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Linking Pig-Tailed Macaque Major Histocompatibility Complex Class I Haplotypes and Cytotoxic T Lymphocyte Escape Mutations in Simian Immunodeficiency Virus Infection

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ABSTRACT

The influence of major histocompatibility complex class I (MHC-I) alleles on human immunodeficiency virus (HIV) diversity in humans has been well characterized at the population level. MHC-I alleles likely affect viral diversity in the simian immunodeficiency virus (SIV)-infected pig-tailed macaque (*Macaca nemestrina*) model, but this is poorly characterized. We studied the evolution of SIV in pig-tailed macaques with a range of MHC-I haplotypes. SIV_{mac251} genomes were amplified from the plasma of 44 pig-tailed macaques infected with SIV_{mac251} at 4 to 10 months after infection and characterized by Illumina deep sequencing. MHC-I typing was performed on cellular RNA using Roche/454 pyrosequencing. MHC-I haplotypes and viral sequence polymorphisms at both individual mutations and groups of mutations spanning 10-amino-acid segments were linked using in-house bioinformatics pipelines, since cytotoxic T lymphocyte (CTL) escape can occur at different amino acids within the same epitope in different animals. The approach successfully identified 6 known CTL escape mutations within 3 Mane-A1*084-restricted epitopes. The approach also identified over 70 new SIV polymorphisms linked to a variety of MHC-I haplotypes. Using functional CD8 T cell assays, we confirmed that one of these associations, a Mane-B028 haplotype-linked mutation in Nef, corresponded to a CTL epitope. We also identified mutations associated with the Mane-B017 haplotype that were previously described to be CTL epitopes restricted by Mamu-B*017:01 in rhesus macaques. This detailed study of pig-tailed macaque MHC-I genetics and SIV polymorphisms will enable a refined level of analysis for future vaccine design and strategies for treatment of HIV infection.

IMPORTANCE

Cytotoxic T lymphocytes select for virus escape mutants of HIV and SIV, and this limits the effectiveness of vaccines and immunotherapies against these viruses. Patterns of immune escape variants are similar in HIV type 1-infected human subjects that share the same MHC-I genes, but this has not been studied for SIV infection of macaques. By studying SIV sequence diversity in 44 MHC-typed SIV-infected pigtail macaques, we defined over 70 sites within SIV where mutations were common in macaques sharing particular MHC-I genes. Further, pigtail macaques sharing nearly identical MHC-I genes with rhesus macaques responded to the same CTL epitope and forced immune escape. This allows many reagents developed to study rhesus macaques to also be used to study pigtail macaques. Overall, our study defines sites of immune escape in SIV in pigtailed macaques, and this enables a more refined level of analysis of future vaccine design and strategies for treatment of HIV infection.

There is a need for a highly effective vaccine or immune-based therapies for human immunodeficiency virus (HIV) infection. The immune pressure on the virus by cytotoxic T lymphocytes (CTLs) can result in reduced virus levels (1) but usually leads to the emergence of escape mutations (2). Some of these mutations exact a fitness cost on the virus (3), which can result in a lowered viral load (VL) in the host (4). However, compensatory mutations that occur together with immune escape mutations are able to at least partially negate the fitness cost resulting from escape mutations (5, 6). Compensatory mutations can become fixed in viral sequences, since returning to the wild type requires multiple simultaneous changes (7). Although studies of HIV polymorphisms in humans have been illuminating and have led to the successful identification of important CTL epitopes (8–10), the infecting virus strain and timing of infection are not always known in humans, and this lack of knowledge complicates analyses. Simian immunodeficiency virus (SIV) infection of pig-tailed macaques (*Macaca nemestrina*) is a very useful model for the study of HIV pathogenesis. Recent studies suggest that SIV-infected pig-tailed macaques have a disease progression that is similar to if not more predictable than that in the more commonly used Indian rhesus macaque (*Macaca mulatta*) (11, 12).

The influence of major histocompatibility complex (MHC) class I (MHC-I) alleles on infection progression is not well char-

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TABLE 1 Details of SIV_{mac251}-infected pig-tailed macaques used in this study[a]

Study	No. of macaques	Vaccine	ART	Challenge[b]	Outcome of vaccine	Reference
1	11	Kunjin SIV	No	SIV_{mac251}, intravenously	No effect	29
2	31	Peptide immunotherapy ($n = 21$) or control ($n = 10$)	Yes[c]	SIV_{mac251}, intravenously	Immunotherapy effective	31
3	2	Influenza virus-SIV	No	SIV_{mac251}, intrarectally	No effect	30

[a] A total of 44 macaques were studied.
[b] The same stock of SIV_{mac251}, supplied by R. Pal and N. Miller, was used for all infections.
[c] The animals received ART from weeks 3 to 10 after infection.

acterized in the SIV-infected pig-tailed macaque model, and few CTL epitopes have been described. The most extensively studied CTL responses in pig-tailed macaques and their escape mutations are KP9 in Gag (13, 14) and KSA10 and KVA10 in Tat (15), all of which are restricted by the Mane-A1*084 allele. A compensatory mutation of the canonical monomorphic KP9 mutation K165R has also been recently identified (5). Immune escape at KP9 occurs early. However, the K165R escape mutation extracts a high fitness cost, and viruses which have escaped at the KP9 epitope rapidly revert to the wild type upon passage to naive Mane-A1*084-negative animals (16–18). Escape at KSA10 occurs more slowly than escape at KP9, while the escape kinetics of KVA10 are similar to those of KP9 (15). Escape mutations at the KVA10 and KSA10 Tat epitopes occur at multiple amino acid positions within the epitope, in contrast to the escape mutations at the KP9 Gag epitope. On the basis of the findings for 8 infected animals, it was reported that Mane-A1*084-positive animals maintain a lower SIV load, although this has not been rigorously tested with larger numbers of animals (13). Additional CTL epitopes in pig-tailed macaques have been described in the Env protein, but the restricting MHC-I alleles have not been identified, and the mutations did not appear to affect the replicative capacity of the virus adversely (19, 20).

Broadly reactive CTL responses against multiple SIV epitopes likely contribute toward the efficient control of viremia in macaques. Broader CTL responses, particularly to Gag, are associated with improved control of HIV-1 (21). Conversely, a vaccine directing CTL responses toward a smaller number of critical epitopes restricted by Mamu-B*008 in Indian rhesus macaques was able to control SIV infection efficiently (22).

There is a need to identify a broader range of SIV-specific CTL epitopes, their MHC restriction, and their role in controlling SIV infection in the pig-tailed macaque model. Incisive human studies have linked HIV-1 mutations with particular human leukocyte antigen (HLA) class I alleles, revealing important clues to CTL immunity, epitope discovery, and the control and evolution of HIV (8, 9, 23–25). Several studies have also linked hepatitis C virus (HCV) immune system-driven adaptations to HLA class I alleles, and elegant studies have estimated the false discovery rates of such linkage analyses (10, 26–28). Linking CTL escape mutations with pig-tailed macaque MHC-I haplotypes both will improve this animal model and could in turn facilitate the design of efficacious CTL vaccines that limit CTL escape. We conducted a large analysis of MHC-I typing and SIV deep sequencing in pig-tailed macaques. We amplified whole virus from plasma samples from 44 pig-tailed macaques challenged with SIV_{mac251} at approximately 15 weeks postinfection using Illumina deep sequencing. The same 44 macaques were MHC-I genotyped using Roche/454 pyrosequencing. The MHC typing and SIV sequencing data sets were analyzed using in-house bioinformatics pipelines to associate CTL escape mutations with specific MHC-I haplotypes. We identified a series of SIV polymorphisms linked to particular MHC-I haplotypes and validated that a subset of these polymorphisms corresponds to CTL escape mutations.

MATERIALS AND METHODS

Animals and SIV infection. Forty-four SIV_{mac251}-infected pig-tailed macaques from three separate trials were studied in total (Table 1). Eleven macaques received a Kunjin virus vector SIV or HIV vaccine that was ineffective in controlling SIV infection (29). Two macaques received an influenza virus that expressed KP9 as a vaccine that was also ineffective (30). Thirty-one macaques were infected with SIV_{mac251} and then received antiretroviral therapy (ART) (tenofovir and emtricitabine from weeks 3 to 10 after infection). Of these 31 macaques, 10 macaques remained unvaccinated, 10 macaques received a peptide-based SIV vaccine based on overlapping SIV Gag peptides, and another 11 macaques were given a peptide-based SIV vaccine based on overlapping peptides spanning all 9 SIV proteins (31). The vaccinations were administered from weeks 4 to 10 after SIV_{mac251} infection. All experiments were conducted in compliance with the ethics approval obtained from the University of Melbourne and the Commonwealth Scientific and Industrial Research Organization (CSIRO) Livestock Industries Animal Ethics Committees. Animals were sedated with 10 mg/kg of body weight of ketamine prior to withdrawing blood from the femoral vein into EDTA- and sodium heparin-coated Vacuette tubes. Macaques were euthanized before developing simian AIDS, as determined by their peripheral $CD4^+$ T cell counts and plasma viral loads. Macaques were infected intrarectally or intravenously with the same stock of SIV_{mac251} (kindly provided by R. Pal and N. Miller through the NIH AIDS Research and Reference Reagent Repository), as shown in Table 1.

Amplification of the whole SIV genome. The whole viral RNA genome in plasma samples from SIV_{mac251}-infected macaques and of the SIV_{mac251} challenge stock was amplified by reverse transcriptase PCR (RT-PCR) using specifically designed primers in four overlapping fragments for Illumina deep sequencing. Briefly, 140 μl of frozen plasma from whole blood collected in EDTA-coated Vacuette tubes was used to extract viral RNA using a QIAamp viral RNA minikit (Qiagen, USA). Viral RNA was reverse transcribed to DNA and amplified using a SuperScript III one-step RT-PCR kit with Platinum *Taq* high-fidelity DNA polymerase (Life Technologies, USA) with the following primer pairs (Sigma-Aldrich, Australia): 5′-AGATAGAGTGGGAGATGGG and 5′-CTCTAATTAACCTACAGAGATGTTTGG, 5′-AAAATTGAAGCAGTGGCCATTAT and 5′-CCTCTACTGTTCTGTTTAGCC, 5′-ATAGGGGATATGACTCCAGCAGAAAGATTA and 5′-AGTCCTATTATCCCTACCATGCCAGTAAAT, and 5′-CTTGCTTTTAAGTGTCTACGGGATCTATTG and 5′-GAATACAGAGCGAAATGCAGTG.

RT-PCR was performed in an Eppendorf Realplex Mastercycler (Eppendorf, Germany) under the following conditions: 50°C for 60 min; 94°C for 2 min; 2 cycles of 94°C for 15 s, 60°C for 1 min, and 68°C for 4 min; 2 cycles of 94°C for 15 s, 58°C for 1 min, and 68°C for 4 min; 41 cycles

of 94°C for 15 s, 55°C for 1 min, and 68°C for 4 min; and 68°C for 10 min. PCR products were run on a 0.7% agarose gel (Promega, USA) alongside a 1-kb DNA ladder (New England BioLabs, USA), bands of the correct size were excised, and DNA was extracted using a QIAquick gel extraction kit (Qiagen, USA).

Illumina SIV sequencing. Amplified plasma SIV DNA concentrations were measured using a Qubit double-stranded DNA high-sensitivity (HS) assay kit and a Qubit (version 2.0) fluorometer (Life Technologies, USA). Pools containing the complete SIV_{mac251} genome from each animal were created by combining 4×10^8 copies from each of the four PCR products. A NexteraXT DNA sample preparation kit (Illumina, USA) was used to prepare libraries for indexed paired-end sequencing, and these were sequenced with a MiSeq instrument (Illumina, USA). SIV deep sequencing data are available upon request.

MHC-I typing. MHC-I genotyping was performed as previously described (32, 33). Briefly, cellular RNA was extracted from frozen macaque peripheral blood mononuclear cells (PBMCs) using an RNeasy minikit (Qiagen, USA). cDNA was synthesized using a SuperScript III first-strand synthesis system (Life Technologies, USA). Primary amplicons consisting of 568-bp fragments, including most of exon 2, all of exon 3, and part of exon 4, were generated by PCR using primers that bind highly conserved regions and universally amplify macaque MHC-I sequences. In addition, each primer contained a Roche/454 GS FLX titanium adaptor A or B and a distinct 10-bp multiplex identifier for each animal to facilitate pooling of amplicons for emulsion PCR and bidirectional pyrosequencing with a Roche/454 GS Junior instrument (Roche/454, USA). The resulting sequencing data were binned by multiplex identifier, and sequence reads from each animal were assembled into unidirectional contigs having 100% identity using a custom genotyping analysis work flow (LabKey, USA). The number of sequence reads comprising each unidirectional contig was enumerated, and the resulting consensus sequences were mapped against an in-house database containing known pig-tailed MHC-I allele sequences using the BLASTn program (33–35). Putative Mane-A and Mane-B haplotypes were inferred by identifying combinations of shared alleles between animals as described previously for rhesus macaques (35). MHC-I typing data for 5 out of the 44 macaques have been previously reported (33). The MHC-I typing data are available on request.

Identifying SNPs associated with Mane alleles. The frequency of mutations in plasma virus was identified by comparison of the plasma virus sequence with a reference SIV_{mac251} sequence (GenBank accession number M19499). Sequence reads from Illumina sequencing were assembled using Geneious software (version 7.0.2; Biomatters, Auckland, New Zealand). We assembled the SIV_{mac251} sequence reads obtained from all 44 macaques against the stock reference sequence to call single nucleotide polymorphisms (SNPs). Using an in-house pattern-mining approach, we identified, in all animals, SIV sequence positions with nonsynonymous mutations present at a frequency of >5% compared to the reference sequence (Fig. 1). In order to identify if a mutation was associated with a particular Mane haplotype, we then compared the frequency of the mutation in animals with a particular Mane haplotype with the frequency in those without that haplotype, using Fisher's exact test (Fig. 1). Fisher's exact test then provided a *P* value for the significance of the difference in the frequency of mutations in Mane haplotype-positive animals versus Mane haplotype-negative animals. Spreadsheets evaluating the linkage between SIV polymorphisms and MHC-I alleles are available upon request.

A sliding window to identify mutated regions. The method described above identifies only mutations where the same nonsynonymous mutation is present in multiple animals. For some escape mutations, animals may harbor different mutations within the same epitope. To identify such variable escape patterns, we also analyzed the frequency of mutations within a sliding window of 30 bp (in 3-bp increments), using in-house codes in Matlab and VB.NET (Fig. 1). This sliding window analysis is similar to previous work performed in linking mutations in HCV infection with HLA class I alleles in humans (10). We identified a sequence to be mutated (within a given window) if at least 1 nucleotide had a nonsynonymous mutation present at a >5% frequency. We then used Fisher's exact test to identify associations between mutated windows and Mane haplotypes and obtain a *P* value for each association. Spreadsheets evaluating the linkage between SIV polymorphisms across a 10-amino-acid window and MHC-I alleles are available upon request.

Permutation approach to evaluate the significance of associations. The large number of sequence positions and Mane haplotypes means that there is a high risk of discovery of false associations, as is noted with similar linkage analyses (23, 28). In order to estimate the probability that any identified association was real, we sought to identify the probability of false associations being identified in our data. To do this, we utilized a permutation approach. That is, each animal has a set of Mane haplotypes associated with a set of viral sequences in the same animal. The question that we wish to ask is, how many random associations would we expect to see, given the Mane haplotype structure (i.e., the number of animals with the different Mane alleles) and the virus structure (number of sites mutated)? In order to keep the data structure of the Mane haplotypes and viral mutations, we need to permute all the Mane haplotypes of a given animal *en bloc*, as well as all the viral sequences from a given animal *en bloc*. Therefore, we permuted the order of the animals (and all of the associated Mane haplotypes), leaving the order of viral sequences intact. For each permutation, we had a new order of Mane haplotype sets randomly matched to viral sequence sets. We then again performed Fisher's exact test for the permuted population to identify the *P* values that we would obtain studying the associations between Mane alleles and viral mutations from this random matching of the Mane haplotype and virus sequences (Fig. 1). By performing this permutation 1,000 times, we observed the frequency with which associations of different strength (*P* value) would be identified in our data by chance. This was performed for both the SNP and sliding window analyses to obtain the expected frequency of associations of different strengths for each. By comparing, for a given *P* value, the observed number of associations in our data with the observed frequency in the permuted data sets, we identified the proportion of associations that we observed that were likely to have arisen by chance. By comparing, for a given threshold *P* value, the observed number of associations in our data with the observed frequency in the permuted data sets, we identified the proportion of associations that we observed that were likely to have arisen by chance (28). We note that in previous studies of MHC-viral mutation associations in HIV (8, 9, 36) and HCV (10, 26, 27), it was also necessary to account for phylogenetic relationships in the data (23). However, in our study, animals were infected with the same stock of virus. In addition, mutations were identified not by comparison to the sequences of other macaques but by comparison to this reference sequence. Therefore, we did not investigate the effects of phylogenetic linkage in our analysis.

ICS assays. To validate that our approach can correctly identify CTL epitopes, we infected 3 additional pig-tailed macaques, one of which expressed the Mane-B028 haplotype, with SIV_{mac239}, which was associated with a polymorphism in SIV Nef. We confirmed that infection had occurred (peak viral load, 10^8 to 10^9 copies/ml 2 weeks after infection) and obtained fresh blood 2 to 15 weeks after infection to analyze the responses to the 3 15-mer peptides (NIH AIDS Research and Reference Reagent Repository, USA) spanning the SIV Nef polymorphism by intracellular cytokine staining (ICS), as previously described (37). Briefly, 190 μl of whole blood was stimulated with specific 15-mer peptides at 1 μg/ml in the presence of anti-CD28 and anti-CD49d (BD, USA) and 10 μg/ml brefeldin A (Sigma, USA). Negative and positive controls were included in the assay with an equivalent concentration of dimethyl sulfoxide (DMSO; Sigma, USA) and 1 μg/ml staphylococcal enterotoxin B (Sigma, USA). Cells were incubated for 5.5 h at 37°C in 5% CO_2. The cell surface was stained with CD3-Pacific Blue clone SP34-2 (BD, USA) and CD8-peridinin chlorophyll protein (PerCP) clone SK1 (BD, USA) for 30 min. Red blood cells were lysed using 1× BD fluorescence-activated cell sorting lysing solution, and cells were permeabilized using 1× BD permeabilizing solution 2 (BD, USA). Cells were incubated with anti-gamma interferon

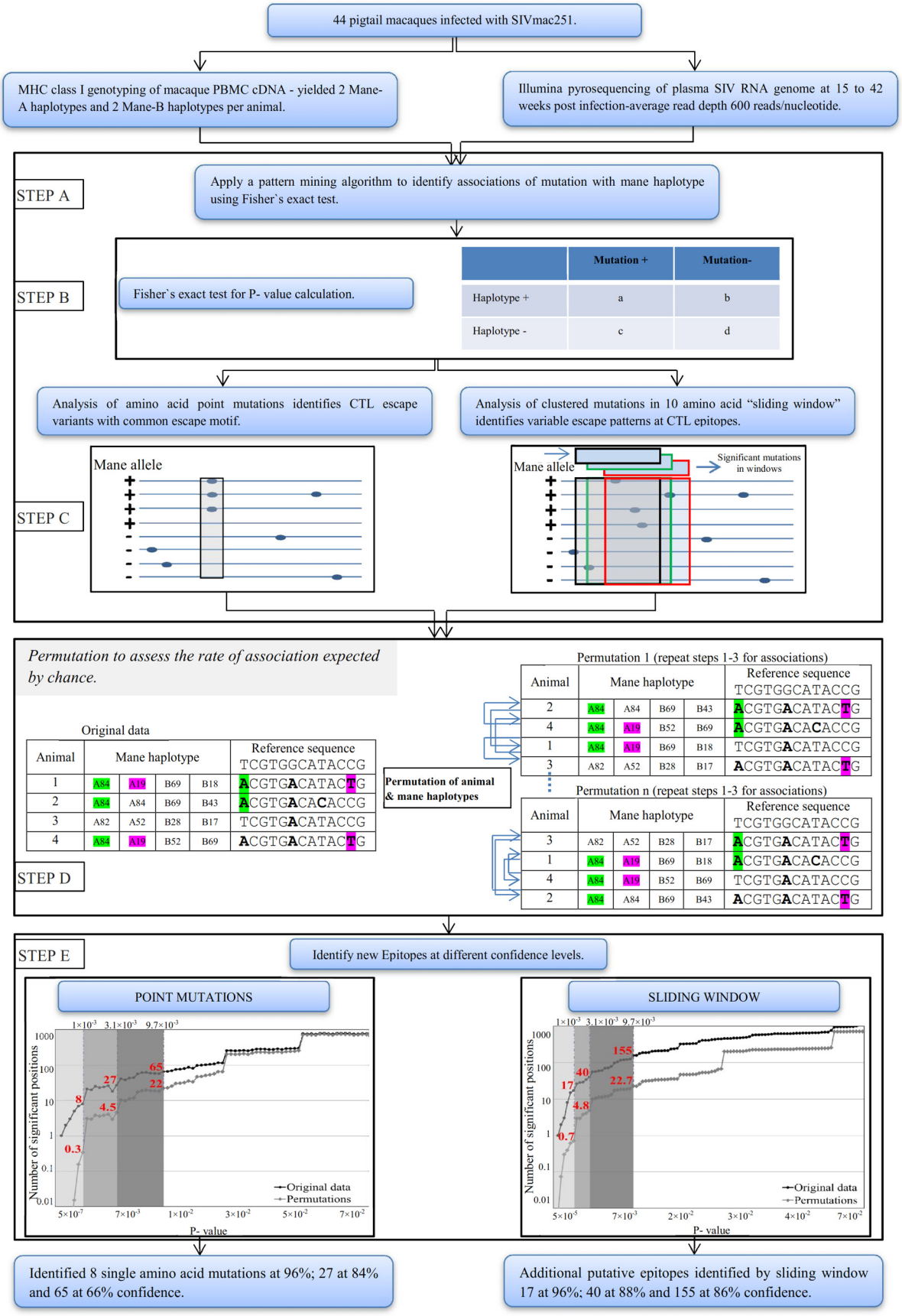

FIG 1 Flowchart of study used for identification of linkages between MHC-I haplotypes and SIV mutations in 44 SIV_{mac251}-infected pig-tailed macaques.

(IFN-γ)-allophycocyanin (APC) (BD, USA) for 45 min. A minimal epitope was mapped by obtaining individual 9- to 11-amino-acid peptides (Purar Chemicals, China) spanning the region of the overlapping 15-mer peptides and performing ICS as described above using dilutions of peptides from a 1-μg/ml stock. Once the minimal epitope was identified, whole blood was stimulated separately with dilutions of peptides from a 1-μg/ml stock of the wild-type and escape mutation 9-mer peptides (Purar Chemicals, China) as described above. Cells were washed and fixed with 1% paraformaldehyde (Sigma). Cells were acquired using a BD LSR II flow cytometer, and at least 2×10^5 lymphocytes were collected. Flow cytometry data were analyzed using FlowJo software (version 9.1; TreeStar, USA).

Tetramer staining for macaques positive for the Mane-B017 haplotype. To identify that CD8 T cells specific for particular Mamu-B017 epitopes could be recovered from Mane-B017 macaques and identified using Mamu-B*017:01 tetramers, frozen PBMCs were thawed and washed in RPMI (Life Technologies, USA) supplemented with 10% fetal calf serum (DKSH, Australia) and expanded as previously described (38). Briefly, PBMCs were split into stimulators and responders at a 1:2 ratio. Stimulators were incubated with 15-mer peptides that span the desired CTL epitope region (NIH AIDS Research and Reference Reagent Repository, USA) at 1 μg/ml for 90 min in 5% CO_2 at 37°C. Stimulators were washed two times and added to the responders. Cells were grown over 10 days at 37°C in 5% CO_2. On day 3 and day 5, fresh medium containing interleukin-2 (IL-2) and interleukin-7 (IL-7) at 50 U/ml and 10 ng/ml, respectively (PeproTech, USA), was added. The same procedure was followed for control samples, whereby stimulators were pulsed with peptides restricted by a different Mane haplotype identified in our MHC-I and SNP correlation analysis. PBMCs were stained with the Live/Dead Fixable Aqua dead cell stain (Invitrogen, USA) for 30 min at room temperature and washed. Cells were stained with Env FW9-phycoerythrin and Nef IW9-APC using a titration-optimized volume of a Mamu-B*017:01 tetramer folded around the FW9 or IW9 epitope (kindly provided by N. A. Wilson Schlei, University of Miami [39]) for 60 min at room temperature. The cell surface was stained with CD3-Alexa Fluor 700 clone SP34-2, CD8-PerCP clone SK1, and CD20-fluorescein isothiocyanate (BD, USA) for 30 min. Cells were washed and fixed with 1% paraformaldehyde (Sigma, USA). Cells were acquired using a BD Canto II flow cytometer, and at least 2×10^5 lymphocytes were collected. Flow cytometry data were analyzed using FlowJo software (version 9.1; TreeStar, USA).

RESULTS

MHC-I typing of 44 pig-tailed macaques by pyrosequencing. Multiple MHC-I alleles and/or haplotypes have been characterized in a limited number of pig-tailed macaques (33, 34, 40, 41). Macaques can express more than 20 MHC-I transcripts from a variable number of Mane-A and Mane-B loci that have undergone gene duplication, in contrast to their human counterparts (34, 40). Each macaque carries a pair of Mane-A and Mane-B haplotypes inherited from the parents. Abbreviated haplotype designations for Mane-A were based on the presence of a transcriptionally abundant Mane-A1 allele, while Mane-B haplotypes were assigned on the basis of a diagnostic major Mane-B transcript that is characteristic for a group of linked Mane-B alleles, as previously described for rhesus macaques (34, 42). We identified a diverse range of Mane-A and Mane-B haplotypes in our cohort of 44 SIV_{mac251}-infected pig-tailed macaques, as shown in Table 2. The most common Mane-A and Mane-B haplotypes in this cohort were Mane-A084, Mane-A082, and Mane-B118, which represented 23%, 15%, and 15% of the MHC-I chromosomal regions, respectively.

Association between MHC-I and VL. We first assessed the influence of Mane haplotypes with levels of chronic SIV viremia across our 44-macaque cohort. We calculated an average SIV plasma RNA level (viral load) for each animal during steady-state viremia from week 6 onwards. Of the 44 animals studied, 21 animals received ART and vaccination, 10 animals received ART only, and 13 animals received neither ART nor vaccination. To account for these variables, a two-way analysis of variance (ANOVA) was employed to study the link between viral load and ART, vaccination, and MHC-I haplotype (Table 3). In an analysis not corrected for the number of associations studied, of the individual MHC-I haplotypes, only Mane-A082 and Mane-B118 showed an association with modestly lower overall viral loads and Mane-B120 showed an association with modestly higher viral loads. However, the results after correction for multiple correlations showed no significant link between specific MHC-I haplotypes and a decrease or increase in viral load. Importantly, we did not find a significant association between Mane-A084 and lower viral loads in this study of 44 macaques (16 of which were Mane-A084 positive [Mane-A084$^+$]), in contrast to the findings of a previous much smaller study (13). We also analyzed whether the Mane-A084$^+$ macaques that mutate at the KP9 Gag CTL epitope have lower viral loads than those that remain wild type but did not detect significant differences. The 11 Mane-A084$^+$ macaques that generated the K165R escape mutant had an overall mean VL of 4.535 $\log_{10}$ copies of RNA/ml plasma, whereas the 5 Mane-A084$^+$ macaques that did not generate this mutant had a mean VL of 4.690 $\log_{10}$ copies of RNA/ml plasma ($P = 0.740$, Mann-Whitney test). However, we acknowledge that compensatory mutations may confound this analysis, and the use of larger numbers of animals would be helpful to study this more fully.

Validation of the method employed to associate pig-tailed macaque MHC-I haplotypes with SNPs in the SIV genome. A previous study conducted in Mauritian cynomolgus macaques, together with a retrospective analysis of studies with Indian-origin rhesus macaques, demonstrated that transcriptionally abundant MHC-I alleles play a crucial role in presenting CTL epitopes in SIV infection (43). We developed an in-house bioinformatics pipeline to study associations between the four MHC-I haplotypes for each macaque across the nearly 10,000-bp-long SIV genome, as described in Materials and Methods and shown in Fig. 1. We first validated our approach by analyzing the data for previously characterized KP9, KSA10, and KVA10 CTL epitopes restricted by Mane-A1*084 (14, 15). We compared SIV sequences from animals with and without the Mane-A084 haplotype. The strongest association was with the previously identified KP9 epitope in SIV Gag (Table 4). In position 165 of Gag, we observed this mutation in 11 of 16 Mane-A084-positive animals and 0 of 28 Mane-A084 negative animals ($P = 5.7 \times 10^{-7}$). The second most significant set of mutations was also observed in a previously identified epitope, the KVA10 epitope in Tat, in which 9 and 7 of 16 Mane-A084-positive animals had a K-to-E mutation at position 1 and position 2, respectively, whereas 0 of 28 Mane A084-negative animals had these mutations ($P = 1.6 \times 10^{-5}$ and 3×10^{-4}, respectively). We also identified 2 mutations within the KSA10 epitope of Tat (in positions 3 and 9 of the epitope) as being significantly linked with Mane-A084 ($P = 1.1 \times 10^{-3}$ and 4×10^{-3}, respectively). Thus, our method identified the three known Mane-A1*084-restricted SIV CTL epitopes and their previously described escape mutations, confirming the ability of this approach to identify MHC-I-restricted CTL escape mutations.

Identification of novel MHC-I-linked mutations in SIV. Using the approach described above, we searched for associations for

TABLE 2 MHC-I genotyping of the 44 pigtail macaques infected with SIV_{mac251}

Mane-A or -B haplotype	No. of haplotypes observed	Haplotype frequency (%)	Major alleles					Minor alleles		
			Diagnostic	2	3	4	5	1	2	3
A084	20	22.7	*A1*084*					*A3*13*		
A082	13	14.8	*A1*082*					*A3*13*		
A052	6	6.8	*A1*052*					*A3*13*	*A5*30*	
A019	5	5.7	*A1*019*					*A2*05*	*A4*14*	
A010	4	4.5	*A1*010*					*A3*13*	*A4*14*	
A006	4	4.5	*A1*006*					*A2*05*		
A009	3	3.4	*A1*009*					*A2*05*		
A031	3	3.4	*A1*031*					*A4*14*		
A072	2	2.3	*A1*072*					*A3*13*		
A016	2	2.3	*A1*016*					*A4*14*		
A032	2	2.3	*A1*032*					*A4*14*		
A047	2	2.3	*A1*047*					*A2*05*	*A4*01*	
A053	2	2.3	*A1*053*					*A2*05*	*A4*14*	
A066	2	2.3	*A1*066*					*A3*13*		
A083	2	2.3	*A1*083*					*A2*05*	*A3*13*	
A114	2	2.3	*A1*114*					*A2*05*	*A3*13*	
A003	2	2.3	*A1*003*					*A2*05*		
A004	1	1.1	*A1*004*					*A4*14*		
A007	1	1.1	*A1*007*					*A2*05*		
A085	1	1.1	*A1*085*					*A2*05*	*A4*14*	
A018	1	1.1	*A1*018*					*A2*05*	*A4*14*	
A unknown	8	9.1	*A1* novel							
B118	13	14.8	*B*118*	*B*122*	*B*027*	*B*030*		*B*082*		
B043	8	9.1	*B*043*	*B*030*	*B*045*			*B*072*		
B015	8	9.1	*B*015*	*B*068*				*B*064*	*B*088*	
B028	7	8.0	*B*028*	*B*061*	*B*124*	*B*068*	*B*021*	*B*088*	*B*079*	*B*045*
B016	7	8.0	*B*016*	*B*041*				*B*089*	*B*088*	
B120	6	6.8	*B*120*	*B*107*	*B*082*			*B*078*		
B047	6	6.8	*B*047*	*B*068*				*B*112*		
B039	5	5.7	*B*039*	*B*108*				*B*088*		
B069	4	4.5	*B*069*	*B*081*	*B*107*			*B*082*	*B*079*	*B*051*
B119	3	3.4	*B*119*	*B*068*						
B150	3	3.4	*B*150*	*B*068*				*B*057*	*B*089*	*B*072*
B104	3	3.4	*B*104*	*B*144*				*B*057*	*B*046*	
B004	2	2.3	*B*004*	*B*004*						
B110	2	2.3	*B*110*	*B*123*	*B*082*	*B*068*		*B*072*		
B017	2	2.3	*B*017*					*B*060*		
B056	2	2.3	*B*056*	*B*017*	*B*041*			*B*082*	*B*079*	
B052	2	2.3	*B*052*	*B*058*						
B008	1	1.1	*B*008*	*B*125*	*B*082*			*B*079*	*B*072*	
B019	1	1.1	*B*019*	*B*014*				*B*057*	*B*116*	
B024	1	1.1	*B*024*	*B*068*				*B*089*	*B*082*	
B099	1	1.1	*B*099*	*B*058*				*B*072*	*B*054*	*B*063*
B101	1	1.1	*B*101*	*B*068*				*B*060*	*B*089*	

the more common Mane haplotypes present in our cohort. The analysis of associations between four Mane-A and -B haplotypes for each macaque across the nearly 10,000 bp of the SIV genome results in a very large number of associations, many of which are random. Thus, the *P* values from our Fisher's exact test are not a precise indication of the probability that a mutation is genuine or random. To assess how often we might expect to see an association of a particular strength simply by chance, we can randomly sort the animal identities (and their Mane-A and -B haplotypes) with the viral mutation patterns. That is, we can permute the animal identities, leaving the order of viral sequences intact, so that, for example, the Mane haplotypes of animal 1 are a match with the viral sequences of animal 18, the Mane haplotypes of animal 2 are a match with the viral sequences of animal 6, and so on. Each time that we permute the animal identities, we can then perform the same analysis of associations between Mane haplotypes and mutations and observe the *P* values that arise from these random associations. By performing the permutation 1,000 times, we obtain the expected distribution of *P* values arising from chance associations (Fig. 1). Thus, for a given *P* value for the association, we observed a certain proportion of associations in the actual data set and another for the permuted data set. We were then able to identify threshold values below which we had a given level of confidence in the associations (Fig. 1). For example, we first identified a *P* value below which we estimated that 95% of observed associations were genuine. That is, at a *P* value cutoff of 1.0×10^{-3}, there

TABLE 3 Influence of MHC-I genotype on SIV load

Haplotype	No. of macaques		VL[a]						Uncorrected *P* value[b]
			No ART, not vaccinated		Early ART, not vaccinated		Early ART, vaccinated		
	Haplotype positive	Haplotype negative	Mane positive	Mane negative	Mane positive	Mane negative	Mane positive	Mane negative	
Mane-A006	3	41	5.96	5.74	NA	4.84	3.31	4.43	0.56
Mane-A082	13	31	5.96	5.62	5.48	4.57	4.7	4.3	0.04
Mane-A084	16	28	5.61	5.83	4.55	5.04	4.25	4.48	0.22
Mane-A114	2	42	NA	5.78	5.42	4.78	3.31	4.43	0.62
Mane-B015	8	36	5.54	5.88	4.9	4.83	5.13	4.3	0.72
Mane-B016	7	37	5.98	5.69	4.75	4.85	5.14	4.3	0.3
Mane-B017	2	42	5.38	5.81	NA	4.84	4.7	4.36	0.94
Mane-B028	7	37	5.39	5.85	4.28	4.98	4.18	4.41	0.12
Mane-B043	7	37	5.67	5.82	NA	4.84	4.33	4.38	0.75
Mane-B047	6	38	6.07	5.75	4.9	4.84	3.8	4.51	0.24
Mane-B069	4	40	5.61	5.79	4.32	4.97	4.34	4.38	0.36
Mane-B118	13	31	6.06	5.75	5.63	4.65	4.65	4.13	0.02
Mane-B120	6	38	6.13	5.75	4.31	5.07	3.26	4.49	0.03

[a] The VL ($\log_{10}$ number of copies of RNA/ml plasma) is the mean of all available VL measurements after week 6. NA, not applicable.
[b] After correction for multiple comparisons, no MHC-I alleles were associated with a significant change in VL.

were 8 associations with lower *P* values in our data versus an average of 0.3 association in the permuted data (3 associations with *P* values of $<1 \times 10^{-3}$ out of 1,000 permutations). Similarly, we identified 2 thresholds of *P* values where either 84% of associations or 66% of associations were likely to be genuine (Fig. 1). The associations between SIV SNPs and MHC-I alleles are shown in Table 5.

Using this approach, we identified a total of 46 novel nonsynonymous mutations within SIV that were significantly associated with particular Mane class I A or B haplotypes at a level of at least 66% and likely to be genuine associations using our permutations analyses ($P < 9.7 \times 10^{-3}$; Table 5). An example of a polymorphism detected in Nef that was linked to the Mane-B028 haplotype is shown in Fig. 2. Within the 46 associations identified, 27 had *P* values sufficiently low ($P < 3.1 \times 10^{-3}$; Table 5) that they were 84% likely to be genuine associations using our permutation analyses. The associations identified were distributed across 18 Mane class I haplotypes and were in 7 SIV proteins (5 in Gag, 6 in Pol, 22 in Env, 6 in Nef, 3 in Vif, 3 in Tat, and 1 in Vpr).

Identification of mutated regions using a sliding window. The identification of individual mutations that are associated with a particular MHC-I haplotype requires that escape occur in the same way in multiple animals. However, for some epitopes, escape occurs in different ways in different animals. In this case, we would see clusters of mutations within the epitope, but no single mutation may occur at a sufficient frequency to be significantly associated with a particular MHC-I haplotype. Indeed, we noted in the single nucleotide mutation analysis described above that some identified mutations linked to the same MHC-I haplotype were closely spaced, suggesting the likelihood that a single epitope spanned both associations. For example, the multiple Mane-A*010 associations in Pol and Env (Table 5) were <30 nucleotides apart. This was also observed in the validation exercise, where multiple mutations in the KVA10 and KSA10 epitopes were linked to the restricting MHC-I allele Mane-A1*084.

Thus, we developed a sliding window approach to link closely spaced mutations (<10 amino acids apart) with MHC-I haplotypes. This approach is similar to an approach previously used to link HCV mutations in humans with HLA class I alleles (10). This approach identifies all the closely spaced mutations linked to the same haplotype identified in the single nucleotide analyses presented in Table 5 but also identifies new sites where no single mutation is of sufficient frequency to be significantly associated with an MHC-I haplotype. The schematic of this approach is

TABLE 4 Validation of the study approach to link pig-tailed macaque MHC-I haplotypes with CTL escape mutations by using previously defined CTL responses restricted by the Mane-A1*084 allele

Protein	Epitope	Region of epitope within protein	No. of Mane-positive mutation-positive animals/total no. of Mane-positive animals	No. of Mane-negative mutation-positive animals/total no. of Mane-negative animals	*P* value (two-tailed)	Amino acid change	Escape motif[a]
Gag	KP9	164–172	11/16	0/28	5.7E−07	K → R	K**R**FGAEVVP
Tat	KSA10	87–96	6/16	0/28	1.1E−03	A → T	KKAK**T**NTSSA
	KSA10	87–96	6/16	0/28	1.1E−03	S → T or S → P	KKAKANTS**T**A or KKAKANTS**P**A
	KSA10	87–96	5/16	0/28	4.0E−03	A → T	KK**T**KANTSSA
Tat	KVA10	114–123	9/16	0/28	1.6E−05	K → E	**E**KETVEKAVA
	KVA10	114–123	7/16	0/28	3.0E−04	K → E	K**E**ETVEKAVA

[a] The mutated amino acids are in boldface and underlined.

TABLE 5 Novel SIV_{mac251} SNPs linked to MHC-I haplotypes in pig-tailed macaques

Haplotype	Protein	Nucleotide position	No. of Mane-positive mutation-positive animals/total no of Mane-positive animals	No. of Mane-negative mutation-positive animals/Total no. of Mane-animals	*P* value[a] (two tailed)	Amino acid change(s)	Wild-type motif[b]
Mane-A*010	Gag	2179	2/4	0/40	0.0064	P → S	EALKEALAPV**P**IPFAAAQQRG
	Pol	4405	2/4	0/40	0.0064	N → S	CPTESESRLV**N**QIIEEMIKKS
	Pol	4408	2/4	0/40	0.0064	Q → R	PTESESRLVN**Q**IIEEMIKKSE
	Env	7483	2/4	0/40	0.0064	D → E	NRTYIYWHGR**D**NRTIISLNKY
	Env	7493	2/4	0/40	0.0064	I → V	IYWHGRDNRT**I**ISLNKYYNLT
	Env	8019	2/4	0/40	0.0064	T → I	VTSLIANIDW**T**DGNQTNITMS
Mane-A*032	Gag	2179	2/2	0/42	0.0011	P → S	EALKEALAPV**P**IPFAAAQQRG
	Pol	4405	2/2	0/42	0.0011	N → S	CPTESESRLV**N**QIIEEMIKKS
	Pol	4408	2/2	0/42	0.0011	Q → R	PTESESRLVN**Q**IIEEMIKKSE
	Env	7483	2/2	0/42	0.0011	D → E	NRTYIYWHGR**D**NRTIISLNKY
	Env	7493	2/2	0/42	0.0011	I → V	IYWHGRDNRT**I**ISLNKYYNLT
	Vif	5863	2/2	0/42	0.0064	N → D	NPTWKQWRRD**N**RRGLRMAKQN
Mane-A*053	Nef	9676	2/2	0/42	0.0011	S → Y, S → F	RHYLMQPAQT**S**KWDDPWGEVL
Mane-A*082	Env	7001	5/13	0/31	0.0012	T → A, T → S, T → P	LTKSSTTTAP**T**TTTAASKIDM
	Env	7033	8/13	5/31	0.0087	M → I	TTTTAASKID**M**VNETSSCITH
Mane-A*114	Gag	2477	2/2	0/42	0.0011	Q → R	PAVDLLKNYM**Q**LGKQQREKQR
Mane-B004	Vif	5935	2/2	1/41	0.0032	K → E	DKQRGGKPPT**K**GANFPGLAKV
	Env	6996	2/2	1/41	0.0032	A → V	WGLTKSSTTT**A**PTTTTAASKI
	Env	7017	2/2	2/42	0.0064	A → G A → V	TTTAPTTTTA**A**SKIDMVNETS
Mane-B015	Env	9236	4/8	1/36	0.0024	L → V	RIRQGLELTL**L**
	Env	7280	3/8	0/36	0.0042	T → A	QESCDKHYWD**T**IRFRYCAPPG
Mane-B016	Gag	1360	3/6	0/38	0.0015	I → V	KVKHTEEAKQ**I**VQRHLVVETG
	Env	6789	3/6	0/38	0.0015	G → D	WGTTQCLPDN**G**DYSELALNVT
Mane-B017	Nef	9568	2/2	0/42	0.0011	I → T	DWQDYTSGPG**I**RYPKTFGWLW
Mane-B028	Env	7049	3/7	0/37	0.0027	S → P	SKIDMVNETS**S**CITHDNCTGL
	Env	9105	4/7	3/37	0.0075	Q → R	YGWSYFQEAV**Q**AGWRSATETL
	Nef	9105	4/7	3/37	0.0075	K → E	MGGAISRRRS**K**PAGDLRQRLL
Mane-B039	Env	7027	3/5	2/39	0.0070	I → M	APTTTTAASK**I**DMVNETSSCI
Mane-B043	Vif	5917	3/8	0/36	0.0043	G → S	KQNSRGDKQR**G**GKPPTKGANF
	Nef	9402	5/8	5/36	0.0091	T → A	VPVMPRVPLR**T**MSYKLAIDMS
Mane-B056	Pol	4618	2/2	1/42	0.0032	R → K	KELVFKFGLP**R**IVARQIVDTC
Mane-B069	Gag	1880	2/4	0/40	0.0064	R → K	WIQLGLQKCV**R**MYNPTNILDV
	Tat	8808	2/4	0/40	0.0064	I → V, I → L	KP**I**SNRTRHCQPE
	Env	8804	2/4	0/40	0.0064	T → A	FSSPPSYFQQ**T**HIQQDPALPT
	Env	8808	2/4	0/40	0.0064	H → R, H → P	SSPPSYFQQT**H**IQQDPALPTR
Mane-B104	Tat	6408	2/3	0/41	0.0032	G → R	DATTPESANL**G**EEILSQLYRP
	Env	8019	2/3	0/41	0.0032	T → I	VTSLIANIDW**T**DGNQTNITMS
Mane-B118	Env	8981	4/13	0/31	0.0053	G →A	FSNCRTLLSR**A**YQILQPILQR
Mane-B119	Pol	2538	2/3	0/41	0.0032	D → N	RKQREALQGG**D**RGFAAPQFSL
	Env	9089	2/3	0/41	0.0032	F → V	LTYLQYGWSY**F**QEAVQAGWRS
	Env	9093	2/3	0/41	0.0032	Q → R	TYLQYGWSYF**Q**EAVQAGWRSA

(Continued on following page)

TABLE 5 (Continued)

Haplotype	Protein	Nucleotide position	No. of Mane-positive mutation-positive animals/total no of Mane-positive animals	No. of Mane-negative mutation-positive animals/Total no. of Mane-animals	*P* value[a] (two tailed)	Amino acid change(s)	Wild-type motif[b]
	Nef	9089	2/3	0/41	0.0032	I → M	MGGA**I**SRRRSKPAGD
	Nef	9093	2/3	0/41	0.0032	R → G	MGGAIS**R**RRSKPAGDLR
	Tat	6364	2/3	1/41	0.0094	C → Y	SLESSNERSS**C**ISEADATTPE
	Env	7931	2/3	1/41	0.0094	V → I	IRQIINTWHK**V**GKNVYLPPRE
Mane-B150	Vpr	6055	2/3	0/41	0.0032	M → I	YLCLIQKALF**M**HCKKGCRCLG

[a] *P* values are uncorrected (Fisher's exact test).
[b] The mutated amino acids are in boldface.

shown in Fig. 1, and a specific example of an association identified using this methodology is shown in Fig. 3. In the example shown in Fig. 3, 5 of 16 Mane-A1*084$^+$ animals but none of 28 Mane-A1*084-negaitve animals have various mutations across a stretch of 5 amino acids in Gag.

This sliding window approach identified 142 windows where there was an association that we estimated had a >86% probability of being real and did not overlap the mutations observed in our previous analysis of individual sequence positions. This corresponds to a *P* value by Fisher's exact test of $<3.1 \times 10^{-3}$. Many of the windows overlapped each other in the same region (as would be expected around an epitope). Once we identified the unique sites covered, we found 32 sites within the SIV genome where mutations were linked to Mane-A or -B haplotypes that were not discovered when only individual SNPs were analyzed (Table 6). Four of the associations were in Gag, 9 were in Pol, 11 were in Env, 2 were in Tat, 4 were in Vif, and 1 each was in Nef and Vpx.

Validation that a novel Mane-B028-associated polymorphism in Nef represents a CTL escape mutation. To validate that our approach successfully identified novel CTL escape mutations, we infected 3 additional pig-tailed macaques with the closely related strain SIV_{mac239}. One macaque expressed the Mane-B028 haplotype that was linked to a polymorphism in Nef (shown in Fig. 2). We obtained blood samples to analyze CD8 T cell responses to specific 15-mer SIV peptides across the Nef polymorphism linked to Mane-B028 in the analyses described above. We found that ~0.3% of CD8 T cells obtained 10 weeks after infection expressed IFN-γ in response to stimulation with the Nef peptides (Fig. 4A). This response was not identified in the other 2 Mane-B028-negative SIV-infected pig-tailed macaques studied. We subsequently obtained 9- to 11-mer peptides spanning this region and identified the minimal epitope to be a RE9 peptide (Fig. 4B). When the escape mutation identified in the linkage analyses was inserted into the minimal epitope (a mutation of R to E at the first position), a substantial loss of CD8 T cell recognition was observed (Fig. 4C). Thus, our approach successfully identified an escape mutation within a novel CD8$^+$ CTL epitope within SIV in pig-tailed macaques.

Analysis of Mane-B017-associated polymorphisms and CTL responses. Studies conducted in the Indian rhesus macaque model have demonstrated that Mamu-B017 is able to assist in the control of SIV replication in a subset of animals (39, 44). Furthermore, several CTL epitopes, which include epitopes in Env and Nef, have been shown to occur in association with the Mamu-B017 haplotype in rhesus macaques (39). The pig-tailed macaque Mane-B*017 allelic variants that were present in 2 of our SIV-infected animals differed from Mamu-B*017 by only single conservative amino acid substitutions in exons 2 and 4. Interestingly, although the cohort is very small, the 2 Mane-B017-positive pig-tailed macaques did not control SIV_{mac251} to low levels (Table 3).

We examined our sequencing data for the presence of mutations within known Mamu-B*017-restricted Env and Nef epitopes. We found that the Nef IW9 epitope (identified in Mamu-B017-positive [Mane-B017$^+$] rhesus macaques) contained mutations within our Mane-B017$^+$ pig-tailed macaques that were similar to those described in SIV-infected Mamu-B017$^+$ rhesus macaques (Fig. 5A). We also found a mutation within the Env FW9 epitope in one Mane-B017$^+$ pig-tailed macaque, although the particular mutation was different from that reported in Mamu-B017$^+$ rhesus macaques (Fig. 5B). To validate that Mane-B017 presents the Nef IW9 epitope and Env FW9 epitope identified in Mamu-B017-positive rhesus macaques, we thawed frozen PBMCs from one of the Mane-B*017-positive macaques obtained 24 weeks after infection with SIV_{mac251} and stimulated the PBMCs with peptides containing the IW9 and FW9 epitopes. The cultures were stained with Mamu-B017 tetramers folded around the Nef IW9 and Env FW9 epitopes. We identified a tetramer-positive population for Env FW9 (Fig. 5C), although we did not identify such a population for Nef IW9 (not shown).

DISCUSSION

The important role that MHC-I alleles and CTL responses play in the progression of HIV infection has been well documented by linking HIV-1 polymorphisms with MHC-I alleles (24, 25, 45). Although a number of SIV-specific CTL epitopes have been described in most macaque models of SIV infection, a broad linking of MHC-I haplotypes and SIV mutations in macaques has not previously been performed, despite the advantage of knowing the time of infection and identity of the infecting strain in this animal model. In the study described in this report, we sequenced the plasma viral genome of 44 SIV_{mac251}-infected pig-tailed macaques and determined their MHC-I haplotypes. The MHC-I haplotypes and the viral sequencing databases were analyzed using specifically designed in-house pipelines to identify an association between viral mutations and Mane-A and -B haplotypes. The methodology that we used was first validated using the well-described KP9, KSA10, and KVA10 escape mutations associated with Mane-A1*084. We then used this method to identify novel potential

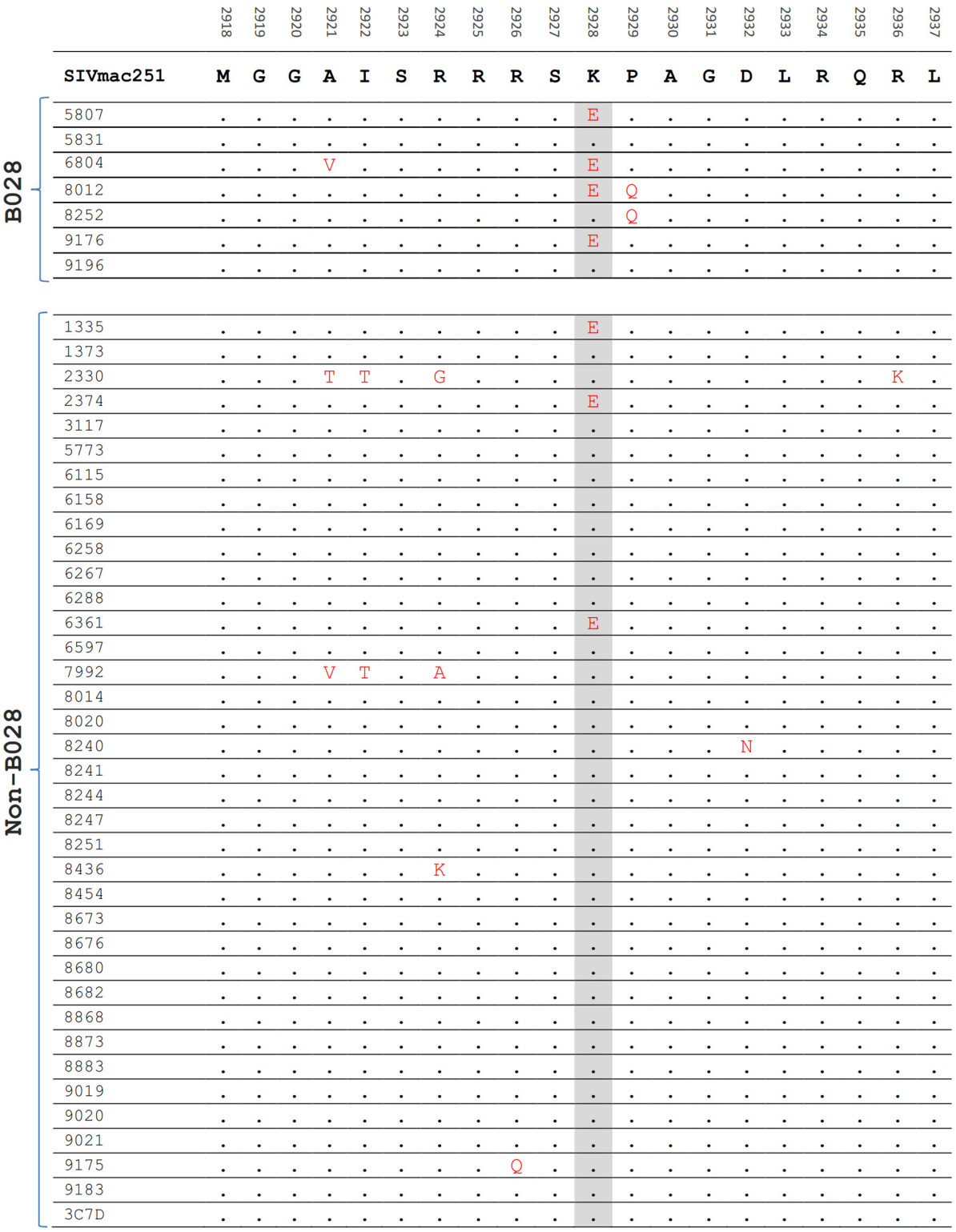

FIG 2 Example of linkage analysis to identify mutations within the SIV proteome which are more common in macaques with specific MHC-I haplotypes. Shown are SIV sequences across Nef in all 44 macaques. Mutations at the shaded amino acid were observed in 4 of 7 Mane-B028^{+} macaques and 3 of the Mane-B028-negative macaques ($P = 0.0075$, Fisher's exact test).

escape mutations, and we were able to identify 46 novel single nucleotide mutations throughout the SIV_{mac251} proteome linked to diverse Mane class I haplotypes with a reasonable likelihood of being genuine associations (we estimated >86% and >66% likelihoods in sliding window and point mutation analyses, respectively). Further, a novel sliding window methodology was used to identify 10-amino-acid regions of the SIV_{mac251} genome where closely spaced amino acids (potentially within the same CTL

						Window													
	SIVmac251	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
A084	1335	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	A	D	H	V	M	A
	8454	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	V	D	H	V	M	A
	9175	C	R	A	P	R	R	Q	G	C	W	K	C	G	R	M	G	H	V	M	A
	2374	C	R	A	P	R	R	Q	G	C	W	K	C	G	R	M	D	H	V	M	A
	8244	C	R	A	P	R	R	Q	G	C	W	E	C	G	K	M	G	H	V	M	A
	3C7D	C	R	T	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8240	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8241	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8020	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	9176	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8014	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	9020	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	9021	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	1373	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8252	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	9183	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
Non-A084	9019	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	6597	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	6258	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	G	H	V	M	A
	6804	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8251	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8682	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8680	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8247	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8868	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	5831	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	6267	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	6169	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	2330	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8676	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	5773	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8012	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	9196	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	6288	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	3117	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8873	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	5807	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	6361	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	6115	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	6158	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8436	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	7992	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8673	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A
	8883	C	R	A	P	R	R	Q	G	C	W	K	C	G	K	M	D	H	V	M	A

FIG 3 Example of sliding window analysis to identify 10-amino-acid regions of the SIV proteome which undergo changes at different positions but which are more common in macaques with specific MHC-I haplotypes. Shown are SIV sequences across Gag in all 44 macaques. Five of the 16 Mane-A084^{+} macaques showed mutations, identified in the shaded 10-amino-acid window, that were not observed in any of the 28 Mane-A084-negative macaques.

epitope) were mutated at different places in different animals. This analysis identified a further 32 potential MHC-restricted CTL epitopes undergoing escape. The novel potential epitopes identified will allow scientists to study in finer detail the CTL responses induced by vaccination or infection in this model and potentially better understand the principles of T cell control of SIV in this model.

The total of 78 sites in SIV undergoing mutational escape that we identified through this MHC-I linkage analysis likely represents an overestimate of the true number of CTL epitopes and

TABLE 6 Identification of putative SIV-specific polymorphisms linked to pig-tailed macaque MHC-I haplotypes using a 10-amino-acid sliding window analysis

Haplotype	Protein	Nucleotide region		No. of Mane-positive mutation-positive animals/total no. of Mane-positive animals	No. of Mane-negative mutation-positive animals/total no. of Mane-negative animals	*P* value (two-tailed)	No. of windows positive for amino acid sequence	Amino acid sequence spanning positive sliding windows
		Start	End					
Mane-A*006	Gag	2182	2226	2/3	1/41	0.0094	6	IPFAAAQQRGPRKPI
	Pol	4047	4076	2/3	1/41	0.0094	1	QWWTDYWQVT
	Env	7538	7588	3/3	6/41	0.0063	8	RPGNKTVLPVTIMSGLV
Mane-A*019	Pol	3036	3095	4/5	2/39	0.0005	11	AIKKKDKNKWRMLIDFRELN
	Env	9023	9091	4/5	2/39	0.0005	10	ALQRIREVLRTELTYLSYF
Mane-A*032	Tat	6393	6425	2/2	2/42	0.0063	2	ESANLGEEILS
Mane-A*052	Env	6911	6958	3/5	2/39	0.0070	7	KLSPLCITMRCNKSET
Mane-A*082	Env	7256	7309	4/13	0/31	0.0053	9	SCDKHYWDTIRFRYCAPP
Mane-A1*084	Gag	2284	2313	5/16	0/28	0.0040	1	RQGCWKCGKM
	Env	8795	8836	13/16	7/28	0.0005	5	FQQTHIQQDPALPT
Mane-A*114	Gag	2446	2475	2/2	1/42	0.0032	1	PAVDLLKNYM
	Pol	4044	4073	2/2	2/42	0.0063	1	EQWWTDYWQV
	Vif	5701	5748	2/2	2/42	0.0063	7	EVRRAIRGEQLLSCCK
Mane-B017	Pol	3426	3455	2/2	2/42	0.0063	1	DRTDLEHDRV
Mane-B039	Tat	8862	8900	3/5	1/39	0.0029	4	EKAVATAPGLGR
Mane-B043	Pol	5277	5306	3/5	0/36	0.0042	1	ILKVGTDIKV
	Vif	5533	5562	4/8	1/35	0.0023	1	SHLEVQGYWH
	Vif	5548	5589	4/8	2/36	0.0065	5	QGYWHLTPERGWLS
	Env	9137	9190	5/8	2/36	0.0009	8	AGAWGDLWETLRRGRWI
Mane-B047	Env	8837	8866	6/6	14/38	0.0055	1	REGKEGDGGE
	Nef	9279	9314	5/6	9/38	0.0089	3	PWRNPAEEREKL
Mane-B052	Pol	2751	2780	2/2	2/42	0.0063	1	VLGKRIKGTI
	Pol	3351	3413	2/2	1/42	0.0032	12	VLEPFRKANPDVTLVQYMDDI
	Vif	5869	5904	2/2	2/42	0.0063	3	RGLRMAKQNSRG
Mane-B069	Gag	2272	2304	2/4	0/40	0.0063	2	RAPRRQGCWKC
	Pol	2568	2603	2/4	0/40	0.0063	3	LWRRPVVTAHIE
	Env	6815	6853	2/4	0/40	0.0063	4	VTESFDAWENTVT
Mane-B104	Vpx	6619	6657	3/3	7/41	0.0091	4	ESAAYRHLAFKCL
	Env	7142	7189	2/3	1/41	0.0094	7	KEYNETWYSTDLVCEQ
	Env	8291	8341	2/3	1/41	0.0094	8	QQQQLLDVVKRQQELLR
Mane-B119	Pol	3822	3857	2/3	1/41	0.0094	3	PLEATVIKSQDN
	Env	7907	7939	2/3	1/41	0.0094	2	QIINTWHKVGK

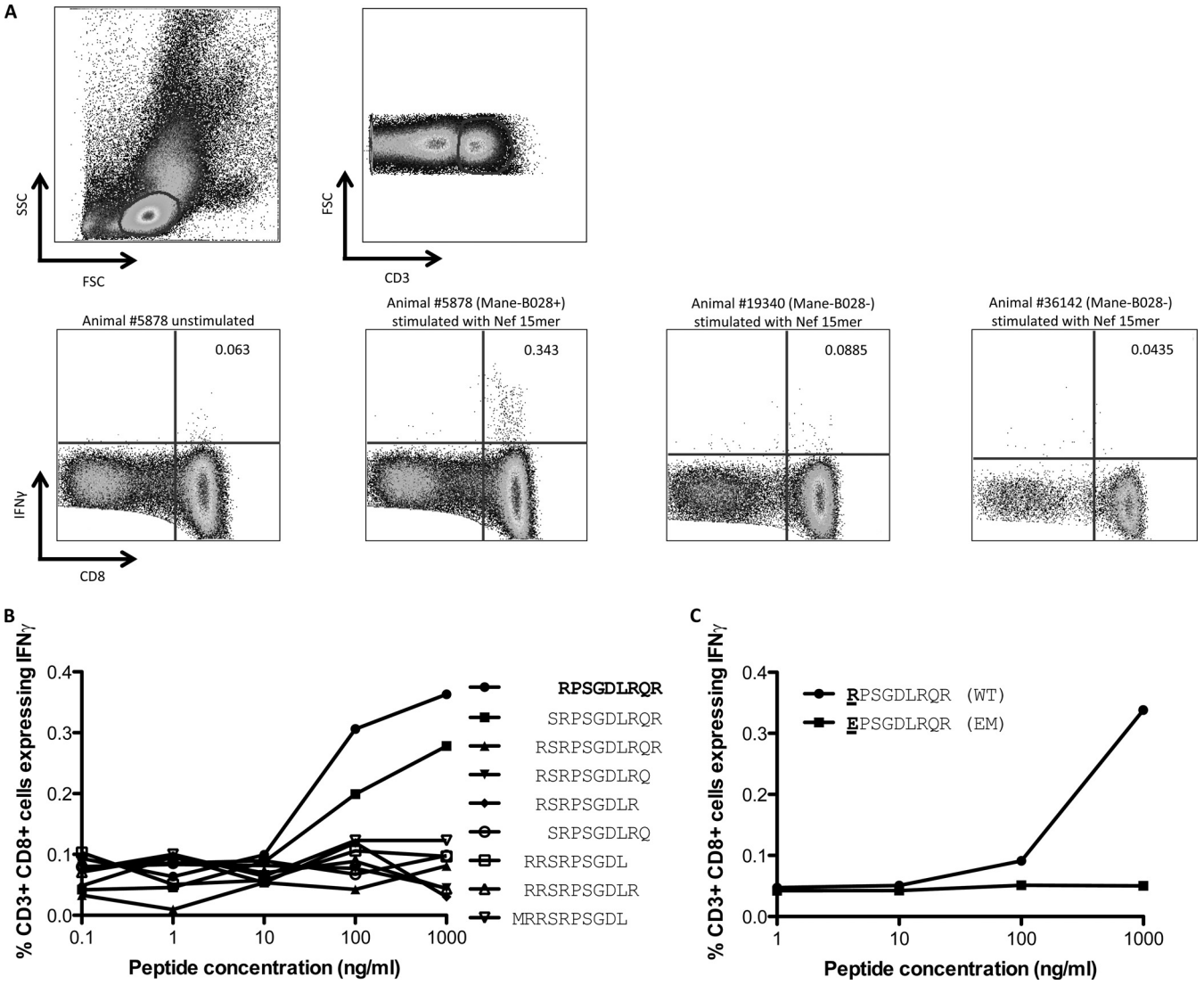

FIG 4 SIV Nef-specific CD8 T cell response predicted by linkage analysis. (A) Whole blood from 3 SIV-infected macaques was stimulated by 3 SIV Nef 15-mer peptides spanning the site of a putative Mane-B028-restricted CTL response identified by the MHC-I–SIV mutation linkage analysis. Gated $CD3^+$ $CD8^+$ lymphocytes were studied for IFN-γ expression, with the 1 Mane-B028-positive macaque showing a positive response and 2 Mane-B028-negative macaques showing no response. SSC, side scatter; FSC, forward scatter. (B) The minimal Nef-specific CTL epitope was mapped to a 9-mer peptide using serial dilutions of overlapping 9- to 11-mer peptides. (C) The putative CTL escape mutation (R to E at position 1) identified in the linkage analysis was evaluated for whether it abrogated CTL recognition.

their escape motifs at these sites, since (i) some of the observed associations may have occurred by chance, (ii) some mutations may reflect compensatory changes for CTL mutations elsewhere, and (iii) some mutations may reflect adaptation to high-avidity CD8 T cell responses rather than escape mutations (8, 46). We conducted a permutation analysis to estimate the likelihood of a false discovery rate, and we report only associations with greater than 86% and 66% likelihoods of being a true association by sliding window and point mutation analyses, respectively.

Interestingly, our analyses found a relatively large number of associations within the variable Env protein (33 of 78 associations). These likely reflect the higher levels of variability in Env than in other SIV proteins. Nonetheless, we also identified a total of 24 associations within the less variable Gag and Pol proteins. Given the associations between Gag CTL responses and control of both HIV and SIV (47), many of these new potential epitopes will be of interest to those studying CTL-based control of SIV in this model. As a proof of principle, our studies to date validated that our approach (i) correctly identifies immune escape mutations within 3 known pig-tailed macaque CTL epitopes restricted by Mane-A1*084, (ii) detected using functional T cell assays that a CTL epitope identified in our linkage and sequencing analyses was indeed directed to a Mane-B028-restricted Nef CTL epitope, and (iii) identified Mane-B017-linked mutations in epitopes previously defined in Mamu-$B017^+$ rhesus macaques (39). However, more work remains to be done to validate a larger proportion of the putative CTL responses identified in this report, and we are currently infecting additional pig-tailed macaques to begin those studies. Identifying a wider suite of SIV-specific CTL epitopes and their escape patterns should assist with identification of which

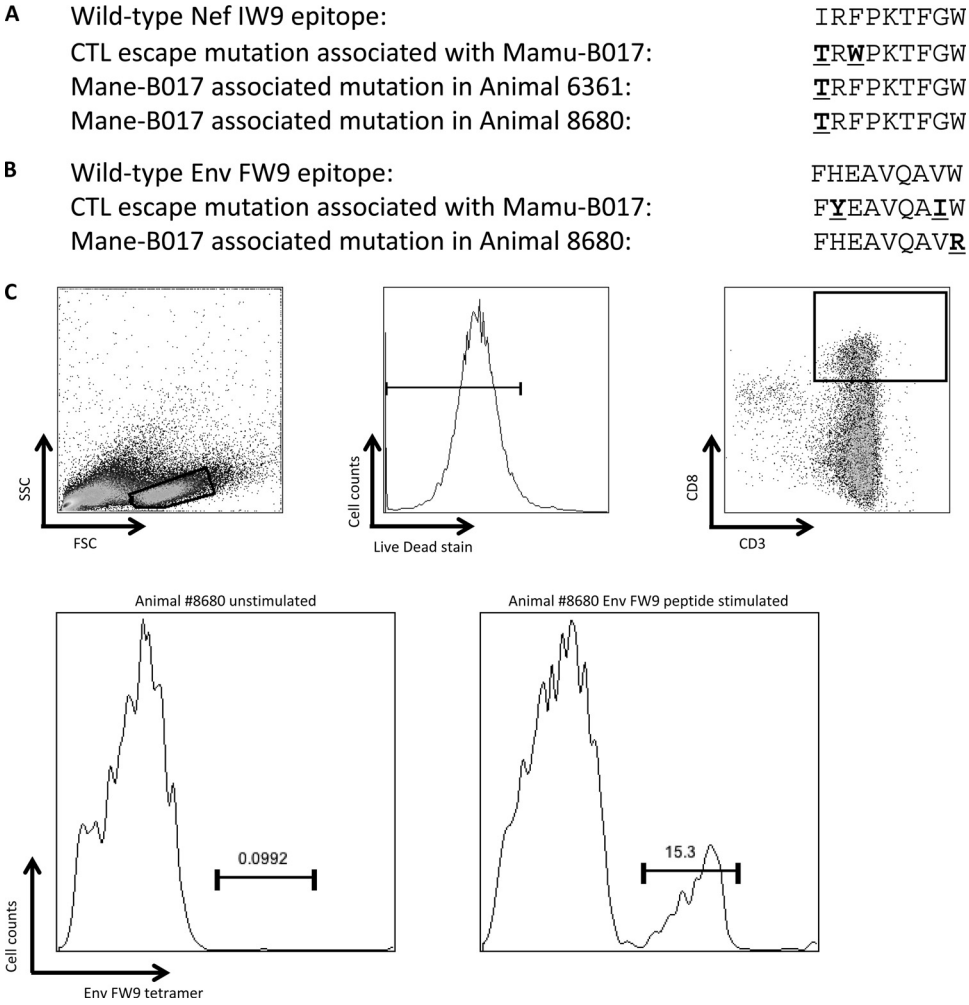

FIG 5 Recognition of known Mamu-B017-restricted CTL epitopes identified in rhesus macaques by Mane-B017$^+$ pig-tailed macaques. (A and B) The SIV sequences of 2 CTL epitopes previously identified in Mamu-B017$^+$ rhesus macaques (Nef IW9 and Env FW9) were studied in Mane-B017$^+$ pig-tailed macaques and compared to common mutations observed in Mamu-B017$^+$ rhesus macaques. The mutations identified are underlined and in bold. (C) Identification of an Env FW9/Mane-B017 tetramer-specific CD8 T cell population in PBMCs from a Mane-B017$^+$ pig-tailed macaque. PBMCs were unstimulated or stimulated for 10 days with the Env epitope, and gated CD3$^+$ CD8$^+$ lymphocytes were stained with an Env FW9/Mane-B017 tetramer.

CTL responses or combinations of CTL responses (either by vaccination or by natural infection) are best able to assist with control of SIV. It is notable that our experiments validated that rhesus macaque MHC reagents, such as Mamu-B*017 tetramers, can detect T cell responses in pig-tailed macaques sharing very similar MHC-I alleles, which will allow some of the tools developed for rhesus macaque research to be used for the study of pig-tailed macaques.

Our use of a cohort of 44 SIV-infected pig-tailed macaques allowed us to ask if any particular MHC-I haplotypes were strongly associated with better control of SIV. Only weak VL associations with 3 Mane class I haplotypes were observed, and none were significant after adjusting for multiple comparisons. There are caveats in our analyses, since (i) there were only 44 animals studied, so we did not have a robust power to detect VL differences, and (ii) the 44 animals underwent a variety of treatments prior to and during SIV infection, although we attempted to control for these variables using a two-way ANOVA. We accept that there may well be MHC-I haplotypes that are associated with at least the modest control of SIV in pig-tailed macaques and that might be picked up with a much larger and more stringently selected cohort of animals. An important negative finding was that the presence of Mane-A084 was not associated with a significantly improved control of SIV, which contrasts with a preliminary report from our group studying a much smaller ($n = 8$) cohort (13), although the findings of the analysis described in this report are in agreement with those of another group who found no affect of Mane-A084 on plasma SIV load across 32 pig-tailed macaques (11 expressing Mane-A084) (48). We have developed a number of reagents for Mane-A1*084 (including MHC tetramers and genetic techniques to screen animals for this allele [14]) and have previously characterized the escape patterns at the known Mane-A1*084-restricted KP9, KVA10, and KSA10 CTL epitopes. Our analyses should allow scientists to proceed with studies using Mane-A084$^+$ pig-tailed macaques without undue concern that these animals will control SIV to a much greater extent than Mane-A084-negative animals.

In summary, we employed a linkage analysis between MHC-I

alleles and SIV sequences in SIV-infected pig-tailed macaques to identify potential CTL epitopes undergoing mutational escape. This work provides a basis to improve the pig-tailed macaque model of SIV infection and ultimately better understand the CTL-based control of SIV and HIV infection.

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