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Screening and confirmatory testing of MHC class I alleles in pig-tailed macaques

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Abstract Pig-tailed macaques (*Macaca nemestrina*) are a commonly studied primate model of human AIDS. The *Mane-A1*084:01* MHC class I allele (previously named *Mane-A*10*) is important for the control of SIV infection by CD8+ T cells in this model. Validated methods to detect this allele in large numbers of macaques are lacking. We studied this MHC allele using sequence-specific PCRs in 217 pig-tailed macaques and identified 75 (35%) positive animals. We then performed massively parallel pyrosequencing with a universal 568-bp MHC class I cDNA-PCR amplicon for 50 of these 75 macaques. All 50 animals expressed *Mane-A1*084:01* or closely related variants of the *Mane-A1*084* lineage. *Mane-A1*084* transcripts accounted for an average of 20.9% of all class I sequences identified per animal. SIV infection of a subset of these macaques resulted in the induction of SIV-specific CD8+ T cell responses detected by Mane-A1*084:01 tetramers. An average of 19 distinct class I transcripts were identified per animal by pyrosequencing. This analysis revealed 89 new *Mane* class I sequences as well as 32 previously described sequences that were extended with the longer amplicons employed in the current study. In addition, multiple *Mane* class I haplotypes that had been inferred previously based on shared transcript profiles between unrelated animals were confirmed for a subset of animals where pedigree information was available. We conclude that sequence-specific PCR is useful to screen pig-tailed macaques for *Mane-A1*084:01*, although pyrosequencing permits a much broader identification of the repertoire of MHC class I sequences and haplotypes expressed by individual animals.

Keywords Pig-tailed macaques · MHC class I · Pyrosequencing · CD8+ T cell · *Mane-A1*084:01* · Cytotoxic T lymphocyte (CTL)

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Introduction

The development of an effective vaccine against HIV is an urgent global priority. Critical to these efforts will be a better understanding of animal models of protective immunity. Particular MHC class I alleles are associated with slower progression to disease in both humans and chimpanzees and multiple macaque models (Altfeld et al. 2006; de Groot et al. 2010). CD8+ T cell responses to conserved regions of Gag generally result in a large reduction in viral replicative capacity if they undergo mutational escape (Altfeld and Allen 2006).

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Several macaque models are widely studied in HIV vaccine research, including rhesus (*Macaca mulatta*), cynomolgus (*Macaca fascicularis*) and pig-tailed macaques (*Macaca nemestrina*). There is, however, a limited supply of Indian rhesus macaques, the most well-studied model, particularly those subsets of Indian rhesus macaques with defined protective class I alleles (Cohen 2000). Cynomolgus macaques are also widely used, but most SIV strains are less pathogenic in this species (Reimann et al. 2005). Pig-tailed macaques have become an increasingly important model of SIV/SHIV pathogenesis that is suitable for vaccine studies (Batten et al. 2006; Dale et al. 2004; Kent et al. 2001). Infection of pig-tailed macaques with SIV results in damage to the intestinal epithelia and high levels of immune activation (Alcantara et al. 2009; Klatt et al. 2010; Kuwata et al. 2009). Recent work demonstrating robust replication of minimally modified HIV-1 derivatives containing only SIV-derived *Vif* sequences has stimulated additional interest in the use of pig-tailed macaques as an AIDS model (Hatziioannou et al. 2009).

We have studied in depth the pig-tailed MHC class I allele *Mane-A*10*, recently renamed *Mane-A1*084:01* (O'Leary et al. 2009). This class I allele presents the SIV Gag CTL epitope KP9 and is associated with delayed progressive SIV disease (Smith et al. 2005a). The KP9 epitope is the homologue of the HLA-B*57-restricted CTL epitope KF11, which is associated with delayed HIV-1 disease (Crawford et al. 2007; Dinges et al. 2010). The KP9 epitope typically undergoes immune escape with a mutation at position 2 within the epitope (Fernandez et al. 2005; Loh et al. 2008). This mutation reverts back to wild type rapidly when passaged to *Mane-A1*084:01*-negative animals, suggesting the mutation incurs a significant fitness cost (Fernandez et al. 2005). Our group has developed *Mane-A1*084:01* tetramer reagents that allow us to study KP9-specific CD8+ T cells in great detail to elucidate their impact on SIV disease. In addition, we recently defined two *Mane-A1*084:01*-restricted CD8+ T cell responses against epitopes termed KSA10 and KVA10 in the SIV Tat protein that allows us to study multiple CTL responses within individual *Mane-A1*084:01*-positive animals (Mason et al. 2009). Further, we have developed real-time PCR assays to accurately quantify escape mutants in the KP9 epitope (Loh et al. 2008).

These are powerful tools to study protective immunity to SIV but rely on robust MHC typing of animals. In 2005, we identified a series of three sets of PCRs with sequence-specific primers (PCR-SSP) to amplify *Mane-A1*084:01* by focussing on sequences known at that time to be unique for the *Mane-A1*084:01* allele (Smith et al. 2005b). The PCR-SSP technique is relatively simple and inexpensive PCRs and can be readily performed in most laboratories. We evaluated these three sets of primers in a small number of pig-tailed macaques in our 2005 study (Smith et al. 2005b). Although they correctly identified *Mane-A1*084:01* in well-characterized animals, only a limited number of *Mane* class I alleles had been identified in pig-tailed macaques at that time. It is possible that other related (but at that time unknown) class I alleles could also be amplified with these PCR-SSP reactions. In recent years, there has been an explosion of new pig-tailed macaque class I sequences identified by cDNA cloning and sequencing as well as pyrosequencing efforts (O'Leary et al. 2009; Wiseman et al. 2009). The latest advances in pyrosequencing technologies allow even longer transcripts to be studied, which should further improve the accuracy of this approach for MHC typing (Budde et al. 2010). Here, we evaluate our original PCR-SSP reactions compared to pyrosequencing with Roche/454 Titanium technology for the identification of *Mane-A1*084:01* and additional pig-tailed macaque class I sequences.

Methods

Animals

The majority of pig-tailed macaques (186) screened for *Mane-A1*084:01* were housed at the Primate Research Centre in Bogor, Indonesia (Pamungkas et al. 2005), whilst the remaining 31 macaques were obtained from the Australian National Macaque Facility (Monash University, Churchill, Australia). The macaques were free from HIV-1/SIV/simian retrovirus infection and anaesthetised with ketamine (10 mg/kg, intramuscular) prior to the procedures. All experiments were approved by the relevant Animal Experimentation and Ethics Committees. The animals studied were all different to those we have previously reported (O'Leary et al. 2009; Pratt et al. 2006).

PCR-sequence-specific primer assay

The design of three PCR-SSP assays for *Mane-A1*084:01* has previously been described (Smith et al. 2005b). Briefly, regions of *Mane-A1*084:01* which did not share exact sequence homology with other pig-tailed macaque class I alleles known in 2005 were identified, and three sets of primers were designed to amplify fragments of 161, 134 and 174 bp, respectively (Smith et al. 2005b). First strand cDNA (SuperScript III reverse transcriptase, Invitrogen) was prepared from total RNA extracted (RNeasy mini kit, Qiagen) from macaque peripheral blood or PBMC. PCR amplification of 0.9 μl of cDNA was carried out with 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) in a 15-μl total reaction containing 1× PCR buffer (Applied Biosystems), 1.5 mM $MgCl_2$ (Applied Biosystems), 0.2 mM

dNTPs (Applied Biosystems) and 0.8 μM of each sequence-specific forward and reverse primers. The primers used were: SSP1 forward 5′-GGCCAACACACA-GACCTACCGAGAGAG-3′, SSP1 reverse 5′-CCCTGCCGTCGTAGGCGTACTGGCTATAT-3′, SSP2 forward 5′-CGGGTCTCACACCTTCCAGAGGATGTAT-3′, SSP2 reverse 5′-CGGTCCAGGAACGCAGGTCCC-3′, SSP3 forward: 5′-GGCGCCTCCTCCGCGGATATAG-3′, SSP3 reverse: 5′-GGCACTCGCCCTCCACGTAGGT-3′. The amplification conditions were: 95°C for 10 min, followed by 30 cycles of 94°C for 20 s and 72°C for 40 s, with a final extension at 72°C for 10 min. Amplicons (5 μl) were visualised on 2.0% TAE agarose gels and sized against a 100-bp DNA ladder (Invitrogen).

Pyrosequencing of class I alleles

Massively parallel pyrosequencing for high-resolution class I genotyping was carried out essentially as previously described (O'Leary et al. 2009; Wiseman et al. 2009). The key difference between this and previous studies was that we utilized a new 568-bp cDNA-PCR amplicon that allowed us to take advantage of recent advances in Roche/454 Titanium pyrosequencing chemistry that routinely yields read length in excess of 400 bp. Each cDNA-PCR primer contained a Roche/454 GS FLX Titanium (Lib-A) adaptor A or B followed by a 10-bp multiplex identifier (MID) or molecular bar code and a highly conserved class I sequence that allows universal amplification of macaque class I sequences. The following class I-specific sequences were included in each pair of primers: SBT568F, 5′-GTGGGCTACGTGGACGAC and SBT568R, 5′-TGATCTCCGCAGGGTAGAAG. Incorporation of distinct MIDs for each animal allowed the pooling of primary cDNA-PCR amplicons from 12 to 14 individuals for emulsion PCR and bidirectional pyrosequencing in 1/16th regions of a 70×75 PicoTiterPlate on a GS FLX instrument (Roche/454 Life Sciences). After image processing, the resulting data were binned by MID and analysed by assembling sequencing reads from each animal into unidirectional contigs with 100% identity using SeqMan Pro software (DNASTAR, Madison, WI). This process typically condensed >1,000 individual reads per animal down to ~150 consensus sequences for BLAST analysis against an in-house database of known/novel pig-tailed *Mane* class I sequences. Sequence contigs that appeared to have putative frameshifts relative to known MHC class I sequences were filtered out of this analysis to avoid insertion/deletion artefacts that commonly arise in homopolymer sequences during pyrosequencing (Wiseman et al. 2009).

For a subset of the novel class I transcripts identified with the 568-bp genotyping amplicon described above, additional sequences were obtained by pyrosequencing of independent pig-tailed macaque cohorts using the overlapping amplicon strategy recently described by our group (Budde et al. 2010). In total, an additional 5′ sequences that included putative start codons were obtained for 51 of 121 *Mane* class I sequences described in this report. Likewise, 11 of these sequences were extended towards the 3′ end and cover the complete class I coding region in most cases. All cDNA sequences obtained here have been deposited in GenBank. Class I sequences containing complete exons 2 and 3 sequence have been submitted to the Immuno Polymorphism Database to obtain official nomenclature designations (Robinson et al. 2010). Transcripts limited to the 568-bp amplicon have been assigned working allele names based on homology to previously described macaque class I sequences.

SIV_{mac251} infection and Mane-A1*084:01 tetramer studies

To confirm that *Mane-A1*084:01* present in the macaques was functional and presented SIV-specific CD8+ T cell epitopes, we infected a subset of the pig-tailed macaques with SIV_{mac251} and assessed peripheral blood for the presence of CD8+ T cells capable of binding *Mane-A1*084:01* tetramers folded around either the KP9, KVA10 or KSA10 CD8+ T cell epitopes (Mason et al. 2009; Smith et al. 2005b). SIV_{mac251} infection was performed intravaginally by atraumatic inoculation as previously described with a stock kindly provided by Drs. R. Pal and N. Miller through the NIH AIDS Research and Reference Reagent Repository (Kent et al. 2005). Infection was confirmed by the presence of SIV_{mac251} viremia by real-time PCR as previously described (Dale et al. 2002). Assessment of the Mane-A1*084:01 tetramers folded around the KP9, KVA10 or KSA10 epitopes was performed as previously described (Mason et al. 2009). Briefly, 200 μl fresh whole blood was stained with a 1:400 dilution of the *Mane-A1*084:01* tetramers for 45 min at room temperature in the dark. Counter-staining with anti-CD3-AF700 (BD Biosciences, San Jose, CA; clone SP34-2), anti-CD4-PECy7 (BD, clone SK3) and anti-CD8-APC-H7 (BD, clone SK1) was then conducted at room temperature in the dark for 45 min. Erythrocytes were lysed with 2 ml 1× FACS Lysing Solution (BD) for 10 min at room temperature incubation and washed twice in FACS buffer. Acquisition and analyses were performed as previously described (Mason et al. 2009).

Results

PCR-sequence-specific primer typing of 217 pig-tailed macaques for Mane-A1*084:01

Pig-tailed macaques expressing the *Mane-A1*084:01* allele are particularly useful for SIV vaccine and pathogenesis studies as there are defined immunology reagents available,

and reliable patterns of immune escape are observed. Robust screening techniques are needed to identify these animals, but previously developed PCR-SSP studies have only been validated in very limited numbers of animals (Smith et al. 2005b). We extracted RNA from peripheral blood samples obtained from 217 unselected pig-tailed macaques and performed PCR-SSP reactions using three separate primer pairs designed to be relatively specific for *Mane-A1*084:01*. Two hundred and three macaques were tested with all three reactions, and 14 macaques were tested with only two reactions—these 14 macaques had negative results in both PCR-SSP1 and PCR-SSP3 reactions.

Seventy-five macaques (34.6%) were positive for *Mane-A1*084:01* by all three PCR-SSP reactions (Table 1). This level of *Mane-A1*084:01* prevalence is similar to our previous studies using alternate typing technologies on smaller numbers of unselected pig-tailed macaques, which found a prevalence of 32.1% in 109 macaques using reference strand conformational analysis typing (Pratt et al. 2006) and 37.5% in 24 macaques tested using a 367-bp pyrosequencing amplicon (O'Leary et al. 2009). Each individual PCR-SSP reaction had much higher proportions of animals responding; 50% for PCR-SSP1, 76% for PCR-SSP2 and 45% for PCR-SSP3. The PCR-SSP2 reaction was least discriminatory, identifying only 24% of macaques as negative.

Class I genotyping by massively parallel pyrosequencing

There is a growing interest in the identification of all class I transcripts within macaque cohorts to assess the protective or detrimental effects of multiple class I sequences in response to infections such as SIV. Our group pioneered the pyrosequencing of cDNA amplicons for macaque class I genotyping, initially using the GS FLX platform developed by Roche/454 Life Sciences (O'Leary et al. 2009; Wiseman et al. 2009). The introduction of long-read GS FLX Titanium chemistry allowed us to replace the 190- and 367-bp amplicons described previously with a new 568-bp amplicon that extends between exons 2 and 4. This longer class I amplicon covers essentially the entire highly polymorphic peptide-binding domain of class I gene products and provides a significantly higher resolution of sequence variants than was possible previously. We randomly selected 50 of the 75 pig-tailed macaques positive by all three PCR-SSP reactions for pyrosequencing analyses. In this study, we identified an average of 1,052 sequence reads per animal that corresponded to an average of 19 distinct class I transcripts or closely related variants of allele lineages per animal (Supplemental Fig. 1). Further analysis of the wealth of data obtained here revealed 89 new *Mane* class I sequences as well as 32 previously described sequences that were extended with the longer amplicons employed in the current study (Table 2). The majority of these new sequences represent allelic variants of previously described *Mane* transcripts or class I lineages defined in rhesus and cynomolgus macaques.

As expected, *Mane-A1*084:01*, or a closely related variant, was expressed as a major transcript, accounting for an average of 20.9% of all class I transcripts identified in each of the 50 pig-tailed macaques examined (Supplemental Fig. 1). *Mane-A1*084:01* transcripts cannot be distinguished from *Mane-A1*084:nov:03* or *Mane-A1*084:nov:04* sequences with the 568-bp amplicon employed in this study since these class I sequences differ outside the region evaluated. *Mane-A1*084:01:02* only differs from *Mane-A1*084:01* by a single synonymous nucleotide substitution while *Mane-A1*084:03* contains a conservative Ala18Val substitution and a pair of synonymous nucleotide substitutions in the signal peptide. Two animals expressed transcripts for *Mane-A1*084:nov:05*; this allele differs from *Mane-A1*084:01* by a Ser101Asn substitution in the alpha 1 domain encoded by exon 2. Macaque C3754 was heterozygous for *Mane-A1*084:01* and *Mane-A1*084:nov:05*, while macaque B0517 lacked *Mane-A1*084:01* but expressed *Mane-A1*084:nov:05 I* and *Mane-A1*061:nov:01* (Supplemental Fig. 1). Ideally, this latter animal should have been excluded from this cohort by the PCR-SSP1 assay due to a single nucleotide mismatch with the terminal 3′ nucleotide of the SSP1 forward primer. Finally, a third animal was found to be heterozygous for *Mane-A1*084:01* and *Mane-A1*084:04*; this latter allele differs from *Mane-A1*084:01* by six amino acid substitutions in the peptide-binding domain.

Since each of these pig-tailed macaques shared *Mane-A1*084:01* or a closely related variant, we next asked if class I alleles that are commonly co-inherited with this allele could be identified by pyrosequencing. Partial pedigree information was available for a subset of the animals examined; at least three pairs of full sibs and two

Table 1 PCR-SSP screening of pig-tailed macaques for *Mane-A1*084:01*

PCR:	SSP1	SSP2[a]	SSP3	SSP1 + SSP2	SSP1 + SSP3	SSP2 + SSP3	All 3 +
# positive/	109/217	154/203	98/217	97/203	80/217	91/203	75/217
%	50.2	75.9	45.2	47.8	36.9	44.8	34.6

[a] PCR-SSP2 reaction was run in 171 of the 186 samples; the 15 other samples were negative in both PCR-SSP1 and PCR-SSP3 reactions

Animal ID	18869	19341	19351	19340
Total Reads Identified	1565	905	1572	1651
# Alleles Identified	20	23	20	18
Paternal Haplotype	9191a	9191a	9191a	9191a
Maternal Haplotype	8869a	9341a	9351a	9340a
Mane-A1				
*A1*004:01*	11.2			
*A1*006:nov:01*		15.1		
*A1*031:01*			14.4	
*A1*038:nov:01*				3.5
*A1*084:01;A1*084:01:02;A1*084:03*	14.2	13.7	13.5	15.9
*A1*108:nov:01*				0.7
Mane-A2				
*A2*05:15*		0.9		
*A2*05g*			0.1	
Mane-A4				
*A4*01:01:01;A4*01:01:02;A4*14:03*				1.0
*A4*14g*			0.8	
Mane-A6				
*A6*01:02*	0.1	0.6	0.4	0.3
Mane-E				
*E*01:01;E*01:02*				10.5
*E*01:nov:03;E*02:01;E*02:02*	1.9		0.9	
*E*02:01;E*02:02*		0.9		
*E*02:nov:01*	0.5	0.4	0.4	0.7
*E*03:01*	28.1	26.4	31.8	25.9
*E*05:nov:03*			0.3	
Mane-B				
*B*017:nov:02*	6.5			
*B*021:01*		2.7		
*B*027:03:01;B*027:03:02*	2.1	1.7	3.4	5.8
*B*028:01*		6.0		
*B*030:03*	3.1	2.1	2.7	1.9
*B*047:01*			2.5	
*B*056:nov:02*	7.3			
*B*060:02*	0.7			
*B*061:02*		2.1		
*B*068:02:02*			4.2	
*B*068:02:03*				3.8
*B*068:05*		2.0		
*B*079:01;B*079:05;B*079:07*		0.2		
*B*082:01;B*082:02;B*082:03*	0.6			
*B*088:01*		0.3		
*B*088:02*	1.1	2.9	1.7	2.1
*B*091:01;B*091:02*				5.2
*B*098:01*		0.4		
*B*103:01*	5.1	7.4	7.1	5.1
*B*112:01*			2.9	
*B*113:01*				1.0
*B*118:01;B*118:02;B*118:03*	9.4	6.0	7.3	11.4
*B*122:01*	5.3	2.1	2.9	4.2
*B*124:01*		3.6		
*B*162:01*	0.3		0.1	
*B11L*01:nov:01*	0.1			
Mane-I				
*I*01:04*	1.5	1.3	1.1	1.0
*I*01g*	0.7	1.2	1.2	

Fig. 1 Representative class I genotypes for pig-tailed macaque half sibs determined by pyrosequencing. High-resolution class I genotypes are illustrated for four offspring derived from sire 9191. The relative abundance of each class I transcript is presented as a percentage of the total sequence reads identified per animal. *Black boxes* highlight class I transcripts that were expressed by all four offspring and constitute the paternal haplotype inherited from sire 9191. This haplotype includes one of three closely related members of the *Mane-A1*084* allele lineage that are ambiguous due to sequence identity within the 568-bp region examined. Transcripts unique to each offspring (highlighted with *dots* or *shades of grey*) are inferred to reflect the distinct maternal haplotypes that were inherited from the respective dams

independent groups of half sibs that shared a common sire or dam were available for this analysis (Supplemental Fig. 1). Class I genotypes for four such half sibs that were derived from sire 9191 and four independent dams are illustrated in Fig. 1. In this example, each of the offspring inherited a common *Mane-A1*084:01* haplotype from 9191 (sequences highlighted in black boxes). In addition to *Mane-A1*084:01*, this haplotype includes major transcripts for *Mane-E*03:01*, six distinct *Mane-B* sequences and *Mane-I*01:04*, as well as minor transcripts for *Mane-A6*01:02* and *Mane-E*02:nov:01*. Cosegregation of these *Mane-B* sequences from 9191 was observed previously in multiple unrelated pig-tailed macaques from two independent cohorts and designated as haplotype Pt4b (O'Leary et al. 2009). The remaining class I sequences in each of these offspring can be inferred to reflect the maternal haplotype that was inherited from the respective dams (Fig. 1). The maternal haplotypes observed in macaques 19341 and 19351 were also observed previously and designated as Pt11 and Pt12, respectively (O'Leary et al. 2009). Animal 19340 displayed a novel class I haplotype while the maternal Mane-B haplotype containing the *Mane-B*017:nov:02* transcript carried by macaque 18869 was shared with another animal in this cohort (see macaque 7448 in Supplemental Fig. 1).

In contrast to this closely related group of siblings, there is a remarkable amount of diversity in the patterns of class I transcripts when this cohort of *Mane-A1*084:01*-positive animals is considered as a whole (Supplemental Fig. 1). Overall, expression of more than 200 distinct class I transcripts or closely related allele lineages was detected by pyrosequencing. Surprisingly, we were unable to define a common Mane-A haplotype across this cohort as would be expected from analogous studies of cynomolgus and rhesus macaques (Wiseman et al. 2009; Budde et al. 2010; Doxiadis et al. 2010). Although the *Mane-A6*01:02* transcript clearly segregates with the *Mane-A1*084:01* haplotype in certain animals (Fig. 1), transcripts of the *Mane-A6*01* lineage were only detected in five of the remaining 46 animals in this cohort. Likewise, members of the *Mane-A3* and/or *Mane-A4* lineages were detected in nearly all animals, but ten or more distinct transcripts were observed (Supplemental Fig. 1). Similar observations extend to linkage of *Mane-A1*084:01* with specific *Mane-B* haplotypes. Eighteen of the 50 animals (36%)

Table 2 Novel *Mane* class I sequences identified by pyrosequencing

Class I sequence	Length (bp)[a]	GenBank ID	Immuno polymorphism database ID	Representative animal(s)
Mane-A1*003:nov:01	530	HQ609857		25377, 26359
*Mane-A1*006:03*	693	HQ609862	70007029	7448, C3749
Mane-A1*006:nov:01	530	HQ609858		19341, C3752
Mane-A1*010:nov:02	530	HQ609859		B0527
Mane-A1*010:nov:03	530	HQ609860		B0399
Mane-A1*010:nov:04	530	HQ609861		3364
Mane-A1*038:nov:01	530	HQ609873		19340
Mane-A1*047:nov:01	530	HQ609863		B0547, C3765
Mane-A1*053:nov:01	530	HQ609864		5259, 2467
Mane-A1*061:nov:01	530	GQ274881		B0517, 36121
Mane-A1*063:nov:01	530	GQ274882		B0517
Mane-A1*063:nov:02	530	HQ609865		C3770
Mane-A1*063:nov:03	530	HQ609866		26301, C3763
Mane-A1*066:nov:01	530	HQ609867		B0443
Mane-A1*072:nov:01	530	HQ609868		C3767
*Mane-A1*084:01:02*	1,108	HQ609869	70006957	PT048
*Mane-A1*084:03*	692	HQ609870	70007030	PT019
*Mane-A1*084:04*	1,108	GQ281746	70007068	B0519
Mane-A1*084:nov:05	530	HQ609871		C3754, B0517
Mane-A1*108:nov:01	530	GQ153457		19340
Mane-A1*114:nov:01	530	HQ609872		35377, 45418
Mane-A2*01:nov:01	530	HQ609874		B0547, C3765
Mane-A3*13:nov:01	530	GQ153453		45610, 36121
Mane-A3*13:nov:03	530	GQ281748		19530, B0526
Mane-A3*13:nov:05	530	HQ609875		PT050
Mane-A3*13:nov:07	530	HQ609876		1442, 1024
*Mane-A4*01:01:01*	692	HQ609877	70007031	PT046
*Mane-A4*01:01:02*	693	HQ609880	70007033	PT052
*Mane-A4*01:02*	692	HQ609878	70007032	5878
*Mane-A4*01:03*	549	HQ609879	70007075	C3752, C3765
Mane-A4*14:nov:01	526	GQ153459		PT052
Mane-A4*14:nov:06	530	HQ609881		B0399, B0547
*Mane-A5*30:01*	1,074	GQ153514	70007076	5878
*Mane-A6*01:02*	693	HQ609882	70007034	C3751, 18869
Mane-A6*01:nov:02	530	HQ609883		C3767
*Mane-B*004:01*	692	GQ281753	70007035	5259, B0443
Mane-B*004:nov:02	530	HQ609884		C0933
Mane-B*008:nov:01	530	HQ609885		45418, 35377
*Mane-B*017:04*	691	GQ281756	70007036	PT050
Mane-B*017:nov:02	530	HQ609886		7448, 18869
Mane-B*019:nov:01	530	HQ609887		5259, 2467
*Mane-B*027:03:01*	1,106	GQ153499	70007069	PT046
*Mane-B*027:03:02*	1,106	HQ609888	70007070	PT048
Mane-B*027:nov:03	530	HQ609889		C3770, C3765
Mane-B*030:nov:02	530	HQ609890		C3770, C3765
Mane-B*031:nov:01	530	HQ609891		16570, B0429
*Mane-B*039:02*	1,107	GQ281754	70007071	26783
Mane-B*041:nov:01	530	HQ609892		3364
Mane-B*044:nov:01	530	HQ609893		7448, B0527

Table 2 (continued)

Class I sequence	Length (bp)[a]	GenBank ID	Immuno polymorphism database ID	Representative animal(s)
*Mane-B*045:02*	691	GQ153500	70007037	25377, C3751
*Mane-B*046:01:01*	690	GQ153469	70007038	PT044
*Mane-B*046:01:02*	691	HQ609894	70007039	PT044
*Mane-B*051:02:02*	691	HQ609895	70007043	PT046
*Mane-B*051:03*	574	GQ153487	70007040	PT048
*Mane-B*051:04:01*	693	GQ153501	70007041	PT048
*Mane-B*051:04:02*	690	GQ153504	70007042	PT047
*Mane-B*051:04:03*	578	HQ609896	70007044	PT053
*Mane-B*054:02*	690	HQ609897	70007045	PT048
Mane-B*056:nov:01	530	HQ609898		3364
Mane-B*056:nov:02	530	HQ609899		B0519, 18869
Mane-B*057:nov:02	530	HQ609900		5259, C3763
Mane-B*058:nov:01	530	HQ609901		B0399
*Mane-B*060:02*	691	HQ609902	70007046	PT049
Mane-B*060:nov:01	530	GQ153503		C3751, 45610
Mane-B*060:nov:02	530	GQ274888		C0942
*Mane-B*068:02:02*	691	GQ153483	70007047	PT010
*Mane-B*068:02:03*	692	HQ609904	70007050	PT053
*Mane-B*068:05*	976	GQ153510	70007048	PT047
*Mane-B*068:06*	692	HQ609903	70007049	PT049
*Mane-B*068:07*	692	HQ609905	70007051	PT020
Mane-B*068:nov:03	530	GQ274889		5259, 1024
Mane-B*068:nov:07	530	HQ609906		C0942
Mane-B*069:nov:01	530	HQ609907		B0517, B0547
*Mane-B*072:02:02*	691	HQ609910	70007053	PT048
*Mane-B*072:04:01*	691	HQ609909	70007052	PT047
*Mane-B*072:04:02*	1,107	HQ609908	70007072	PT044
*Mane-B*072:05*	692	HQ609911	70007054	PT053
*Mane-B*078:03*	692	HQ609912	70007056	PT055
Mane-B*078:nov:07	530	HQ609913		C0933, 45610
*Mane-B*079:02:02*	691	HQ609915	70007058	PT050
*Mane-B*079:05*	691	HQ609916	70007059	PT053
*Mane-B*079:07*	690	HQ609914	70007057	PT044
Mane-B*079:nov:06	530	HQ609917		B0399
Mane-B*080:nov:01	530	HQ609918		C0933
Mane-B*081:nov:01	530	HQ609919		B0547, C3765
*Mane-B*088:02*	692	GQ153502	70007062	PT045
*Mane-B*088:03*	691	GQ153488	70007060	PT048
*Mane-B*088:04*	692	HQ609920	70007061	PT050
*Mane-B*089:03*	692	HQ609921	70007063	PT049
*Mane-B*089:04*	692	HQ609922	70007064	PT020
Mane-B*089:nov:03	530	HQ609923		1442, 1024
Mane-B*089:nov:04	530	HQ609924		26301, C3763
*Mane-B*091:02*	692	HQ609925	70007065	PT053
Mane-B*099:nov:01	530	HQ609926		B0399
Mane-B*104:nov:02	530	HQ609927		25377, C3752
*Mane-B*108:02*	1,105	HQ609928	70007073	PT048
Mane-B*111:nov:03	530	HQ609929		7448
*Mane-B*112:02*	692	HQ609930	70007066	PT049

Table 2 (continued)

Class I sequence	Length (bp)[a]	GenBank ID	Immuno polymorphism database ID	Representative animal(s)
Mane-B*118:nov:01	530	HQ609931		36271, C3751
Mane-B*120:nov:01	530	HQ609932		5259, 2467
Mane-B*120:nov:02	530	HQ609933		C0942
Mane-B*122:nov:01	530	HQ609934		19530
*Mane-B*123:01:02*	1,107	HQ609935	70007074	PT046
Mane-B*125:nov:01	530	HQ609936		35377, 45418
*Mane-B*144:01*	1,074	GQ153464	70007077	PT044
Mane-B*150:nov:01	530	HQ609937		5259
Mane-B*150:nov:02	530	HQ609938		2467, 1442
*Mane-B*162:01*	691	GQ153507	70007067	PT044
*Mane-B*171:01*	692	GQ153508	70007055	PT050
Mane-B11L*01:nov:01	530	GQ153473		B0508
Mane-E*01:nov:01	530	HQ609939		B0517, C3752
Mane-E*01:nov:03	530	HQ609940		5259, 1442
Mane-E*01:nov:08	530	HQ609941		B0429, B0527
Mane-E*02:nov:01	530	GQ153521		18869, 19340
Mane-E*02:nov:02	530	GQ153524		45610, 36121
Mane-E*02:nov:03	530	HQ609942		5259, 26359
Mane-E*02:nov:04	530	HQ609943		C0942
Mane-E*05:nov:01	530	HQ609944		C0933
Mane-E*05:nov:03	530	GQ153526		26783, 26359
Mane-E*05:nov:07	530	HQ609945		5259
Mane-I*01:nov:01	530	GQ153527		25377, C3752

Alleles in italicized font fulfilled criteria for official allele designations, and these were obtained from the Immuno Polymorphism Database

[a] Sequences greater than 530 bp in length were obtained by pyrosequencing of independent pig-tailed macaque cohorts using the overlapping amplicon strategy recently described by (Budde et al. 2010)

examined here appear to carry a PT4b-like *Mane-B* haplotype (Supplemental Fig. 1). However, variants of the Pt4b haplotype appear to be quite common ancestral *Mane-B* haplotypes; 11 of 36 unrelated animals (30%) examined by pyrosequencing previously also carried these haplotypes despite the fact that only five also expressed *Mane-A1*084:01* transcripts (Wiseman, et al. 2009; O'Leary et al. 2009).

Mane-A1*084:01 tetramer analyses for SIV-specific CD8+ T cell responses

One of our goals for identifying *Mane-A1*084:01* is to be able to study in detail Mane-A1*084:01-restricted CD8+ T cell responses. We have developed Mane-A1*084:01 tetramers folded around three separate SIV epitopes, termed KP9 (in SIV Gag), KSA10 and KVA10 (both in SIV Tat) (Mason et al. 2009). We recently infected 17 the 50 Mane-A1*084:01-positive pig-tailed macaques with SIV_{mac251} and studied peripheral blood samples prior to and after infection for CD8+ T cells capable of binding the labelled Mane-A1*084:01 tetramer molecules by flow cytometry (Fig. 2). The macaques studied became infected with SIV as evidenced by SIV RNA detected in the plasma (not shown). All but one (animal B0517) developed readily detectable CD8+ T cell responses to the three SIV epitopes. Animal B0517 generated responses to the two Tat epitopes, but not to the KP9 Gag epitope. Interestingly, this animal expressed the variant allele *Mane-A1*084:nov:05* that contains a Ser101Asn over the region where sequences are available. This non-conservative substitution alters a key residue in the F pocket of the MHC class I peptide-binding domain (Loffredo et al. 2009) and could potentially affect the spectrum of epitopes presented these gene products. Studies with additional pig-tailed macaques expressing *Mane-A1*084:nov:05* will be required to further assess this.

In general, tetramer responses to KP9 and KVA10 were more generally robust than to KSA10, which is consistent with our previous observation on the early evolution of KP9 and KVA10 CD8+ T cell responses and the later development of KSA10 CD8+ T cell responses following SIV infection in most animals (Mason et al. 2009). Taken together, the presence of Mane-A1*084:01-restricted SIV-

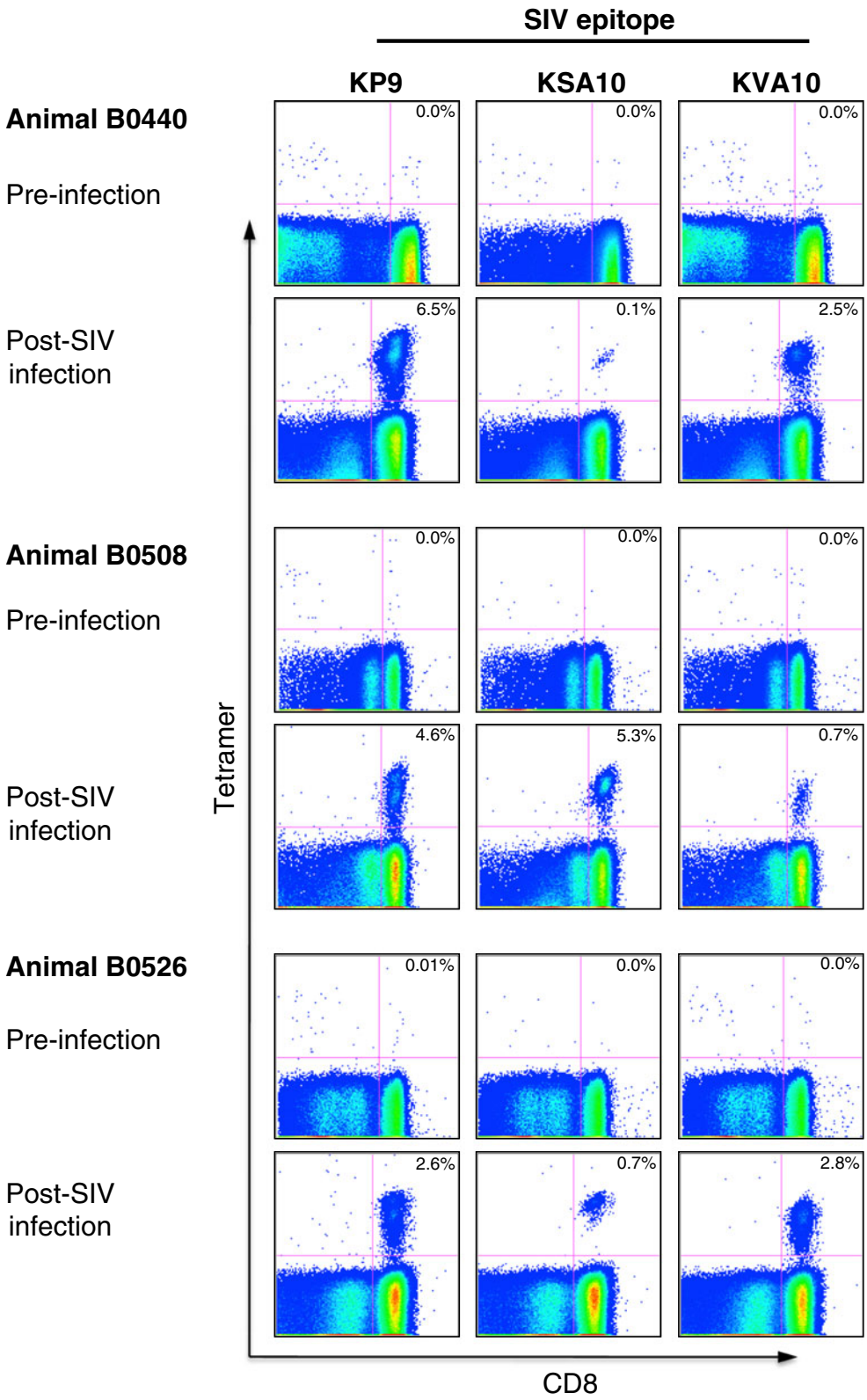

Fig. 2 Mane-A1*084:01-positive macaques generate SIV specific CD8+ T cell responses. Three macaques were infected with SIV_{mac251} and Mane-A1*084:01-restricted CD8+ T cell responses to KP9 (in SIV Gag), KVA10 and KSA10 (both in SIV Tat) studied by Mane-A1*084:01 tetramers before and 3–5 weeks after SIV_{mac251} infection by flow cytometry. The proportion of CD8+ T cells binding the tetramers is shown on each *plot*

specific CD8+ T cell responses confirms the functional expression of *Mane-A1*084:01* as predicted by the class I genotyping using PCR-SSP and pyrosequencing in the subset of macaques studied here.

Conclusions

In this study, we typed 217 pig-tailed macaques for *Mane-A1*084:01* by three PCR-SSP reactions and found an allele

prevalence of 34.6%. This allele frequency accords with MHC typing studies using alternate technology (Pratt et al. 2006). We also performed high-resolution pyrosequencing for class I transcripts in 50 of the 75 positive macaques and detected *Mane-A1*084:01* or closely related *Mane-A1*084* variants in all 50 macaques. Further, a subset of 17 of these 50 macaques have now been infected with SIV_{mac251}, and all but one of the macaques infected to date have mounted SIV-specific CD8+ T cell responses to both Gag and Tat epitopes restricted by Mane-A1*084:01 that are detectable by tetramer assays. Our results suggest these PCR-SSP reactions are a reliable screen for the presence of *Mane-A1*084:01* in pig-tailed macaques that are applicable to a wide range of laboratory settings. Refining the PCR-SSP screening assays, for example starting with the more reliable SSP1 and SSP3 reactions, should lead to further applicability of these assays.

High-resolution pyrosequencing analysis of these 50 pig-tailed macaques provided a wealth of information on new *Mane* class I sequences as well as combinations of alleles that are likely to be co-inherited as haplotypes. One unexpected finding of this pyrosequencing analysis was the diverse range of class I sequences observed in this cohort of pig-tailed macaques despite the fact that all animals had been selected to express *Mane-A1*084:01* or one of several very closely related members of this allele lineage. Although more than a third of the animals examined expressed *Mane-B* transcripts associated with Pt4b haplotypes (*Mane-B*027:03:01/02*, *Mane-B*030:03*, *Mane-B*118:01/02/03* and *Mane-B*122:01*), this ancestral haplotype is also quite common in unselected pig-tailed macaques that are *Mane-A1*084:01* negative (O'Leary et al. 2009; Wiseman et al. 2009). Our observations suggest that CD8+ T cell responses common to multiple *Mane-A1*084:01*-positive macaques are most likely to be restricted by *Mane-A1*084:01* rather than another of the multitude of class I sequences expressed by these animals.

Interestingly, three additional *Mane-B* sequences were identified by pyrosequencing in this study (Table 2) that are variants of the *Mamu-B*008* and *Mamu-B*017* allele lineages which have been associated with superior control of SIV replication in rhesus macaques (Loffredo et al. 2009; Yant et al. 2006). This brings the current totals to five members of the *Mane-B*017* lineage as well as a pair of *Mane-B*008*-like sequences. The pyrosequencing approach described here will allow investigators to prospectively screen pig-tailed macaques and select cohorts of animals expressing these specific class I sequences to rigorously assess their ability to control SIV replication. Given the protective effects observed for the rhesus homologues of these class I sequences, however, it would be prudent for investigators to attempt to balance animals expressing these *Mane* transcripts between vaccine and control groups whenever possible in SIV studies. Given the high degree of conservation of these class I sequences between pig-tailed and rhesus macaques, it may also be possible to make use of panels of *Mamu-B*008* and *Mamu-B*017* tetramer reagents to measure CD8+ T cell responses in SIV-infected pig-tailed macaques that express the *Mane* counterparts of these class I sequences (Friedrich et al. 2007); (Loffredo et al. 2009).

Reliable class I genotyping data make it possible to plan sophisticated immunologic and virologic studies with *Mane-A1*084:01*-positive macaques. For example, we have sorted SIV-specific CD8+ T cells using Mane-A1*084:01 tetramers to study T cell receptor clonotypes and analysed patterns of immune escape at Mane-A1*084:01-restricted CD8+ T cell epitopes (Loh et al. 2008; Smith et al. 2008). Further, we recently identified influenza-specific CD8+ T cells restricted by Mane-A1*084:01 (Sexton et al. 2009), enabling more detailed immunologic studies of influenza immunity in pig-tailed macaque models. Trusted typing technologies are also needed to avoid confounding experimental data through uneven allocation of *Mane-A1*084:01*-positive macaques across vaccine and placebo groups. The ability to genotype pig-tailed macaques reliably for a wide range of class I sequences as well as *Mane-A1*084:01* advances the wider utility of this model for HIV/SIV vaccine research and other applications.

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