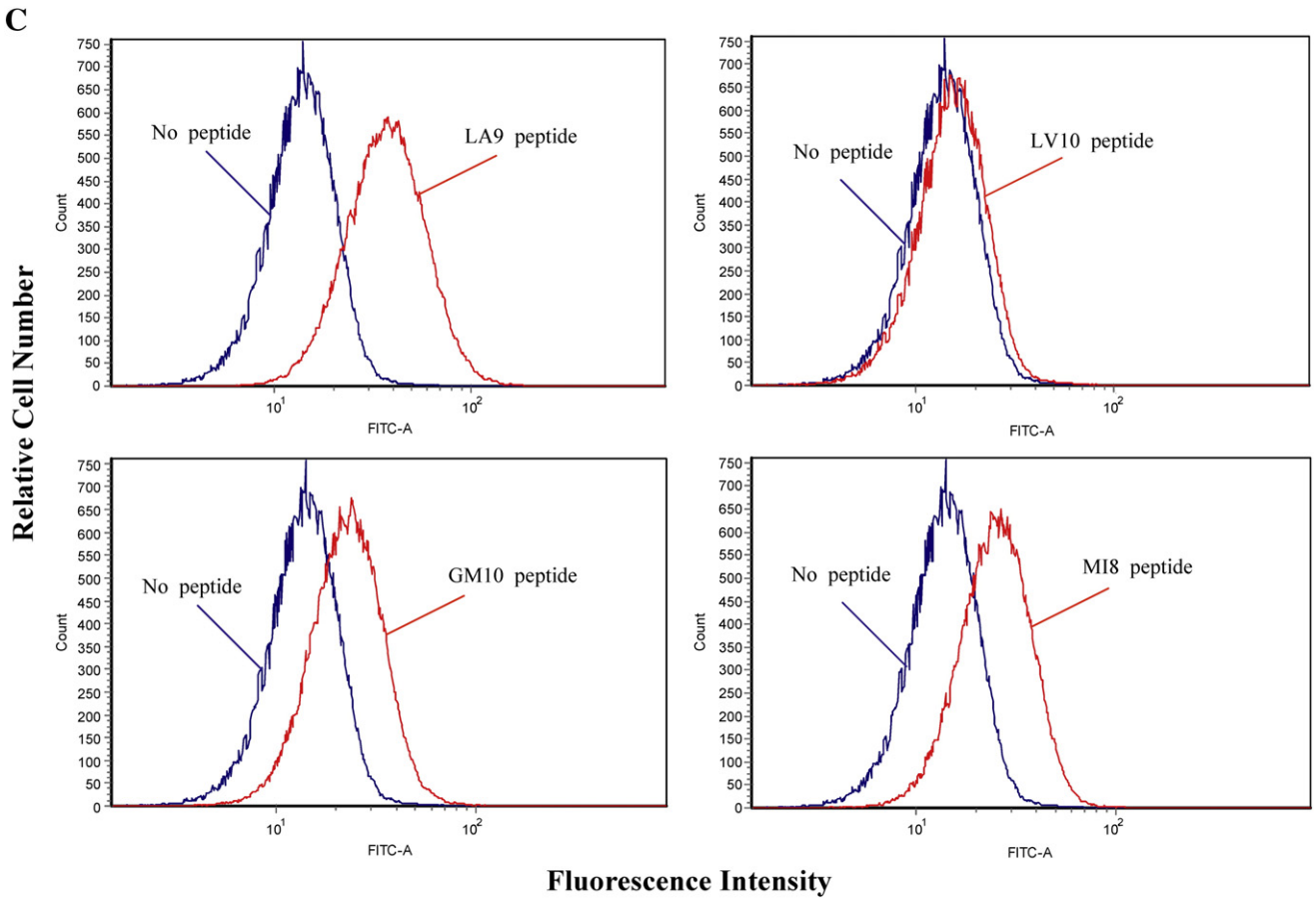

Fig. 1 (*continued*).

Mamu-A*01 gene in a double knockout (H-2K^b/D^b) background. Immunization of the mice with rVV expressing SIV genes successfully caused stimulation of CD8$^+$ T cells recognizing previously known Mamu A*01-restricted CTL epitopes that we quantified using cytokine flow cytometry. Mamu-A*01/Kb mice provides a simple and inexpensive model that may allow further mechanistic insight into EC status (Herberts et al., 2006; Loffredo et al., 2007, 2008). Additional alleles could be used to generate other Tg mouse strains that might inform on the mechanistic basis for suppression of SIV. The similarity of outcome of SIV infection in EC RM and HIV infection in EC humans with selected MHC alleles makes the study of the biology and immunology of these alleles of great interest. A Tg mouse model may improve understanding of the role of specific HLA or RM alleles on infection outcome.

Results

*Structure of Mamu-A*01/K^b transgene and expression in murine cells*

The transgene encoding chimeric Mamu-A*01/K^b was constructed, as illustrated in Fig. 1A. It is comprised of the native H-2K^b gene including the genomic promoter DNA sequence (Fig. 1A, i), followed by the leader sequence through to the third intron derived from Mamu-A*01 genomic DNA, replacing the corresponding murine sequence (Fig. 1A, ii). The chimeric gene terminates with the α3 domain of the H-2K^b gene, followed by transmembrane (TM) and cytoplasmic tail (CT) exons derived from the murine gene (Fig. 1A, iii). To test if the chimeric Mamu-A*01/K^b protein has the capacity for cell surface expression, the chimeric gene construct was introduced into TAP-deficient RMA-S cells using standard approaches described in Materials and methods. These cells are deficient in cell surface MHC Class I expression, because of the defect in endogenous peptide generation that can be overcome by providing exogenous optimal peptide motifs corresponding to MHC alleles carried by RMA-S cells (Ljunggren et al., 1990). The results show that a high level of Mamu-A*01/K^b complex formation is detected by incubation with epitope CM9 (an optimal Mamu-A*01 motif peptide, see Table 1), but not an irrelevant epitope in RMA-S transfectants (Fig. 1B, right panel), and not in untransfected RMA-S cells (Fig. 1B, left panel). Four identified Mamu-A*01 specific SIV epitopes: LA9, LV10, GM10 and MI8 (see Table 1) were also evaluated for their ability to promote cell-surface expression of the Mamu-A*01/K^b MHC protein at 37 °C (Fig. 1C). A relatively high level expression of Mamu-A*01/K^b was observed in the presence of LA9 peptide, and to a lesser extent, expression of Mamu-A*01/K^b was stabilized by incubation with GM10 and MI8 peptides. However, LV10 peptide stabilized a low level of the expression of Mamu-A*01/K^b. These results are evidence that the Mamu-A*01/K^b chimeric protein was translated and processed correctly, and could be brought to the cell surface in the presence of peptides that bound unmodified Mamu-A*01 MHC molecules.

*Expression of Mamu-A*01/K^b in H-2K^b/D^b knockout mice*

Transgene DNA was microinjected into a total of 230 fertilized eggs from DKO mice, however only 17 pups were born and survived because of the reduced viability of the eggs from the DKO strain (data not shown). The Mamu-A*01 gene was detected in three founders by PCR using Mamu-A*01 specific primers on tail DNA from newly weaned mice (Fig. 2A). Tg mice were backcrossed with DKO mice to derive Mamu-A*01 (heterozygous) animals for preliminary immunologic testing and to derive stable homozygous lines of Tg mice. Freshly prepared splenocytes were obtained from heterozygous Mamu-A*01/

Table 1
Summary properties of SIV-derived Mamu-A*01 peptides

SIV gene	Peptide name	Amino acid sequence	Reference	MFI	% IFN-γ CD8$^+$ T cells
Gag	LA9	LAPVPIPFA	Allen et al. (2001)	23.4	9.52
Gag	CM9	CTPYDINQM	Allen et al. (1998)	34.4	67.25
Gag	LW9	LSPRTLNAW	Allen et al. (2001)	1.72	7.81
Pol	LV10	LGPHYTPKIV	Allen et al. (2001)	1.58	4.02
Pol	SV9	STPPLVRLV	Egan et al. (1999)	45.8	10.1
Pol	GM10	GSPAIFQYTM	Allen et al. (2001)	9.06	2.51
Pol	MI8	MTPAERLI	Allen et al. (2001)	11.2	4.65

Seven previously defined Mamu-A*01 restricted SIV Gag and Pol epitopes were tested for stabilization of cell surface expression of Mamu-A*01/K^b in RMA-S cells, and induction of intracellular IFN-γ production in CD8$^+$ T cells after IVS of rVV-SIVGag–Pol immunized Mamu-A*01/Kb Tg mice ($N=2$) for each peptide. Background binding to a control peptide was subtracted for RMA-S assays, and the percentage of IFN-γ$^+$ CD8$^+$ T cells from a mock-stimulated culture was subtracted from all ICC assay results. The mean fluorescence intensity (MFI) and percentage of intracellular IFN-γ positive of CD8$^+$ T cells were determined by flow cytometry using a FACS-Canto™ (BDIS, San Jose, CA). Results shown are an average of $N=2$ mice with <25% standard error from the mean.

K^b Tg mice, though only weak levels of expression of Mamu-A*01 molecules was documented by staining using MAb W6/32, followed by flow cytometry (Fig. 2B, right). Under the same conditions, no signal could be detected on spleen cells from parental DKO mice (Fig. 2B, left). Since the Mamu-A*01/K^b chimeric gene is under the control of H-2K^b promoter, we treated Tg mouse splenocytes with murine IFN-γ for 16 h to activate the promoter (Israel et al., 1986). Consequently the surface expression of Mamu-A*01/K^b increased dramatically in cells from Mamu-A*01/K^b Tg mice (Fig. 2B, right). IFN-γ also enhanced the background, which was detected by MAb W6/32 in cells from parental DKO mice, but the elevated level was much lower than in cells from Mamu-A*01/K^b Tg mice (Fig. 2B, left).

Derivation of rVV and proteolytic processing of SIV Gag–Pol antigen cassette

The central goal of this mouse derivation was to generate a model to evaluate vaccines that would be applicable for testing in RM. As a first step, we evaluated the immunogenicity and processing of an rVV expressing full-length SIVGag–Pol antigens in Mamu-A*01/K^b Tg mice and the parental DKO strain of which they were derived (Fig. 3A). The rVV-SIVGag–Pol was derived by homologous recombination and the expression of the SIV Gag protein was verified by Western Blot. SIV Gag protein is expressed as a 58-kDa polyprotein precursor (p58), which is cleaved in a sequential manner by the SIV-encoded viral protease into matrix, capsid (p27), and nucleocapsid proteins. Western Blot of lysates prepared from Hu TK$^-$ cells infected with rVV-SIVGag–Pol by using anti-p27 antibody confirmed the SIV Gag protein was expressed and correctly proteolytically processed (Fig. 3B).

*CD8$^+$ T cell response induced by Mamu-A*01 restricted epitopes in Mamu-A*01/K^b Tg mice*

Mamu-A*01/K^b Tg ($N=2$) and parental DKO mice ($N=2$) were infected with rVV-SIVGag–Pol by i.p. injection for two weeks. Spleens were removed, and cell suspensions were incubated for 7 days with

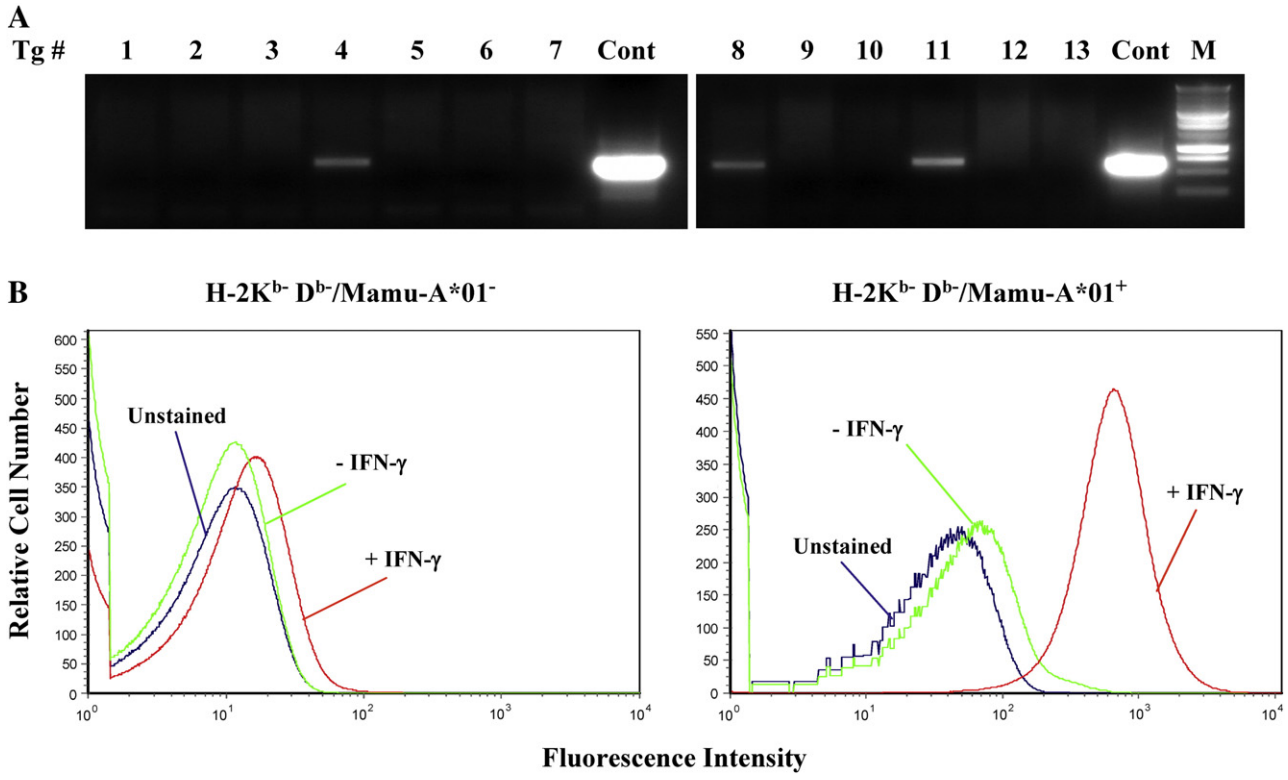

Fig. 2. (A) Detection of Mamu-A*01 DNA in tail samples from 13 Tg mice by PCR. Cont symbolizes control transgene Mamu-A*01/K^b plasmid DNA template. M represents 1 kb DNA ladder molecular size marker. (B) Detection of cell surface expression of Mamu-A*01/K^b MHC antigen in spleen cells. Splenocytes from DKO (left) mice and Mamu-A*01/K^b Tg (right) mice were stained with FITC-conjugated MAb W6/32 followed by flow cytometry. Splenocytes were also treated with 15 ng/ml IFN-γ for 16 h to enhance MHC antigen expression levels.

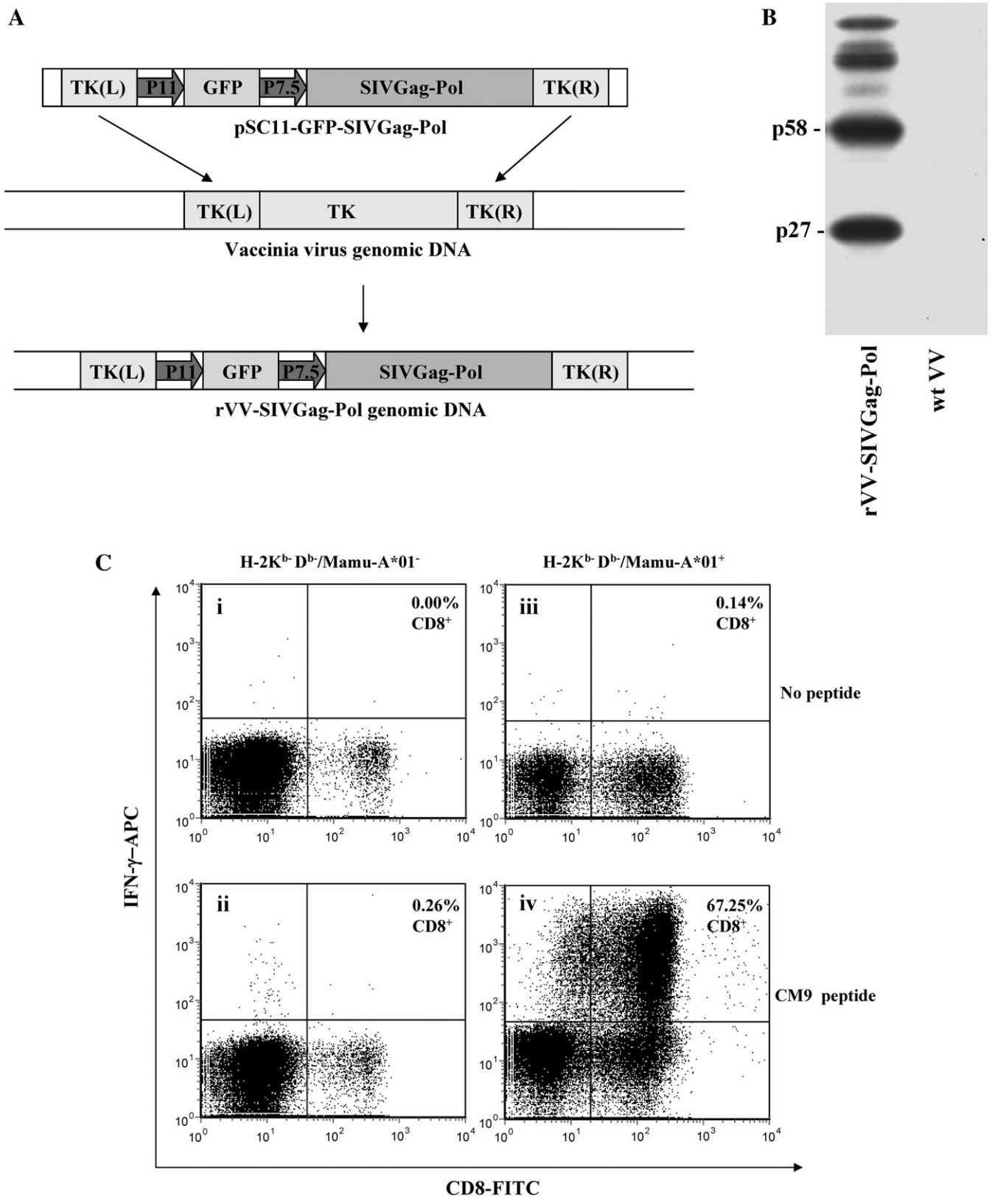

Fig. 3. (A) Schematic map of plasmid transfer vector pSC11-GFP-SIVGag–Pol. It contains two flanking regions; TK(L) and TK(R) which are homologous to the VV-TK gene, the screen marker gene GFP under the control of the VV promoter P11, and the SIVGag–Pol gene from SIVmac239 under control of VV promoter P7.5. rVV-SIVGag–Pol was generated by transfecting pSC11-GFP-SIVGagPol into VV-infected Hu TK$^-$ cells to promote homologous recombination. (B) Western Blot detection of SIV Gag protein in rVV-SIVGag–Pol infected cells. Lysates from rVV-SIVGag–Pol and wild type VV infected Hu TK$^-$ cells were separated on SDS-PAGE and detected with anti-P27 MAb, followed by an HRP conjugated anti-mouse secondary antibody contained in the ECL™ kit (GE Healthcare). (C) ICC of IFN-γ production in CD8$^+$ T lymphocytes from DKO mice (ii) and Mamu-A*01/K^b Tg mice (iv) after IVS and restimulation with CM9 peptide. Plots (i) and (iii) are negative controls without CM9 peptide restimulation. (D) Detection of IFN-γ production in CD8$^+$ T lymphocytes after IVS with four known Mamu-A*01 specific SIV peptides: LA9 (i), GM10 (ii), LV10 (iii) and MI8 (iv). In C and D, IVS followed by overnight peptide restimulation was performed according to Materials and methods followed by staining with anti-CD8 and anti-IFN-γ antibodies, and analyzed by flow cytometry.

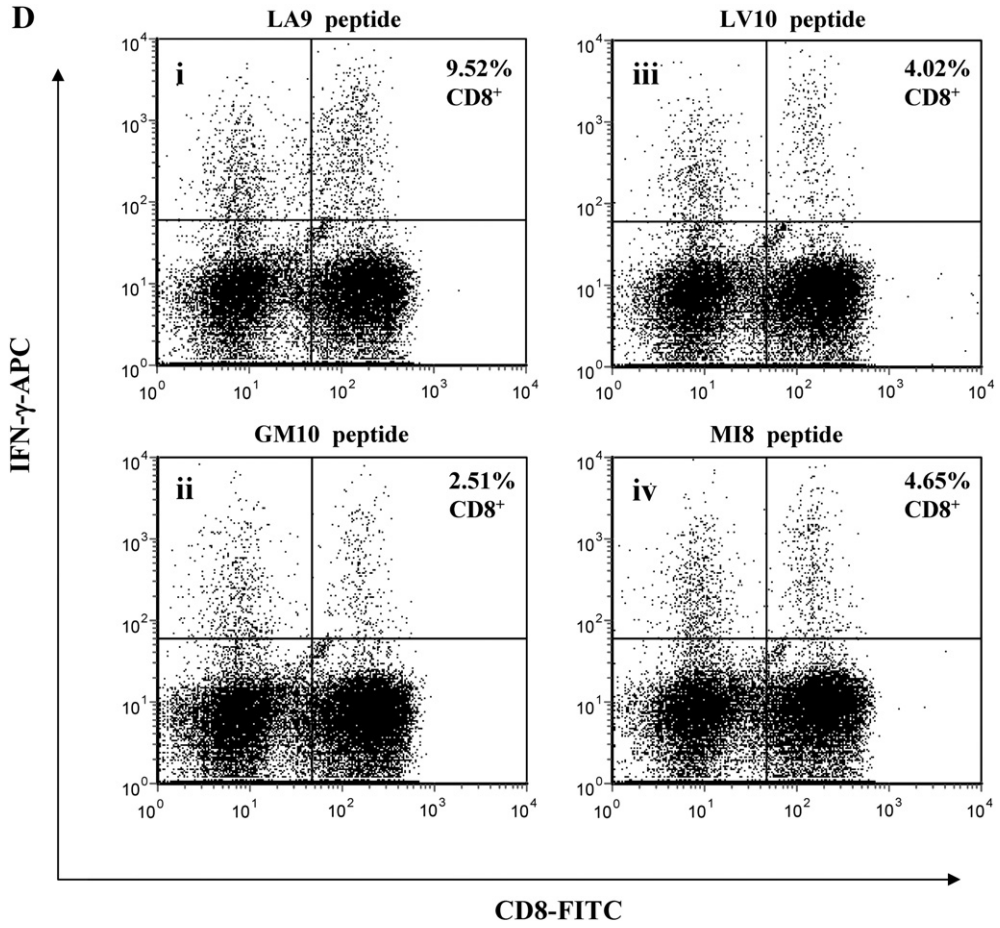

Fig. 3 (*continued*).

peptide CM9 (Table 1), using peptide-pulsed RMA-S/Mamu-A*01/K^b as APC. Analysis of cultured splenocytes by flow cytometry to detect intracellular staining of IFN-γ was performed. Remarkably, the immunodominant CM9 epitope stimulated 67.25% of $CD8^+$ T lymphocytes to produce IFN-γ from the splenocyte culture, shown as one example of the Mamu-A*01/K^b Tg mice we immunized, while only background levels were detected in the absence of the SIV epitope restimulation (Fig. 3C, iii and iv). As expected, splenocytes from parental DKO mice showed minimal recognition of the CM9 epitope (Fig. 3C, i and ii).

*Existing Mamu-A*01-restricted epitopes are recognized after rVV-SIVGag–Pol infection*

We further investigated the breadth of epitope recognition after rVV-SIVGag–Pol immunization of Mamu-A*01/K^b Tg mice, by examining the immune recognition of 4 previously characterized Mamu-A*01 restricted epitopes (LA9, LV10, GM10 and MI8, Table 1) after IVS with these 4 peptides. All of the peptides which were found to stabilize Mamu-A*01 surface staining in RMA-S/Mamu-A*01/K^b cells (Fig. 1C) also induced $CD8^+$ T lymphocytes to produce IFN-γ (Fig. 3D). LA9 which stabilized a high level of the Mamu-A*01 surface staining in RMA-S/Mamu-A*01/K^b cells (Fig. 1C), also induced a high level of IFN-γ producing $CD8^+$ T lymphocytes (9.52%) in Mamu-A*01/K^b splenocyte cultures (Fig. 3D, i). LV10 which minimally stabilized Mamu-A*01 surface staining in RMA-S/Mamu-A*01/K^b cells also induced a similar level of IFN-γ production as the other two peptides GM10 and MI8 which moderately stabilized Mamu-A*01 surface staining. These results are supporting evidence that the Mamu-A*01/K^b Tg mice contain a T cell repertoire that faithfully reproduces T cell recognition comparable to Mamu-A*01 RM, and will be a useful tool for the evaluation of virus-specific, Mamu-A*01 restricted T cell response.

*Screening of SIV Gag peptide library and identification of $CD8^+$ T cell epitopes in Mamu-A*01/K^b Tg mice*

We tested whether Mamu-A*01/K^b Tg mice could be used to distinguish and finely map Mamu-A*01 restricted epitopes to the level of minimal peptides starting with a library of 125 15-mer peptides overlapping by 11 amino acids that encompasses the entire SIVmac239 Gag amino acid sequence. Two Mamu-A*01/K^b Tg mice (Tg line 4) which were previously immunized with rVV-SIVGag–Pol were used for this approach. Immune splenocytes were stimulated with the complete library or 8 peptide subpools, each containing 16 component peptides from the whole library (Fig. 4A). T cell responses induced against each pool of peptides were analyzed by ICC for IFN-γ. Peptide pool 33–48 reproducibly induced high frequencies of IFN-γ expressing $CD8^+$ T cells (46% and 69%), while peptide pools 81–96 and 97–112 also induced moderate levels of IFN-γ expressing $CD8^+$ T cells ranging from 5.45% to 31.8% (Fig. 4A). Each of the three responding pools was further analyzed by testing each of the component peptides individually. Peptides 45 and 46 containing the CM9 epitope sequence (Table 1) induced IFN-γ expressing T cells very efficiently (81.5% and 84.5%, Fig. 4B), similar to the levels stimulated by the minimal 9-mer CM9 epitope (Fig. 3C). In addition, peptides 38 and 39 also induced IFN-γ producing T cells, although at a lower level (16.2% and 9.32%). Peptide 38 contains a previously identified Mamu-A*01 restricted epitope LW9 (Table 1). In addition, as shown in Fig. 4C, both peptides 92 and 93 induced a

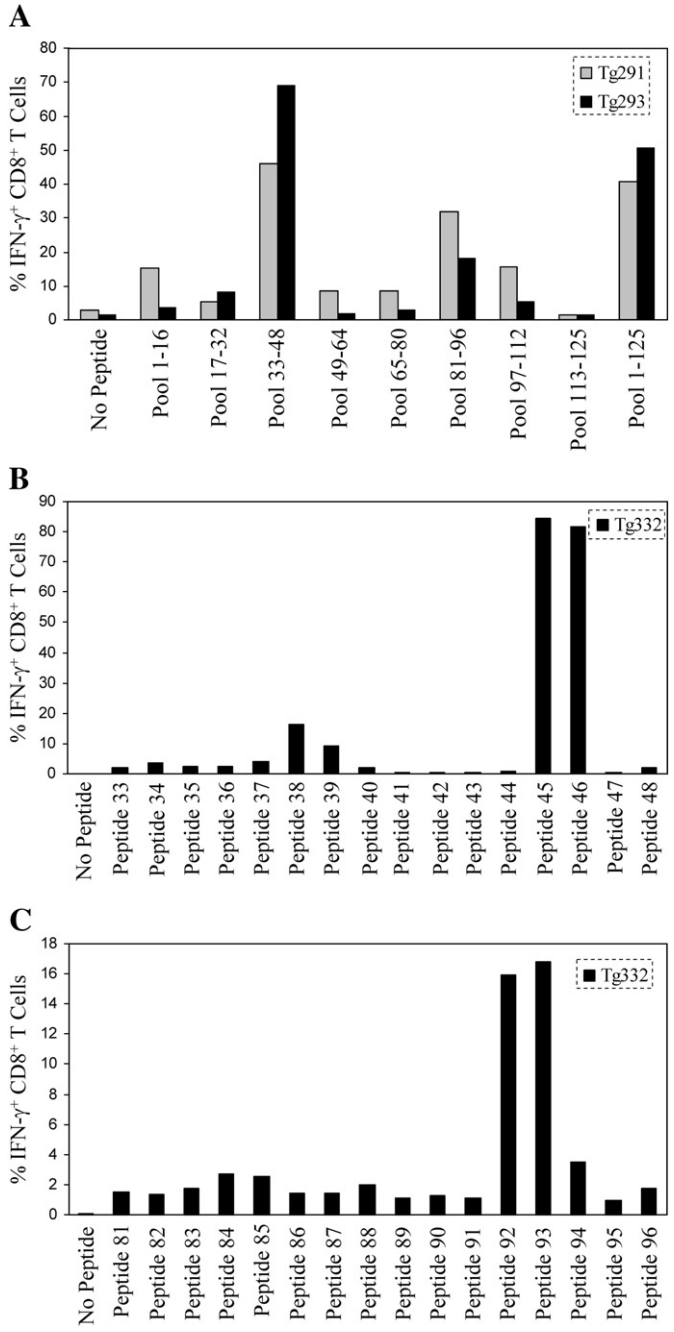

Fig. 4. Mapping Mamu-A*01 restricted epitopes of SIV Gag by intracellular IFN-γ staining in CD8$^+$ T cells after IVS. Splenocytes from rVV-SIVGag–Pol immunized Mamu-A*01/K^b Tg mice were subjected to IVS with 8 SIV Gag peptide subpools or the full-length peptide library. (A) Reactive peptide pools were deconvoluted by testing individual SIV Gag peptides 33 to 48 (B), and 81 to 96 (C).

moderate frequency of IFN-γ producing CD8$^+$ T cells (15.9% and 16.8%). These two 15-mer peptides contain an identified Mamu-A*01 restricted epitope, LA9 (Table 1).

Screening of SIV Pol peptide library and identification of CD8$^+$ T cell epitopes

In SIV or SHIV infected RM, Pol specific CTL responses were observed at low frequencies (Allen et al., 2001; Egan et al., 1999). These observations led us to investigate the processing of the Pol protein sequence in Mamu-A*01/K^b Tg mice. In rVV-SIVGag–Pol infected lysates, SIV Gag polyprotein was proteolytically cleaved precisely according to expected MW, suggesting that the protease encoded in the Pol sequence is active, and that SIV Pol protein was also expressed (Fig. 3B). IVS was conducted on splenocytes from two rVV-SIVGag–Pol immunized Mamu-A*01/K^b Tg mice with eleven peptide subpools, each consisting of 24 15-mer peptides spanning the entire SIVmac239 Pol protein. Seven days later, the IVS-stimulated splenocytes were analyzed for expression of the intracellular cytokine IFN-γ in CD8$^+$ T cells (Fig. 5A). A moderate level of IFN-γ expressing CD8$^+$ T cells were induced by peptide pool 145–168 (7.7% and 16.6%). In addition, low levels of IFN-γ expressing CD8$^+$ T cells were reproducibly induced by peptide pools 1–24, 25–48, 49–72, 169–192 and 217–240 with frequencies ranging from 2.5% to 3.9%. We assessed individual component peptides from pool 145–168 for their ability to induce IFN-γ expressing CD8$^+$ T cells (Fig. 5B). We selected pool 145–168 for further analysis because it induced the highest level of IFN-γ producing CD8$^+$ T cells among all the 11 subpools. Two peptides among the 24 separately evaluated from pool 145–168 showed equally high levels of response using the ICC assay (Fig. 5B). Both peptides 156 and 157 contain the known Mamu-A*01 restricted epitope SV9 were found to induce IFN-γ producing T cells at equivalent levels (12.2% and 12.3%, Fig. 5B) and (Table 1).

Immunization with fusion peptide vaccines

Based on our previous success with the PADRE:CTL fusion peptide vaccine model for HIV vaccines in HLA A2/Kb mice (Daftarian et al., 2003), we synthesized an analogous structure for 3 different CTL epitopes from SIV Gag and Pol shown in Table 2 (MI8, LW9, CM9). The

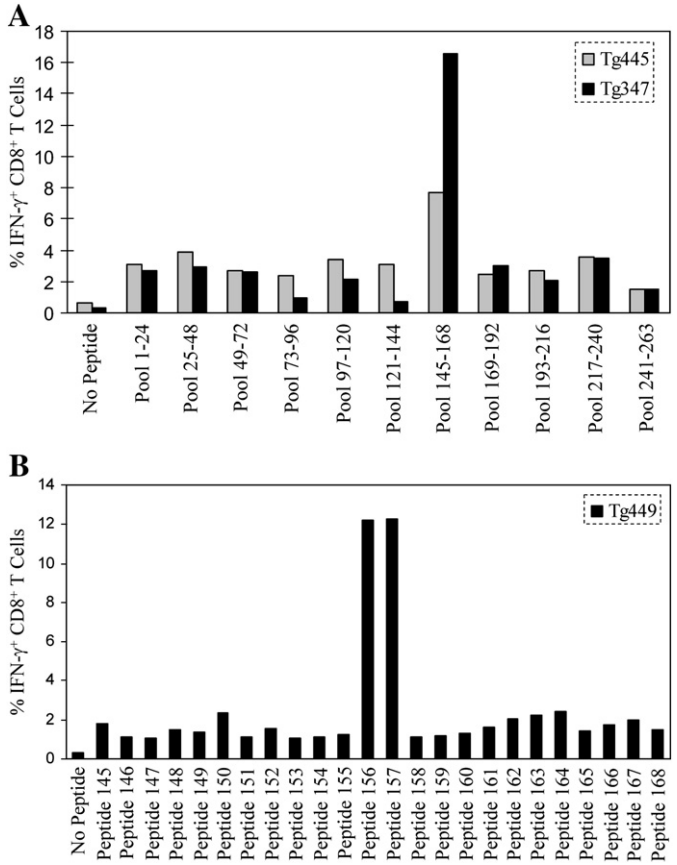

Fig. 5. Mapping Mamu-A*01 restricted epitopes of SIV Pol by intracellular IFN-γ staining in CD8$^+$ T cells after IVS. Splenocytes from rVV-SIVGag–Pol immunized Mamu-A*01/K^b Tg mice were subjected to IVS in similar conditions as described in the legend to Fig. 4 with 11 SIV Pol peptide subpools (A). Individual SIV Pol peptide pools were evaluated between 145 to 168 (B).

Table 2
Nomenclature, molecular weight and amino acid sequence of synthetic minimal CTL and T_H epitopes and epitope fusion peptides

Compound	Name	[a]MW (kDa)	Sequence
Pan DR binding epitope (Alexander et al., 1994)	PADRE	1.354	AK[b]XVAAWTLKAAA
PADRE:SIV$Gag_{181-189}$ fusion	PADRE:CM9	2.419	AK[b]XVAAWTLKAAACTPYDINQM
PADRE:SIV$Gag_{149-157}$ fusion	PADRE:LW9	2.393	AK[b]XVAAWTLKAAALSPRTLNAW
PADRE:SIV$Pol_{961-969}$ fusion	PADRE:MI8	2.266	AK[b]XVAAWTLKAAAMTPAERLI
CpG-ODN #7909 (Hartmann et al., 2000)	CpG-Human	7.900	5′ TCGTCGTTTTGTCGTTTTGTCGTT 3′

The sequences are shown for the components and final products that were used in the fusion peptide immunization. The sequence and names of the CTL epitopes can be found in Table 1. Details of fusion peptides and structure can be found in prior publications (Daftarian et al., 2005;La Rosa et al., 2002). Active CpG motif underlined.
[a] MW = molecular weight confirmed by mass spectroscopy.
[b] X = cyclohexyl-alanine.

vaccination approach we employed for the studies shown in Fig. 6 combined 100 nmol fusion peptide and 25 μg CPG 7909 DNA (Hartmann et al., 2000), administered s.c., similar to our published studies for HIV-specific fusion peptides (Daftarian et al., 2003). Shown are results from two identically immunized mice for each peptide fusion, analyzed for IFN-γ producing T-cells using ICC (Fig. 6A). ICC evaluations of splenocytes were conducted with a non-specific control peptide (grey bars) and the CTL epitope that is part of the fusion peptide (black bars) for each fusion peptide that was studied. Of the three CTL epitopes fused to the PADRE T-help peptide, the MI8 octamer was recognized most inefficiently after rVV-SIVGag–Pol infection, but its fusion peptide was the best at eliciting antigen-specific $CD8^+$ T cells after immunization (Table 1 and Fig. 6A). The LW9 nonamer was recognized with intermediate intensity, but its fusion peptide was poorly immunogenic, whereas the CM9 nonamer that was efficiently recognized after rVV-SIVGag–Pol infection also elicited strong immunogenicity as a fusion peptide (Table 1 and Fig. 6A). Interestingly, the MI8 fusion peptide immunized without CpG 7909, showed an average of only 8.0% IFN-γ^+ $CD8^+$ T-cells in Mamu-A*01 Tg mice ($N=2$), a >90% reduction from the immunization containing CPG 7909 DNA (Fig. 6A).

Challenge with rVV-SIVGag–Pol

The MI8, LW9, and CM9 fusion peptides represent a large range of immunogenicities. All 3 fusion peptides were administered to Mamu-A*01/Kb Tg mice as a series of two immunizations that were spaced two weeks apart, conducted as a limited dose titration between 1 and 100 nmol plus 25 μg of CpG 7909 ODN. Similar to the studies of Berzofsky and collaborators, fusion peptides were introduced s.c. and i.p. for both immunizations (Ahlers et al., 2001). Seven days after the second immunization, immunized mice and a control unimmunized litter-mate were i.p. administered 10 million infectious units (pfu) of rVV-SIVGag–Pol or rVV-GFP. Five days later, ovaries were removed and lysates were prepared, and stored in aliquots at −80 °C. Infection was carried by a series of dilutions of the ovary lysate on CV-1 cell monolayers for a period of 18 h by standard approaches. Results with accompanying *P*-values are presented in Fig. 6B. Plaques were counted by immunostain using a monoclonal antibody to VV as a primary, and an HRP-conjugate as secondary (Wang et al., 2008). The left (L) and right (R) ovaries were separately analyzed, but results of both ovaries were consistent for both immunized and unimmunized mice. At the lowest dilution tested of 10^3 of the ovary lysate from PADRE:MI8-

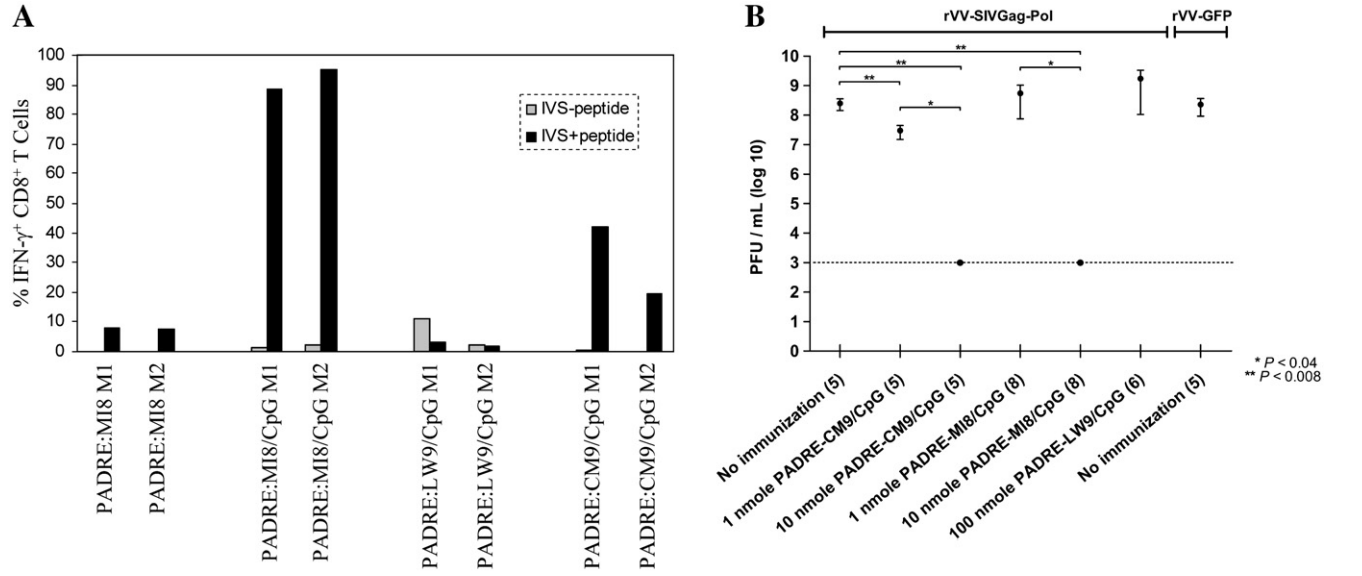

Fig. 6. Immunogenicity (A) and challenge studies (B) conducted with fusion peptide immunized Tg mice. (A) Tg mice ($N=2$) were immunized with 100 nmol of fusion peptides + 25 μg CPG 7909 DNA one time, and were sacrificed after 14 days for processing of splenocytes for ICC studies. ICC assays were carried out as described in Materials and methods using CTL epitope encoded by fusion peptide (filled bars) or without exogenous peptide (grey bars). (B) Tg mice (number of mice indicated in parentheses for each group on the *x*-axis) were immunized with indicated amounts of fusion peptides + 25 μg CPG 7909 DNA twice as described in Materials and methods. Seven days later, rVV-SIVGag–Pol or rVV-GFP were administered i.p. to immunized mice, as indicated in the legend above each data series. Five days later, mice were sacrificed, ovary lysates prepared, and diluted before incubating with monolayers of CV-1 cells as described in Materials and methods. Plaques were visualized using immunostain to VV antigens as described in Materials and methods. Dotted line indicates the limit of detection of VV plaques using the assay. *P*-values (2-sided) were determined using the Wilcoxon rank-sum test, and values which are significant are shown as one ($P<0.04$) or two asterisks ($P<0.008$). Standard error of the mean is shown as horizontal bars, with means indicated as filled circles. Undetectable plaques in all members of a group are represented by a single filled circle without error bars on the dotted line signifying the minimal detection limit.

immunized mice (100 or 10 nmol), no plaques were visible in lysates made of ovaries from rVV-SIVGag–Pol challenged mice ($N=8$). In addition, PADRE:CM9 (10 nmol) immunized mice ($N=5$) also showed no evidence of viral plaques at 10^3 dilution of the ovary lysate, similar to the PADRE:MI8 immunized mice. However, the dose titration down to 1 nmol fusion peptide was only partially effective at controlling rVV-SIVGag–Pol infection for both MI8 ($N=8$) and CM9 ($N=5$) fusion peptides. The weakly immunogenic PADRE:LW9 ($N=6$) immunized mice (100 nmol) only gave partial protection to challenge with rVV-SIVGag–Pol, similar to mice immunized with 1 nmol of either MI8 or CM9 fusion peptides. As expected, non-immunized mice had substantial visible plaques at 10^7–10^8 dilutions of the ovary lysates from either challenge virus (rVV-GFP ($N=5$) or rVV-SIVGag–Pol ($N=5$), Fig. 6B). These results are informative for exploring immunization strategies in RM.

Discussion

Mamu-A*01/K^b Tg derived from DKO mice were successfully generated and employed to investigate recognition of Mamu-A*01 restricted epitopes produced by a model rVV-SIV. We constructed the Mamu-A*01 transgene based on results of previous studies that showed increased immunologic function of chimeric MHC molecules composed of α1α2 domains from human HLA and H-2K^b α3 from mouse (Kalinke et al., 1990; Vitiello et al., 1991). This strategy was used to enable TCR-CDR3 and MHC-α1α2 interactions to more efficiently activate T cells based on Mamu-A*01 restricted peptide specificity. Complex stability between CD8$^+$ T cells and professional APC was enhanced by the interaction of endogenous murine CD8 molecules with the H-2K^b α3 mouse partner as a means to stabilize the tripartite complex (Vitiello et al., 1991). The first construct we developed was composed of the promoter and three first exons of Mamu-A*01, and the α3, transmembrane and cytoplasmic domains from H-2K^b genomic DNA (Fig. 1A and not shown). Following transfection into murine fibroblast NIH3T3 cells, the cell surface expression of Mamu-A*01/K^b monochains was detected by indirect immunofluorescence. However, the cell surface expression of Mamu-A*01/K^b was minimal (data not shown). We attempted to improve the transcriptional efficiency in mouse by replacing the Mamu-A*01 promoter with the H-2K^b promoter (Fig. 1A). This substitution proved successful, because of improved surface expression that could be dramatically enhanced by treatment of splenocytes derived from Mamu-A*01/K^b Tg mice with murine IFN-γ (Fig. 2). This is the first example of a Mamu MHC Tg mouse which may serve as a paradigm for construction of other Mamu Class I allele strains.

We were concerned that differences in TAP specificity of RM and murine Class I MHC would reduce the efficiency of endogenous murine antigen-processing, resulting in reduced levels of peptide-loaded and surface expressed Mamu A*01 MHC molecules. The reduction of surface Mamu A*01, might negatively impact the frequency and antigen specificity of the Mamu A*01-restricted peripheral T cell repertoire in the Mamu-A*01/K^b Tg mice, rendering them not useful for RM-specific vaccine studies. Others have found that human β2-microglobulin (β2m) introduced into Tg mice by covalent linkage to the N-terminus of the transgenic Class I heavy chain constructed analogously, increased the cell surface expression of the chimeric molecule in RMA cells (Pascolo et al., 1997). However, we demonstrated that a relatively high level of Mamu-A*01/K^b expression could be stabilized by exogenous peptides in TAP-deficient mouse RMA-S lymphoma cells expressing Mamu-A*01/K^b without introducing covalently added human or RM β2m. Consistent with previous reports describing HHD Tg mice derived from H-2D^b β2m knockout mice, the expression of Mamu-A*01/K^b molecules on T and B peripheral cells was low(Pascolo et al., 1997). Nevertheless, our results showed efficient usage of the Mamu-A*01/K^b monochain to generate a potent immune response induced by CD8$^+$ T lymphocytes. Once homozygous animals are isolated, greater levels of CD8$^+$ T lymphocytes may be observed. Similar to the case of HHD Tg mice, the expression of Mamu-A*01/K^b partially restores to normal levels, the low levels of CD8$^+$ T lymphocytes found in the periphery before immunization or viral infection, potentially because of defective positive selection in the absence of a classical MHC Class I gene in the DKO mice (data not shown). Our results suggest that Mamu-A*01/K^b monochain is able to replace the missing endogenous K^b or D^b MHC molecules to stabilize CD8 surface expression on circulating T lymphocytes in the Tg mice.

We examined the usefulness of the Mamu-A*01/K^b mice to aid in RM-specific CTL epitope identification. Our strategy was to immunize Mamu-A*01/K^b Tg mice with rVV-SIVGag–Pol that we constructed from SIV genomic sequence containing plasmid DNA. CD8$^+$ T cell reactivity was screened using overlapping 15-mer peptide libraries spanning SIV Gag and Pol proteins. Four CTL epitopes located within the SIV Gag protein were identified. Among them, the previously characterized CM9 peptide, and two other epitopes called LA9 and LW9 (Allen et al., 2001) were identified, because they stimulated IFN-γ expression in splenic CD8$^+$ T cells. A previously described epitope located within SIV Pol, SV9 (Egan et al., 1999) was also identified. Although the Mamu-A*01/K^b Tg mice showed dramatic CD8$^+$ T cell responses to SIV immunodominant epitope CM9, they also recognized a broad distribution of epitopes in SIV Gag and Pol proteins. Both high (CM9) and moderate-low (LA9, LW9, SV9, LV10, GM10 and MI8, Table 1) affinity peptides elicited immunologic recognition in Mamu-A*01/K^b Tg mice. The immunogenicity of the Mamu-A*01 restricted peptides identified through rVV infection and peptide library screening suggests that the CD8$^+$ T cell repertoire in the Mamu-A*01/Kb Tg mice faithfully represents at least a portion of the RM T cell repertoire. We also identified and fine mapped two novel Mamu-A*01-restricted CTL epitopes (data not shown): the SIV Gag-derived epitope RW9 (RAPRRQGCW) and Pol-derived epitope NE8 (NFPIAKVE). To confirm and further characterize these two newly defined epitopes with respect to their stage of appearance after SIV infection, we will infect Mamu-A*01 positive RM with SIV and enumerate antigen-specific CTL response in infected animals.

Tg mice expressing full length HLA Class I molecules were the first examples used to study HLA class I restricted CTL responses (Arnold and Hammerling, 1991). Either through virus infection or allo-stimulation by other HLA Class I alleles, these mice preferentially or exclusively developed H-2-restricted CD8$^+$ T cell responses, rather than the desired HLA-restricted T cell responses (Barra et al., 1989; Engelhard et al., 1991; La Rosa et al., 2001). Consequently, H-2D^b and β2m knockout mice expressing the HHD chimeric Class I molecule were derived to force the CD8$^+$ T cell repertoire to be educated by Tg HLA Class I molecules (Pascolo et al., 1997). As a result, virus infection of these Tg mice generated predominantly HLA-A2.1-restricted CD8$^+$ T cell responses against influenza A and vaccinia viruses as representative examples (Pascolo et al., 1997). We sought to simplify and speed up the process of deriving a Tg Class I strain in the DKO background. The process for deriving HHD mice required several time-consuming steps, and we sought to reduce the process to a single step. By doing so, it reduced the number of additional crosses to just one that would be needed to generate homozygotes. This work represents the first example of a new Tg mouse strain derived by direct transgenesis of a DKO strain. If we can improve the viability of the eggs from the DKO mice during microinjection, it may be the best way to generate Tg mice for other primate and human MHC Class I alleles.

There are many areas of study where these mice will prove useful. Recently, we developed a vaccine consisting of rhesus cytomegalovirus (RhCMV) pp65, gB and IE1 expressed via modified vaccinia Ankara (MVA), and evaluated it in RM, including some which are Mamu-A*01 positive (Yue et al., 2008). Rhesus pp65 and IE1-specific cellular immune responses were detected. To track the evolution of these cellular immune responses using tetramers, especially in CMV-

negative RM, Mamu-A*01 restricted CTL epitopes need to be identified. The Mamu-A*01/Kb Tg mice, represent a facile system to preliminarily identify potential CTL epitopes that can be later verified in the Mamu A*01 RM. Identified epitopes would allow detailed studies including those with tetramers to determine avidity and other functional parameters (Egan et al., 1999). While we found no effect on specificity of epitope detection, all of our studies utilized an intermediate step of IVS to amplify the epitope-specific splenocyte populations prior to conducting ICC. Our approach of employing IVS when using transgenic mice is consistent with other studies from a variety of laboratories (Pascolo et al., 1997; Vitiello et al., 1997). While not optimal, the fact that protection studies correlate with the levels of immunogenicity measured in Fig. 6A suggests minimal disruption of functional properties to distort the good correlation with the challenge results (Fig. 6B).

CTL epitopes can be quickly identified using the mice, and verified using appropriately infected RM with Mamu-A*01 allele. Another area of investigation that will benefit is the testing of vaccine concepts in the relatively inexpensive Tg mice prior to evaluation in the costly RM model. Since all HIV vaccine concepts must pass a macaque feasibility test, using SIV or SHIV as a challenge virus, we envision preliminary testing in this mouse model. Viruses, peptides, or DNA products that elicit T cell response could be first tested for immunogenicity in mice prior to advanced testing in monkeys. Epitope based vaccines are candidates for preliminary evaluation in mice prior to monkey testing, including constructs similar to what we developed for HIV (Daftarian et al., 2005). Our preliminary investigation of 3 Mamu-A*01 epitopes incorporated into fusion peptide vaccines was informative, since the immunogenicity characteristics of CTL epitopes processed from rVV-SIVGag–Pol differed from the ranking of immunogenicity of the peptide fusion vaccines. Whether the immunogenicity ranking predicted by the mice is also similar in RM needs to be evaluated in a separate study. The results of the challenge study reassured and followed the results of the immunogenicity ranking, as LW9 fusion peptide performed poorly in both tests. In contrast, the MI8 and CM9 fusion peptides performed well in both immunogenicity and challenge tests. Many other antigens from pathogens that infect RM could be intensively studied immunologically in these mice to rapidly identify epitopes that could be used to study disease pathogenesis or vaccine strategies in the more costly primate model.

Materials and methods

Media, cell lines and mice

Continuously growing cell lines were maintained in RPMI 1640 (Cellgro, Herndon, CA) containing 10% FBS (Omega, Tarzana, CA), 4 mM L-glutamine (GIBCO, Rockville, MD), 0.1 mM 2-mercaptoethanol, and 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO). 2×10^7 RMA-S cells (Ljunggren et al., 1990) (transporter associated with antigen presentation [TAP] negative, a kind gift from Dr. Martin Kast, USC Keck School of Medicine) in 0.5 ml of PBS were electroporated with 15 μg of linearized pH2K^{b}Mamu-A*01α1α2 DNA at 250 V and 2000 μF using a BTX ECM 600 Electroporation System (BTX, San Diego, USA). After 24 h, cells were transferred in selective medium containing 1.0 mg/ml G418 (Invitrogen, Carlsbard, CA) and cloned by limiting dilution in 96 well plates. Neomycin-resistant stable clones expressing the Mamu-A*01/K^b gene were identified by PCR using the Mamu-A*01 specific primers 5-GACAGCGACGCCGCGAGCCAA-3 and 5-CGCTGCAGCGTCTCCTTCCCC-3. These cells were expanded in the absence of G418 selection, but were periodically examined for expression of Mamu-A*01/K^b protein by indirect immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated W6/32 MAb (Maziarz et al., 1986) (eBioscience, San Diego, USA). Cells were evaluated by incubation overnight at 26 °C with a Mamu-A*01 specific peptide and subsequent at 37 °C, followed by flow cytometry analysis. C57BL/6 H-2K^b/D^b knockout (DKO) mice were obtained from the Institute Pasteur (Paris, France) through an agreement with NIAID and Taconic Laboratories (Germantown, NY). Mice used throughout this study were bred and maintained under standard pathogen-free conditions in the American Association for Laboratory Animal Care approved animal care facility at City of Hope.

Transgene construction

Genomic DNA was isolated from C57BL/6 mouse tails by using DNAzol (Molecular Research Center Inc., Cincinnati, OH). 5-CGCGGATCCTGTGCTGAGGGGACCAGC-3 and 5-ATTTGCGGCCGCGTCTGTCACCAAGTCCACTCCAGGC-3 primers were used to amplify a DNA fragment which contains the H-2K^b α3, transmembrane and cytoplasmic domains. The 1.3 kb PCR product was cloned into BamH I–Not I sites of vector pEGFP-N3 (Clontech, Palo Alto, CA). 5-CGCATTAATGTCGACGGTCAGGGAGGGAGGGAGAGGGTC-3 and 5-CCCAAGCTTGTCGGCGATTCGCGACTTCTGAG-3 primers were used to amplify the H-2K^b promoter. The 1.0 kb PCR product was cloned into Ase I–Hind III sites of pEGFP-N3 vector which we designated as pH2K^{b}Δα1α2 (Fig. 1A). The DNA fragment containing the leader, αl, and α2 domains of Mamu-A*01 was amplified from genomic DNA of Mamu-A*01 positive RM by PCR with primers 5-CCCTCGCGACAGGATGGCGGTCATGGCGC-3 and 5-CGCGGATCCCATTTGTCTCCCCTCC-3. The 1.1 kb PCR product was cloned into Nru I–BamH I sites of pH2K^{b}Δα1α2 vector, and the completed plasmid was designated as pH2K^{b}Mamu-A*01α1α2 (Fig. 1A). DNA sequence of all constructs was confirmed by standard automated DNA sequencing methods at the City of Hope Core Sequencing Facility.

Production of Tg mice

A 3.4 kb Sal I–Not I chimeric monkey-mouse DNA fragment containing the murine H-2K^b promoter, the leader, αl, and α2 domains of rhesus macaque Mamu-A*01 and the α3, transmembrane, and cytoplasmic domains of murine H-2K^b was excised from pH2K^{b}Mamu-A*01α1α2 (Fig. 1A) and purified by sucrose density gradient fractionation, and injected into the pronuclei of fertilized eggs from C57BL/6 H-2K^{b}D^b double MHC knockout (DKO) mice according to established protocols (Mann and McMahon, 1993). The microinjected eggs were transferred surgically to the uteri of 0.5 day-post-coitus pseudo-pregnant recipient female mice (Mann, 1993). Tg mouse founder lines were established by PCR detection of a 300 bp genomic DNA fragment using the Mamu-A*01 specific primers 5-CCCCCACGGACGGCCCGCGTCG-3 and 5-CCGTCGTAGGCGTACTGTTCAT-3. Heterozygous Tg mice from a founder line designated Mamu-A*01/K^b Tg mice line #4 (Fig. 2A) was selected for these studies. Cell surface expression of Mamu-A*01 was detected in spleen cells by staining with mAb W6/32 and analyzing by FACS immediately, or following overnight culture in RPMI-10% FBS with 15 ng/ml murine IFN-γ (PeproTech, Rocky Hill, NJ). All studies involving live animals were conducted following review and approval of all protocols by the City of Hope Institutional Animal Use and Care Committee, and in accordance with all applicable guidelines.

Generation of recombinant vaccinia virus (VV)

The SIVmac239 Gag–Pol genes were amplified using primers: 5-TAAGAATGCGGCCGCGAGATGGGCGTGAGAAACTCC-3 and 5-GACGTCGACCTATGCCACCTCTCTAGCC-3 from plasmid pSHIV-89.6 5′ (NIH AIDS Research and Reference Reagent Program). The resulting 4.4 kb DNA was cloned into pSC11-GFP using Not I and Sal I sites. pSC11-GFP is a modified pSC11 vector in which the β-galactosidase gene is replaced with the GFP gene (Chakrabarti et al., 1985) (kind gift of Dr. Bernard Moss, LVD, NIAID, NIH). The SIVGag–Pol recombinant vaccinia virus (rVV-SIVGag–Pol) was generated by transfecting the plasmid into WR strain VV-infected Hu TK$^-$ cells. rVV-SIVGag–Pol

infected GFP plaques were detected using a Olympus CK40 fluorescence microscope (Olympus, Tokyo, Japan) and simultaneously selected with 0.1 mg/ml bromodeoxyuridine (Sigma-Aldrich, St Louis, MO) for 5 rounds. The expression of SIV Gag protein was detected from cell lysates prepared from rVV-SIVGag–Pol infected Hu TK^- cells by Western Blot analysis with anti-p27 MAb (NIH AIDS Research and Reference Reagent Program).

*Immunization of Mamu-A*01/K^b Tg mice with rVV-SIVGag–Pol and in vitro stimulation (IVS) of spleen T lymphocytes*

8 to 12 week old (*N* varies, see figure legends) mice (Tg line 4) were i.p. injected with 2×10^7 PFU (plaque forming units) rVV-SIVGag–Pol using a 1 ml syringe in a volume of 100 μL of PBS. After 14 days, the mice were killed, spleens were aseptically removed and splenocyte suspensions were produced by teasing the spleen through a sterile nylon mesh. RMA-S/Mamu-A*01/K^b as antigen-presenting cells (APC) were loaded with 100 μM of the SIV Gag or Pol peptides for 3 h in a 37 °C and 5% CO_2 incubator with occasional shaking. The IVS was initiated by co-culture of 10^6 irradiated (5000 rad, Isomedix Model 19 Gammator; Nuclear Canada, Parsippany, NJ) peptide-loaded RMA-S/Mamu-A*01/K^b cells with splenocytes (3×10^6) from VV-infected or control mice for 7 days in 2 ml medium containing 10% T-Stim Culture Supplement (BD Biosciences, San Diego, CA).

Intracellular cytokine staining (ICC)

A total of 1×10^6 cells from 7 day IVS spleen cell cultures were incubated at 37 °C for 1 h with 1 to 10 μM SIV peptides, or a mock stimulation without peptide. Cells were then treated with 10 μg of brefeldin A per ml to inhibit protein trafficking and incubated overnight at 37 °C. Cells were then washed twice with PBS plus 0.1% BSA (Bovine Serum Albumin) and stained with FITC-conjugated CD8 MAb. After fixation and permeabilization with the Cytofix/Cytoperm Plus Kit (BD Biosciences) according to the manufacturer's protocol, cells were washed twice and then incubated with Allophycocyanin-conjugated antibody to IFN-γ (BD Biosciences), and analyzed by FACS on a two laser-6 color FACS-Canto™ (BD-Immunocytometry systems, San Jose, CA). The number of double-positive cells is expressed as a percentage of the $CD8^+$ T-cell population.

Peptide vaccine immunization and in vivo VV challenge assay

Groups of Mamu-A*01 Tg female mice (8- to 12-weeks) were immunized twice in a two week period on days 0 and 14 by a combined s.c. at the base of the tail and i.p. approach with various amounts of peptide vaccine plus 25 μg synthetic oligodeoxynucleotide (ODN) 7909 with CpG motifs in a final volume of 100 μl saline as previously described. Seven days after the second immunization, the mice were challenged i.p. with 1×10^7 PFU of rVV-SIVGag–Pol or rVV-GFP. Five days post-challenge, mice were sacrificed and both ovaries were removed. Following homogenization and three rounds of freeze–thaw, ovary lysates (total volume was about 10 μl per ovary) were assayed for VV titer by a modification of a standard approach. The titer of VV in ovary lysates was determined by plating serial 10-fold dilutions on duplicate six-well plates of CV-1 cells ($\sim10^6$ cells per well). Following overnight incubation, monolayers were immunostained with mAb to VV as primary, and an HRP-conjugate as secondary, and plaques were counted (Wang et al., 2008).

Synthetic peptides

Individual nonamer and fusion vaccine peptides were prepared by the standard solid phase Fmoc procedure using Symphony Peptide Synthesizer (Protein Technologies, Inc., Tucson, AZ). Peptides were purified by standard high performance liquid chromatography methods using Agilent 1200 series (Agilent Technologies, Santa Clara, CA), and molecular weight of peptides was confirmed by matrix assisted laser desorption/ionization (MALDI; Kratos, Chestnut Ridge, NY) as previously described (La Rosa et al., 2002). The complete SIVmac239 Gag and Pol (15-mer) peptides sets were obtained from the NIH AIDS Research and Reference Reagent Program.

Statistical analysis

Results of challenge studies shown in Fig. 6B were analyzed using the Wilcoxon rank-sum test. All *P*-values were calculated as two sided, with significance defined as $P<0.05$. The statistical package Systat 12™ (Cranes Software Int. Ltd) was used for all calculations.

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