Major Histocompatibility Complex Class I Alleles Associated with Slow Simian Immunodeficiency Virus Disease Progression Bind Epitopes Recognized by Dominant Acute-Phase Cytotoxic-T-Lymphocyte Responses

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Certain major histocompatibility complex class I (MHC-I) alleles are associated with delayed disease progression in individuals infected with human immunodeficiency virus (HIV) and in macaques infected with simian immunodeficiency virus (SIV). However, little is known about the influence of these MHC alleles on acute-phase cellular immune responses. Here we follow 51 animals infected with SIV$_{mac}$239 and demonstrate a dramatic association between Mamu-A*01 and -B*17 expression and slowed disease progression. We show that the dominant acute-phase cytotoxic T lymphocyte (CTL) responses in animals expressing these alleles are largely directed against two epitopes restricted by Mamu-A*01 and one epitope restricted by Mamu-B*17. One Mamu-A*01-restricted response (Tat$_{28-35}$SL8) and the Mamu-B*17-restricted response (Nef$_{165-173}$IW9) typically select for viral escape variants in early SIV$_{mac}$239 infection. Interestingly, animals expressing Mamu-A*1 and -B*17 have less variation in the Tat$_{28-35}$SL8 epitope during chronic infection than animals that express only Mamu-A*01. Our results show that MHC-I alleles that are associated with slow progression to AIDS bind epitopes recognized by dominant CTL responses during acute infection and underscore the importance of understanding CTL responses during primary HIV infection.

Several lines of evidence suggest that cellular immunity is critically important in the immune control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). Strong antigen-specific CD8$^+$ and CD4$^+$ T-lymphocyte responses are correlated with improved clinical outcome in HIV-infected individuals (7, 33, 49), while depletion of CD8$^+$ lymphocytes in SIV-infected macaques accelerates disease progression (20, 30, 55). Additionally, expression of certain major histocompatibility complex class I (MHC-I) and MHC-II alleles, such as HLA-B*27 and HLA-B*57, is associated with unusually favorable clinical outcomes (long-term nonprogression) (5, 8, 12–14, 17, 21–24, 35, 50, 53, 57, 58).

Chronic-phase cellular immune responses in HIV long-term nonprogressors expressing HLA-B*27 and -B*57 have been characterized in detail. Immunodominant responses directed against epitopes within Gag p24 are restricted by each of these molecules (11, 12, 15, 16, 42, 43). In addition, strong cytotoxic

T lymphocyte (CTL) responses restricted by these molecules have been mapped in Rev, Env, and Pol (25, 56, 59). Most previous studies of long-term nonprogressors have emphasized the circumstantial link between these dominant chronic-phase CTL and delayed disease progression.

However, these chronic-phase CTL may be only one component of the vigorous chronic-phase immune response that characterizes long-term nonprogression. CD8$^+$ T cells of nonprogressors proliferate, produce perforin, and elaborate soluble virus inhibitors (29, 34, 63, 68). Additionally, strong HIV-specific CD4$^+$ proliferative responses are frequently detected in nonprogressors (49). It is not clear why these antigen-specific responses occur predominantly in individuals with certain MHC-I genotypes. One possibility is that the acute-phase CTL responses initially contain viral replication, allowing for the development of a coordinated and robust antiviral response. In support of this theory, plasma viral load following acute infection is predictive of disease outcome in HIV-infected individuals (viral setpoint) (10, 31, 32). Acute-phase cellular immune responses, which arise coincidently with the decline in primary

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viremia (65), likely play a role in determining this viral set-point.

Unfortunately, it is difficult to study these immune responses during acute HIV infection (66). Synthetic peptides for ex vivo CTL assays need to be tailored to each infected individual, since consensus peptides do not accurately reflect the genotypic variability among infecting virus strains (62). Moreover, most HIV-infected individuals do not know precisely when they were infected, and many remain asymptomatic throughout acute infection. By the time that HIV infection is verified, MHC-I alleles are identified, and autologous viral peptides are synthesized, the narrow window of opportunity for studying the immune responses that are restricted by a patient's MHC-I alleles that might delay disease progression during acute HIV infection is usually closed. Complicating the situation, immune escape variants can be detected as early as 3 weeks postinfection (45) and can dominate the circulating virus population by 4 weeks postinfection (3). Studying the responses that select for these variants requires an exquisite knowledge of the precise timing of infection that is lacking in most cases of HIV.

The hypothesis that important acute-phase CTL responses influence long-term clinical outcome is more easily tested in macaques experimentally infected with SIV, since the precise timing of the acute phase, as well as the genetic composition of the infecting virus, are carefully controlled. We reasoned that the delayed disease progression associated with particular MHC-I alleles results from effective control of viral replication during acute infection. By evaluating acute-phase CTL responses prior to the emergence of viral escape variants, we eliminate the systematic bias against potentially important, but ephemeral, CTL that rapidly select for escape variants. In this study, we identify an association between three MHC-I alleles and positive outcome following challenge with highly pathogenic $SIV_{mac}239$. We then show that the MHC-I alleles associated with improved control bind epitopes recognized by the dominant CTL responses during acute infection.

MATERIALS AND METHODS

Animals, viruses, and infections. Fifty-one macaques were infected with a molecularly cloned virus, $SIV_{mac}239$. Five stocks of virus were used, namely, stocks 1 to 3 ($SIV_{mac}239$/nef open expanded on rhesus peripheral blood lymphocytes), stock 4 ($SIV_{mac}239$/nef stop expanded on rhesus peripheral blood lymphocytes), and stock 5 ($SIV_{mac}239$/nef open expanded on CEMx174). $SIV_{mac}239$/nef stop differs from $SIV_{mac}239$/nef open by a stop codon present in the nef gene (52). There is an intense selection for full-length Nef protein in vivo, and the nef open reading frame is restored within a few weeks of infection. Animals infected with $SIV_{mac}239$/nef open received 10 50% monkey infectious doses, comprising approximately 3,000 50% tissue culture infective doses ($TCID_{50}$), intrarectally. Animals infected with $SIV_{mac}239$/nef stop received 1,000 $TCID_{50}$ intrarectally. Animals infected with $SIV_{mac}239$/nef open expanded on CEMx174 cells received 20 ng of p27 $SIV_{mac}239$ (3,000 $TCID_{50}$) intrarectally. Twenty-one of the 51 macaques, including SIV-controlling (controller) animals 95061 and 95096, were vaccinated as part of previous studies that failed to ameliorate disease progression (1, 2, 19, 61).

Stock 1 was used to challenge 11 animals (including Mamu-A*01-, -B*17-, and -B*29-positive animal 95061), stock 2 was used to challenge 8 animals, and stock 3 was used to challenge 22 animals (including Mamu-A*01-, -B*17-, and -B*29-positive animals 2065, 2095, and 2129). The $SIV_{mac}239$/nef open expanded stock 4 was used to challenge 6 animals (including Mamu-A*01-, -B*17-, and -B*29-positive animals 1937 and 95096), while the $SIV_{mac}239$/nef stop stock was used to challenge 7 animals. SIV-infected animals at the University of Wisconsin were cared for in accordance with an experimental protocol approved by the University of Wisconsin Research Animal Resource Committee.

Viral load analysis. Viral loads were quantitated by using the SIV branched-chain DNA assay (Chiron Diagnostics, Emeryville, Calif.) and the Taqman kinetic reverse transcriptase PCR assay (18, 64). SIV viral RNA levels were measured every week for the first 4 weeks and biweekly thereafter.

Statistical analysis. Viral load differences between groups infected with $SIV_{mac}239$ were tested for statistical significance by using t tests after log transformation of the data to improve normality and homoscedasticity. In addition, Levene's test for homoscedasticity was conducted, and if differences were found to be significant, the Welch correction for unequal variances was employed. Finally, to further examine the robustness of the results, a nonparametric test, the Mann-Whitney U test, was performed. The P values for the nonparametric tests were calculated by exact methods. All the P values are two tailed.

In determination of the extent of variability in $Tat_{28-35}SL8$, mean and median proportions of nonsynonymous nucleotide differences per nonsynonymous site (p_N) in the $Tat_{28-35}SL8$ epitope in comparisons between viruses from monkeys and the inoculum were calculated by using the direct virus sequence shown in Fig. 5B. Proviral sequence from 95061 and 95096 and plasma sequence from the other 16 animals were weighted equally in this analysis. Polymorphic nucleotides were weighted equally in computation of mean p_N between the sequences of virus from each monkey and the inoculum.

MHC-I typing by PCR-SSP. Using the technique of sequence-specific DNA amplification (PCR-SSP), we performed molecular typing of MHC-I alleles which bind SIV-derived peptides, namely, Mamu-A*01, Mamu-A*02, Mamu-A*08, Mamu-A*11, Mamu-B*01, Mamu-B*03, Mamu-B*04, Mamu-B*17, and Mamu-B*29. The 3′-terminal region of PCR-SSP primers targeted nucleotide polymorphism unique to these eight Indian rhesus MHC-I alleles. PCR was performed as previously described (26), and PCR products were electrophoresed on 2% agarose gels (0.5× Tris-borate-EDTA). The agarose gels were analyzed for the presence of an internal control product and each of the specific allele amplicons.

MHC-I and -II cloning and sequencing. From 1 to 5 million herpesvirus papio-transformed B cells or peripheral blood mononuclear cells (PBMC) were used to isolate total cellular RNA with the Qiagen total RNEasy kit (Qiagen, Valencia, Calif.) following the manufacturer's instructions. cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif.). The cDNA was amplified with Pwo DNA polymerase (Roche, Mannheim, Germany) and MHC-I and -II 5′ and 3′ sequence-specific primers (sequences available upon request). The amplified cDNA was purified from an agarose gel by using a gel extraction kit (Qiagen) and was ligated into pCR-Blunt by using the Invitrogen Zero Blunt cloning kit following the manufacturer's protocol. Ninety six clones from each animal were miniprepped and sequenced with T7 and M13 primers on an ABI 377 automated sequencing machine (Applied Biosystems, Foster City, Calif.). Sequences were analyzed using software from Applied Biosystems and Accelrys (Burlington, Mass.).

Neutralization of $SIV_{mac}239$ by SIV control sera. Serum neutralization experiments determined the antibody titer needed to protect 50% of cells from virus-induced killing, as previously described (19, 36). Additional enzyme-linked immunosorbent assays for p27 production were performed as previously described (47) to confirm these results.

Enumeration of SIV-specific $CD8^+$ and $CD4^+$ responses by intracellular IFN-γ cytokine staining. Intracellular staining for gamma interferon (IFN-γ) production was performed as previously described (37).

SIV sequencing. SIV sequencing from blood plasma was performed as previously described (44).

SIV sequences from animals 95061, 95096, 96072, and 96093 were derived from PBMC provirus. PBMC were lysed, and the genomic DNA (gDNA) was isolated by DNeasy spin column purification (Qiagen). PCR amplicons spanning the entire SIV epitopes of interest (3) were generated with the HotStarTaq master mix kit (Qiagen) and 500 ng of gDNA. At least four independent amplifications were performed for time points for animal 95061. The amplified products were purified using QIAquick PCR purification kits (Qiagen) and then directly sequenced as previously described (3). Mixed-base sites within single amplicons and single-site differences in the sequences of multiple amplicons from the same time point were both considered polymorphic. Nucleotide sequences were conceptually translated and aligned to $SIV_{mac}239$ gene products by using MacVector 7.0 trial version (Accelrys, San Diego, Calif.).

RESULTS

Identification of SIV controllers. We first identified MHC-I alleles that are associated with delayed disease progression in rhesus macaques infected with $SIV_{mac}239$. A cohort of 51

rhesus macaques was infected with the same highly pathogenic, molecularly cloned strain of SIV ($SIV_{mac}239$) (41). The animals were infected between 1998 and 2002 in five SIV vaccine trials. The vaccine immunogens were: lipopeptide encoding the $Gag_{181-189}CM9$ CTL epitope (animal 95096), DNA and modified vaccinia virus Ankara (MVA) encoding the $Gag_{181-189}CM9$ CTL epitope (eight vaccinated animals, including animal 95061) (1), DNA and MVA encoding Tat (three vaccinated animals) (2), DNA and MVA encoding seven of the nine SIV proteins (eight vaccinated animals) (19), and DNA and MVA encoding Tat, Rev, and Nef (three vaccinated animals) (T. Vogel, unpublished data). The cohort also included 29 vaccine-naive animals (1, 2, 19, 37) that initially served as controls in these vaccine studies. The geometric mean viral load in the vaccinated animals was approximately 10-fold lower than the geometric mean viral loads in the vaccine-naive controls (Fig. 1A). These differences in viral load did not appear to affect survivorship, as the vaccinated animals had a lower median survivorship than the vaccine-naive controls (Fig. 1A, inset). However, the spectrum of survivorship was broader in the animals that were vaccinated, with several vaccinated animals surviving more than 18 months after SIV challenge. Since only 51 animals were used for these comparisons, it is possible that meaningful effects of the vaccination on suppression of viral replication or disease-free survivorship have been masked.

Nonetheless, viral loads in both groups were typically greater than 10^5 copies of virus per ml of blood plasma during the first year of infection. Animals that survived for more than 1 year postinfection tended to have lower viral loads, a trend that was noticed in both the vaccinated and control groups. Based on this data, we decided that including vaccinated animals in our analysis was reasonable.

Though all of the animals in this study were infected with $SIV_{mac}239$, different $SIV_{mac}239$ stocks were used in the different vaccine studies. To verify that the virus stock did not systemically affect chronic-phase viral load, we compared the viral loads of the animals challenged with each of the five $SIV_{mac}239$ stocks (Fig. 1B). In each challenge, peak viral load exceeded 10^6 copies of virus per ml of plasma, with chronic-phase viral setpoints in excess of 10^5 copies of virus per ml of plasma. All four of the $SIV_{mac}239$ stocks expanded on rhesus macaque PBMC induced similar viral loads. The six animals infected with $SIV_{mac}239$/nef open expanded on CEMx174 had lower viral loads during chronic infection. However, three of the six animals infected with this challenge stock developed simian AIDS within 30 weeks of infection and were sacrificed. Two of the remaining three animals infected with this stock were Mamu-A*01, -B*17, and -B*29 positive, possibly accounting for the lower viral loads observed with this virus stock.

All 51 SIV-infected animals were previously tested for the presence of Mamu-A*01, Mamu-A*02, Mamu-A*08, Mamu-A*11, Mamu-B*01, Mamu-B*03, Mamu-B*04, Mamu-B*17, and Mamu-B*29 by PCR-SSP (26, 39). Notably, Mamu-B*17 and Mamu-B*29 appear to be closely linked, with 39 of 43 Mamu-B*29-positive animals also being positive for Mamu-B*17.

We grouped the animals according to their (PCR-SSP-deduced) MHC genotype and compared the viral loads and sur-

vivorship for each group (39). Of the animals that did not express Mamu-A*01, Mamu-B*17, or Mamu-B*29, median survivorship was less than 50 weeks, with only one animal surviving more than 100 weeks (Fig. 2A). In agreement with previous studies (39, 40, 46, 69), we observed that Mamu-A*01-positive animals controlled SIV replication more effectively than Mamu-A*01-negative animals following resolution of peak viremia ($P = 0.001$; t test) (Fig. 2B). However, these differences in viral load did not significantly prolong survivorship in Mamu-A*01-positive animals ($P = 0.09$) (Fig. 2B). Two vaccine-naive animals that expressed Mamu-B*17 and Mamu-B*29, but not Mamu-A*01, survived for 62 and 68 weeks postinfection, despite persistently high plasma viral loads (Fig. 2C).

Six of the 51 animals expressed Mamu-A*01, Mamu-B*17, and Mamu-B*29. Remarkably, all six animals remain alive as of this writing (Fig. 2D). The vaccination histories, SIV challenge strain, and current survivorship of these six animals are shown in Table 1. Three of these animals, namely, animals 95061, 95096, and 1937, controlled chronic phase viral replication below 5,000 vRNA copies/ml of plasma (Fig. 2D). Though animals 2065, 2095, and 2129 have chronic-phase viral loads in excess of 400,000 copies/ml (48 weeks), they have already exceeded the median survivorship of our cohort (Fig. 1D). The significantly increased survivorship ($P < 0.001$) and lower viral loads of Mamu-A*01-, -B*17-, and -B*29-positive animals infected with $SIV_{mac}239$ correlate this MHC genotype with improved disease outcome.

We verified that Mamu-A*01, Mamu-B*17, and Mamu-B*29 were the only MHC alleles shared among these animals by sequencing individual cDNA clones from all six animals (Table 2). We also typed all of these macaques for their MHC-II *DP*, *DQ*, and *DR* alleles by denaturing gradient gel electrophoresis and direct sequencing. We did not identify any additional shared alleles (data not shown).

Humoral immune responses in SIV controllers. Effective immunity to viruses such as HIV and SIV likely requires the contribution of both cellular and humoral immunity. The rapid onset of disease in most $SIV_{mac}239$-infected animals typically occurs in the absence of neutralizing antibodies (nAb) against $SIV_{mac}239$ (70); moreover, $SIV_{mac}239$ is largely insensitive to nAb. Sera from the Mamu-A*01-, -B*17-, and -B*29-positive animals, as well as sera from typical progressors, neutralized lab-adapted $SIV_{mac}251$ samples (Fig. 3). In contrast, nAb against autologous $SIV_{mac}239$ infection were rare in both groups of animals. Therefore, it is unlikely that viral control in the Mamu-A*01-, -B*17-, and -B*29-positive animals results from unusually potent nAb responses.

Acute-phase cellular immune responses in Mamu-A*01-, -B*17-, and -B*29-positive animals. We evaluated the $CD8^+$ T-lymphocyte response in five of the six Mamu-A*01-, -B*17-, and -B*29-positive animals at 4 weeks postinfection (37) by detecting intracellular cytokine production following PBMC stimulation with peptide pools spanning the $SIV_{mac}239$ proteome. Since animal 95061 was infected with $SIV_{mac}239$ before this method was introduced, we were unable to evaluate its acute-phase cellular immune response. All five animals tested responded to the peptide pools containing the Mamu-A*01-restricted epitopes $Gag_{181-189}CM9$ (4) and $Tat_{28-35}SL8$ (3) (Fig. 4). These responses are always seen in Mamu-A*01-

A. Vaccination
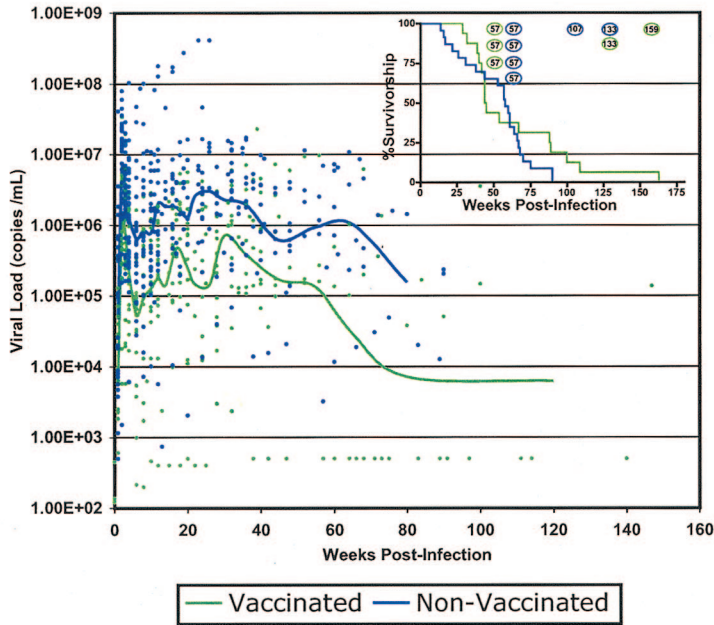
Weeks	Vaccinated	Non-Vaccinated
1.0-1.5	18	28
1.6-2.4	21	30
2.5-3.4	21	29
3.5-4.4	21	30
4.5-6.4	20	25
6.5-8.4	20	27
8.5-10.4	18	21
10.5-12.4	17	22
12.5-16.4	15	23
16.5-19.4	13	21
19.5-20.4	14	9
20.5-24.4	14	17
24.5-28.4	16	14
28.5-32.4	13	18
32.5-40.4	26	25
40.5-50	13	18
51-60	10	13
61-70	7	11
71-90	11	9
91-150	8	0

B. SIV$_{mac}$239 Stock
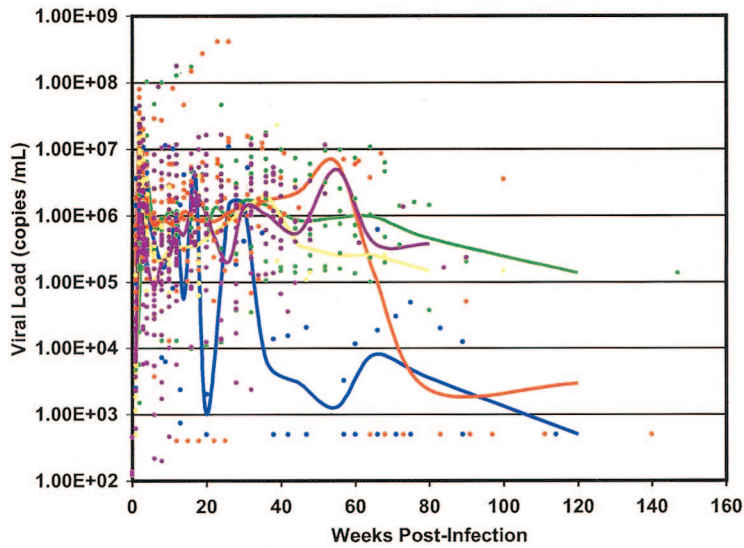
Weeks	Nef open; CEMx174 expanded	Nef stop; Rhesus expanded	Nef open; Rhesus expanded (1)	Nef open; Rhesus expanded (2)	Nef open; Rhesus expanded (3)
1.1-1.5	6	2	10	?	21
1.6-2.4	6	7	10	?	21
2.5-3.4	6	5	10	?	22
3.5-4.4	6	7	10	?	21
4.5-6.4	0	7	10	?	21
6.5-8.4	2	6	10	?	22
8.5-10.4	2	1	8	?	21
10.5-12.4	2	6	10	8	21
12.5-16.4	4	7	16	?	11
16.5-19.4	2	0	10	?	15
19.5-20.4	2	5	0	6	16
20.5-24.4	0	7	10	6	14
24.5-28.4	2	6	11	6	11
28.5-32.4	2	6	7	6	16
32.5-40.4	4	12	9	3	24
40.5-50	4	10	3	3	11
51-60	2	16	2	1	2
61-70	5	8	5	1	1
71-90	8	6	3	1	3
91-150	1	1	5	0	0

— Nef open: CEMx174 expanded
— Nef stop: Rhesus expanded
— Nef open: Rhesus expanded
— Nef open: Rhesus expanded (2)
— Nef open: Rhesus expanded (3)

FIG. 1. Influence of prechallenge vaccination and SIV$_{mac}$239 stock on viral loads. Viral loads were measured using kinetic real-time reverse transcriptase PCR or branched-chain DNA assays. Each data point used to calculate the viral load trend line is indicated as a colored dot. Trend lines were obtained by grouping viral loads into time point intervals and then determining the geometric mean of each interval. The number of samples from each interval is shown in the tables to the right. (A) Prechallenge vaccination does not influence postchallenge viral load. With the exception of the vaccinated macaques 95061 and 95096, all other vaccinated animals have viral loads in excess of 10,000 copies/ml of blood plasma.

TABLE 1. Vaccination and challenge profiles of six Mamu-A*01-*B*17-, and -B*29-positive animals

Animal no.	Vaccine	Challenge stock[a]	Peak viremia (wk)	Survivor-ship (wk)
2065	—[b]	SIVmac239 Nef open: rhesus expanded (stock 3)	4.5×10^7 (2)	57
2095	—	SIVmac239 Nef open: rhesus expanded (stock 3)	2.9×10^7 (3)	57
2129	—	SIVmac239 Nef open: rhesus expanded (stock 3)	2.2×10^7 (2)	57
1937	—	SIVmac239 Nef open: CEMx174 expanded	3.7×10^5 (2)	133
95096	Lipopeptide $Gag_{181-189}$ CM9 CTL epitope[c]	SIVmac239 Nef open: CEMx174 expanded	1.3×10^7 (2)	133
95061	DNA/MVA $Gag_{181-189}$ CM9 CTL epitope[d]	SIVmac239 Nef open: rhesus expanded (stock 1)	2.4×10^7 (2)	159

[a] Multiple stocks of SIVmac239 Nef open were used in this study; the arbitrary stock numbers correspond to the parenthetical stock numbers in Fig. 1.
[b] —, vaccine naive.
[c] Vaccine did not elicit detectable $Gag_{181-189}$CM9-specific CTL in the periphery, and results were not published.
[d] Vaccine results were published by Allen et al. (1).

positive animals, irrespective of their rate of disease progression (37). These peptide pools are not consistently recognized in Mamu-A*01-, -B*17-, and -B*29-negative macaques (see Fig. 1 in online supplemental data, http://yakui.primate.wisc.edu/people/watkins/pdf/SIVcontrollerSupp_Data.pdf).

All five Mamu-A*01-, -B*17-, and -B*29-positive animals also recognized the peptide pool containing the Mamu-B*17-restricted $Nef_{165-173}$IW9 (9, 38) epitope (Fig. 4). The same Nef peptide pool was weakly recognized by two Mamu-A*01-, -B*17-, and -B*29-negative macaques (macaques 92050 and 97086) at 3 weeks postinfection (see Fig. 1 at http://yakui.primate.wisc.edu/people/watkins/pdf/SIVcontrollerSupp_Data.pdf). However, this peptide pool contains epitopes restricted by molecules other than Mamu-B*17 (9, 44), including the Mamu-A*02-restricted $Nef_{159-167}$YY9 epitope (44, 60). Both animals 92050 and 97086 are Mamu-A*02 positive, strongly suggesting that the reactivity is directed against $Nef_{159-167}$YY9 and not $Nef_{165-173}$IW9. Nonetheless, we verified the specificity of $Nef_{165-173}$IW9 recognition in the Mamu-A*01-, -B*17-, and -B*29-positive animals by demonstrating reactivity against the synthetic $Nef_{165-173}$IW9 9-mer (data not shown). Additionally, the peptide pool Vif_{41-91} was recognized in four of the five Mamu-A*01-, -B*17-, and -B*29-positive animals (Fig. 4). Recent work has shown that this peptide pool contains three Mamu-B*17-restricted CTL epitopes (38). Unfortunately, we cannot verify that the Vif reactivity is due to Mamu-B*17-restricted responses, since our acute-phase samples from these animals are exhausted.

On average, responses against the peptide pools containing $Gag_{181-189}$CM9, Tat_{28-35}SL8, and $Nef_{165-173}$IW9 comprised over 50% of the acute-phase cellular immune responses in the Mamu-A*01-, -B*17-, and -B*29-positive animals. This is, to our knowledge, the first demonstration that MHC alleles associated with delayed disease progression bind epitopes recognized by the dominant CD8^{+} T-lymphocyte responses during acute infection.

CTL escape from Tat_{28-35}SL8 in SIV controllers. Escape from HIV- and SIV-specific CD8^{+} T-lymphocyte responses has been widely observed (3, 9, 12, 14, 44, 48, 51). $Gag_{181-189}$CM9-specific CTL can select viral escape variants in animals challenged with SIV_{mac}239 (Fig. 5) and SHIV89.6P (6). We reasoned that strong selective pressure exerted by $Gag_{181-189}$CM9-specific CTL in the Mamu-A*01-, -B*17-, and -B*29-positive animals might accelerate viral escape from this response. While 3 of 11 Mamu-A*01-positive, Mamu-B*17-, and -B*29-negative animals exhibit epitope variation in $Gag_{181-189}$CM9 by approximately 1 year postinfection, only one of the Mamu-A*01-, -B*17-, and -B*29-positive animals has variation in this epitope (Fig. 5A). It is unlikely that this difference is biologically significant, and it is more likely the result of vagaries in the rate of escape from the $Gag_{181-189}$CM9 response.

In striking contrast to the sporadic escape that is observed from $Gag_{181-189}$CM9 responses, viruses from most Mamu-A*01-positive animals escape recognition by Tat_{28-35}SL8-specific CTL by 4 weeks postinfection. Since weak responses to this epitope were detectable during chronic-phase infection of Mamu-A*01-, -B*17-, and -B*29-positive animals (data not shown), we hypothesized that the maintenance of these responses might be correlated with reduced Tat_{28-35}SL8 epitope diversity. We attempted to sequence Tat_{28-35}SL8 from plasma virus obtained 1 year postinfection. Unfortunately, repeated attempts to amplify plasma virus from animals 95096 and 95061 failed, presumably because of the exceptionally low concentration of virus in these plasma samples. However, plasma SIV from the other four SIV controllers was successfully sequenced. Though evidence for Tat_{28-35}SL8 variation was observed, the extent of variation was less dramatic than in typical

The inset shows survivorship of the vaccinated and naive macaques. Each open circle represents one animal that remains alive as of this writing. (B) Dose of SIV_{mac}239 challenge does not influence viral load. While all 51 animals were infected with SIV_{mac}239, different stocks of this virus have been used during the 3-year period when these animals were infected. All stocks of SIV_{mac}239 establish infection, maintain a persistent viral burden in excess of 10,000 copies/ml, and lead to progressive immunodeficiency.
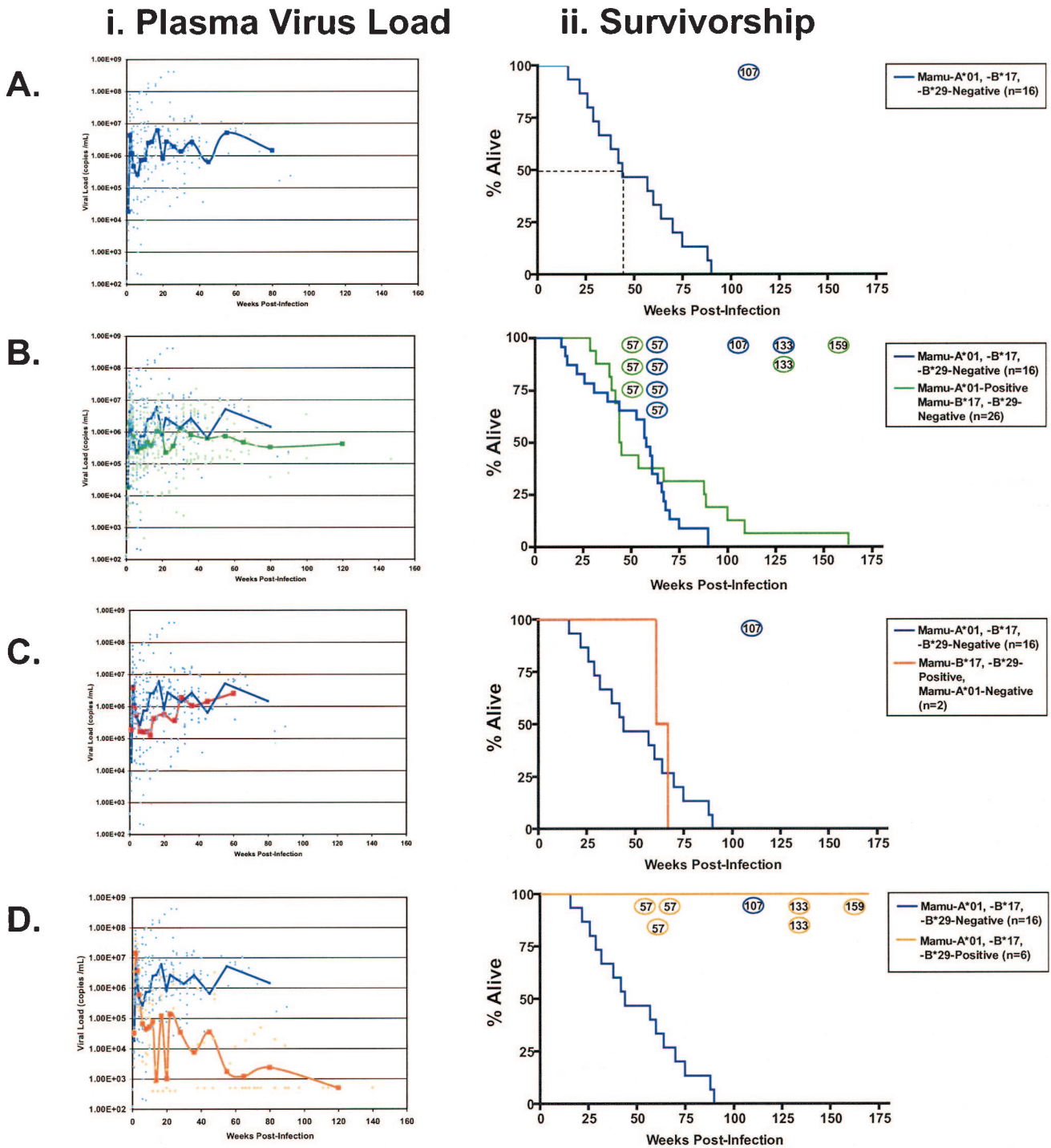
FIG. 2. Animals expressing Mamu-A*01, -B*17, and -B*29 have reduced viral loads and increased survivorship. Plasma viral loads and survivorship were followed in 51 SIV$_{mac}$239-infected rhesus macaques. Viral load trend lines were calculated as described in the legend for Fig. 1. Survivorship curves include only animals that have been sacrificed. Animals that remain alive are shown as open circles. The number of weeks postinfection is shown inside each animal's open circle. There are seven Mamu-A*01-positive animals alive at 57 weeks postinfection, of which only three are Mamu-A*01, -B*17, and -B*29 positive. (A) In 16 Mamu-A*01-, -B*17-, and -B*29-negative macaques, median survivorship was 44 weeks, with a setpoint viral load in excess of 1,000,000 copies per ml of blood plasma. (B) Mamu-A*01-positive, Mamu-B*17-, and -B*29-negative animals have slightly improved outcomes relative to Mamu-A*01-, -B*17-, and -B*29-negative macaques in terms of plasma viral load and survivorship. (C) The two Mamu-B*17- and -B*29-positive, Mamu-A*01-negative animals have slightly improved survivorship relative to the Mamu-A*01-, -B*17-, and -B*29-negative macaques. (D) All six Mamu-A*01-, -B*17-, and -B*29-positive macaques remain alive with significantly lower viral loads than the Mamu-A*01-, -B*17-, and -B*29-negative controls.

TABLE 2. MHC profiles of Mamu-A*01-, -B*17-, and -B*29-positive animals

MHC class	Profile for animal no.:					
	95061	95096	1937	2065	2095	2129
I	A*01	A*01	A*01	A*01	A*01	A*01
	B*17	B*17	B*17	B*17	B*17	B*17
	B*29	B*29	B*29	B*29	B*29	B*29
	A*02	A*11	A*11	A*1302	A*1603	A*26
	B*64	B*48	B*12	B*55	B*66	B*30
						B*65
II	DRB1*1003	DRB*W101	DRB*W2501	DRB*W307	DRB*W201	Not tested
	DRB*W2602	DBR1*0303	DRB*W2002	DRB*W702	DRB1*0309	
	DRB*W602	DRB1*1007	DRB*W2602	DRB1*0303	DRB1*0701	
	DRB1*0306	DRB*W602		DRB1*1007	DRB3*0405	
		DRB*W2501/04			DRB5*0303	
					DRB6*0101	
	DPB1*04	DPB1*06	DPB1*10	DPB1*04	DPB1*03	Not tested
	DPB1*06	DPB1*12		DPB1*06		
	DQA1*01051	DQA1*2601	DQA1*01051	DQA1*2601	DQA1*0104	DQA1*2302
	DQA1*0102			DQA1*2602	DQA1*2603	DQA1*2401
	DQB1*0602	DQB1*1801	DQB1*0602	DQB1*1801	DQB1*0601	DQB1*1804
	DQB1*0605	DQB1*1804	DQB1*1703	DQB1*1811	DQB1*1501	DQB1*1810

Mamu-A*01-positive animals ($P < 0.014$) (Fig. 5B). By 1 year postinfection, viruses from most Mamu-A*01-positive macaques have accumulated at least two dominant nucleotide substitutions within $Tat_{28-35}SL8$. In the Mamu-A*01-, -B*17-, and -B*29-positive animals, only a proline-for-serine substitution at the first position of the epitope was observed. This particular epitope variant binds Mamu-A*01 and is cross-reactive with $Tat_{28-35}SL8$-specific CTL ex vivo (3). In contrast, the double replacements at P1/P5 and P1/P8 that occurred in 8 of the 12 typical progressors do not bind to Mamu-A*01 and are not recognized by $Tat_{28-35}SL8$-specific CTL ex vivo (data not shown and reference 3).

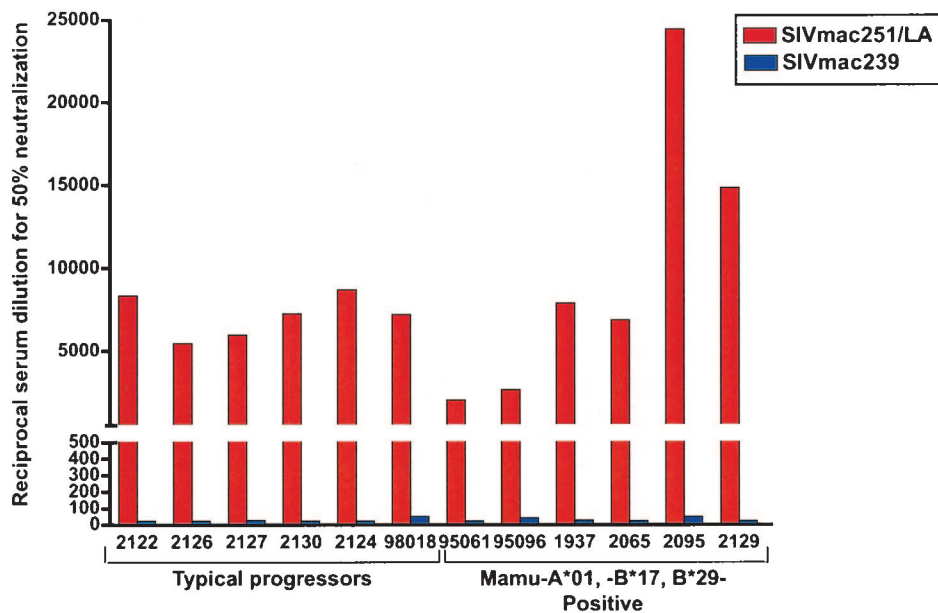FIG. 3. Sera from Mamu-A*01-, -B*17-, and -B*29-positive animals fail to neutralize $SIV_{mac}239$. Dilutions of sera from the six Mamu-A*01-, -B*17-, and -B*29-positive animals were tested for the ability to neutralize $SIV_{mac}239$ and lab-adapted $SIV_{mac}251$. Data from six typical progressors is shown for comparison. No significant neutralization activity against $SIV_{mac}239$ was detected in any of the animals.
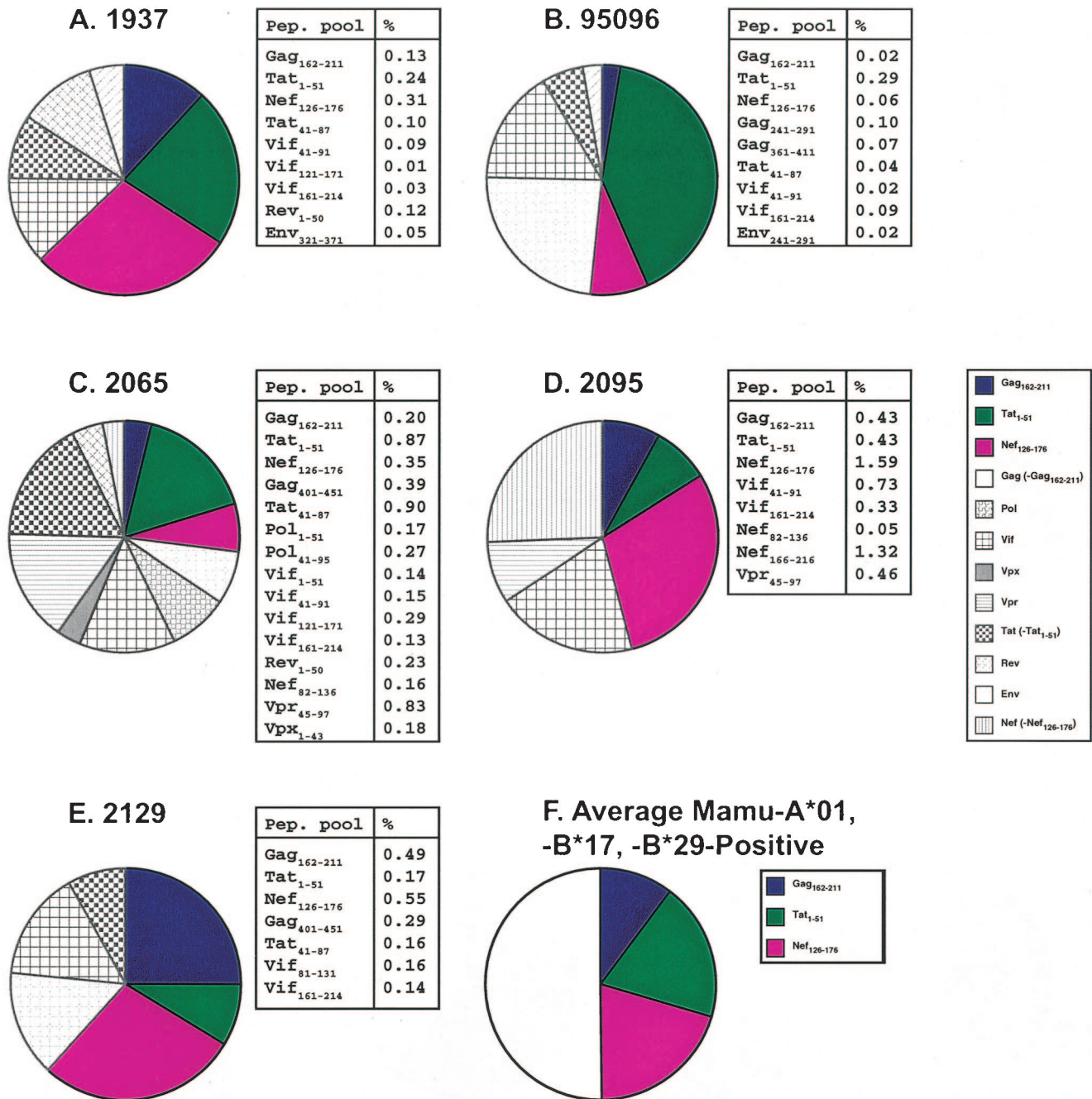
A. 1937

Pep. pool	%
Gag$_{162-211}$	0.13
Tat$_{1-51}$	0.24
Nef$_{126-176}$	0.31
Tat$_{41-87}$	0.10
Vif$_{41-91}$	0.09
Vif$_{121-171}$	0.01
Vif$_{161-214}$	0.03
Rev$_{1-50}$	0.12
Env$_{321-371}$	0.05

B. 95096

Pep. pool	%
Gag$_{162-211}$	0.02
Tat$_{1-51}$	0.29
Nef$_{126-176}$	0.06
Gag$_{241-291}$	0.10
Gag$_{361-411}$	0.07
Tat$_{41-87}$	0.04
Vif$_{41-91}$	0.02
Vif$_{161-214}$	0.09
Env$_{241-291}$	0.02

C. 2065

Pep. pool	%
Gag$_{162-211}$	0.20
Tat$_{1-51}$	0.87
Nef$_{126-176}$	0.35
Gag$_{401-451}$	0.39
Tat$_{41-87}$	0.90
Pol$_{1-51}$	0.17
Pol$_{41-95}$	0.27
Vif$_{1-51}$	0.14
Vif$_{41-91}$	0.15
Vif$_{121-171}$	0.29
Vif$_{161-214}$	0.13
Rev$_{1-50}$	0.23
Nef$_{82-136}$	0.16
Vpr$_{45-97}$	0.83
Vpx$_{1-43}$	0.18

D. 2095

Pep. pool	%
Gag$_{162-211}$	0.43
Tat$_{1-51}$	0.43
Nef$_{126-176}$	1.59
Vif$_{41-91}$	0.73
Vif$_{161-214}$	0.33
Nef$_{82-136}$	0.05
Nef$_{166-216}$	1.32
Vpr$_{45-97}$	0.46

Legend:
- Gag$_{162-211}$
- Tat$_{1-51}$
- Nef$_{126-176}$
- Gag (-Gag$_{162-211}$)
- Pol
- Vif
- Vpx
- Vpr
- Tat (-Tat$_{1-51}$)
- Rev
- Env
- Nef (-Nef$_{126-176}$)

E. 2129

Pep. pool	%
Gag$_{162-211}$	0.49
Tat$_{1-51}$	0.17
Nef$_{126-176}$	0.55
Gag$_{401-451}$	0.29
Tat$_{41-87}$	0.16
Vif$_{81-131}$	0.16
Vif$_{161-214}$	0.14

F. Average Mamu-A*01, -B*17, -B*29-Positive

Legend:
- Gag$_{162-211}$
- Tat$_{1-51}$
- Nef$_{126-176}$

FIG. 4. Peptide pools containing Gag$_{181-189}$CM9, Tat$_{28-35}$SL8, and Nef$_{165-173}$IW9 are well recognized by CD8$^+$ T-lymphocytes from Mamu-A*01-, -B*17-, and -B*29-positive animals during acute infection. (A to E) Acute-phase CD8$^+$ T-lymphocyte responses were monitored in the Mamu-A*01-, -B*17-, and -B*29-positive macaques (identified by animal number) by intracellular staining for IFN-γ production. Percentages of CD3$^+$, CD8$^+$, and IFN-γ-positive PBMC that were stimulated to produce IFN-γ by peptide pools are shown in the tables to the right of each chart. The Gag$_{162-211}$ pool (containing the Gag$_{181-189}$CM9 epitope), the Tat$_{1-51}$ pool (containing the Tat$_{28-35}$SL8 epitope), and the Nef$_{126-176}$ pool (containing the Nef$_{128-135}$IW9 epitope) were recognized in all animals. (F) On average, the three dominant responses constituted 50% of the total acute-phase CD8$^+$ T-lymphocyte response. The acute-phase responses from 1937 and 95096 were previously reported (37).

We also examined viral escape from the Mamu-B*17-restricted Nef$_{165-173}$IW9 response. Both Mamu-B*17- and -B*29-positive, Mamu-A*01-negative animals harbored escape variants in this epitope, in agreement with a previous study (9). Four of the Mamu-A*01-, -B*17-, and -B*29-positive animals also had variation in this epitope by 1 year postinfection (Fig. 5C).

Animals 95061 and 95096, the two Mamu-A*01-, -B*17-, and -B*29-positive animals with viral loads below 1,000 copies/ml of blood plasma, presented a unique opportunity to

A. Gag$_{181-189}$CM9

Mamu-A*01-Positive; Mamu-B*17, -B29-Negative			Mamu-A*01, -B*17, -B*29-Positive		
Animal	Weeks	CTPYDINQM	Animal	Weeks	CTPYDINQM
92077	30	....	2065	51	....
96135	42	....	2095	51	....
95086	44	....	2129	51	....
96123	52	....	1937	139	.C.......
95058	52	....	95061*	140	....
95045	54	....			
85013	58	.A.......			
95084	60	.i.......			
97085	67	....			
93057	68	.I.......			
96031	164	....			

B. Tat$_{28-35}$SL8

Mamu-A*01-Positive; Mamu-B*17, -B29-Negative			Mamu-A*01, -B*17, -B*29-Positive		
Animal	Weeks	STPESANL	Animal	Weeks	STPESANL
92077	30	.i..L...	2065	51	P.......
96135	42	P.......	2095	51	P.......
95086	44	P......P	2129	51	P.......
96123	52	......sp	95096*	98	p.......
95058	52	P..g....	1937	139	P.......
95045	54	P......W	95061*	140	....
85013	58	P.......			
95084	60	P......Q			
97085	67	p...l...			
93057	68	P.......			
96031	164	P......R			

C. Nef$_{165-173}$IW9

Mamu-B*17,-B*29-Positive; Mamu-A*01-Negative			Mamu-A*01, -B*17, -B*29-Positive		
Animal	Weeks	IRYPKTFGW	Animal	Weeks	IRYPKTFGW
96072	67	P........	2065	51	T........
92080	61	t........	2095	51	.....L...
			2129	51	T........
			95096*	92	....
			1937	139	T....H...
*Derived from PBMC gDNA			95061*	118	T........

FIG. 5. Variation in dominant CTL epitopes in chronic SIV$_{mac}$239 infection. The three epitopes were directly sequenced and conceptually translated approximately 1 year postinfection. Codons with identity to the wild-type sequence are shown as dots. Complete amino acid replacements are shown as uppercase single-letter amino acid codes; mixed populations are shown as lowercase single-letter amino acid codes. PBMC proviral sequences were analyzed in animals 95061 and 95096, since repeated attempts to isolate plasma virus RNA from these animals failed. Shown are results for Gag$_{181-189}$CM9 (A), Tat$_{28-35}$SL8 (B), and Nef$_{165-173}$IW9 (C).

evaluate CTL escape in the context of undetectable plasma virus and durable immune control. Despite the lack of plasma virus, integrated proviruses from these animals could be consistently amplified from PBMC-derived genomic DNA. Though proviral sequence is not as representative of the replication-competent viral population as plasma virus (54), we determined that bulk provirus and plasma sequences are similar during chronic SIV$_{mac}$239 infection (see Fig. 2 at http://yakui .primate.wisc.edu/people/watkins/pdf/SIVcontrollerSupp _Data.pdf). Wild-type Tat$_{28-35}$SL8 and Nef$_{165-173}$IW9 epitope sequences dominated in animals 95061 and 95096 throughout chronic infection (Fig. 5B and C).

To ensure that the wild-type virus in these animals remained replication competent, we cocultured CD8$^+$-depleted PBMC from animal 95061 with uninfected CEMx174 cells for 3 weeks. Viral RNA was recovered from the supernatant and sequenced. Remarkably, the outgrown virus differed from wild-type SIV$_{mac}$239 by only a single nucleotide (see Fig. 3 at http://yakui.primate.wisc.edu/people/watkins /pdf/SIVcontrollerSupp_Data.pdf). This data provides evidence that wild-type SIV$_{mac}$239 is still present in animal

95061 and suggests that the ongoing immune responses are preventing SIV$_{mac}$239 recrudescence.

DISCUSSION

For the first time, we have established a link between SIV survivorship, particular MHC-I alleles, and acute-phase CTL responses. Acute-phase CTL responses are extremely difficult to measure in HIV-infected individuals, since investigators rarely have synthetic peptides autologous to the infecting virus. Moreover, even closely related strains of HIV can differ in residues that are critical for CTL recognition. This problem may be exacerbated if viruses harboring variation in CTL responses are transmitted among individuals (12, 67). We overcame this issue by infecting all 51 animals in our cohort with the clonal virus SIV$_{mac}$239 and by assaying immune responses during the acute phase with synthetic peptides identical to the infecting virus.

It is possible that other genes linked to Mamu-A*01 or Mamu-B*17 and -B*29 influence SIV viral replication during chronic infection. Unidentified gene products could exert their effect by reducing the susceptibility of host cells to HIV infection or by slowing the production of new HIV virions in infected cells. However, during the first 2 weeks of infection, prior to the development of the CTL response, SIV$_{mac}$239 replicated to high titer in all six Mamu-A*01-, -B*17-, and -B*29-positive animals. Indeed, the levels of peak viremia in the Mamu-A*01-, -B*17-, and -B*29-positive animals were indistinguishable from those of animals that progress to simian AIDS with typical kinetics. If non-MHC host cell genes were contributing to the control of viral replication, we would have expected a reduction in peak viremia.

Alternately, it is possible that acute-phase immune responses that are first directed against epitopes bound by Mamu-A*01 and Mamu-B*17 have long-lasting effects on the chronic-phase immune response to SIV$_{mac}$239. In five of the six SIV Mamu-A*01-, -B*17-, and -B*29-positive animals, SIV-specific CD4$^+$ responses were detected during chronic infection (data not shown). HIV-infected individuals with low viral setpoints frequently mount strong HIV-specific CD4$^+$ T-lymphocyte responses (49), while HIV-infected individuals with higher viral loads and macaques with normal SIV disease progression typically do not. These CD4$^+$ responses may act in concert with the CTL responses by coordinating the immune response, suppressing viral replication, and increasing disease-free survival.

Vigorous acute-phase CTL responses in Mamu-A*01-, -B*17-, and -B*29-positive animals may also reduce the extent of viral escape during acute and chronic infection. If extremely effective CTL rapidly curb viral replication following peak viremia, the virus's capacity to spawn viable escape variants may be diminished. This is consistent with our observation that Mamu-A*01-, -B*17-, and -B*29-positive animals have less chronic-phase variation in the Tat$_{28-35}$SL8 epitope than typical Mamu-A*01-positive animals. The reduction in diversification, in turn, may preserve the ability of CTL to combat the infection. In the longest-lived Mamu-A*01-, -B*17-, and -B*29-positive animal, animal 95061, low-level Tat$_{28-35}$SL8 responses have been transiently detected throughout chronic infection by tetramer staining, IFN-γ ELISPOT, and IFN-γ intracellular cy-

tokine staining (data not shown). Additionally, the rescue of virus from animal 95061 that differs from SIV$_{mac}$239 by only a single nucleotide after 140 weeks of infection suggests that viral diversification has been reduced and that viral resurgence is actively prevented by ongoing immune responses. We hypothesize that containment of low-level SIV replication and reduction of viral diversification during chronic infection does not require the same CTL response magnitude that is required for initial viral containment during acute SIV infection. Results observed in animals challenged with SIVsmE660 and immediately treated with antiretroviral therapy indirectly support this hypothesis (27, 28). In these animals, undetectable plasma virus loads were associated with modest lymphoproliferative responses. Upon depletion of CD8$^+$ cells by in vivo treatment with monoclonal antibody, viral replication could be detected in cultures of lymph node mononuclear cells.

Interestingly, the two SIV Mamu-A*01-, -B*17-, and -B*29-positive animals with the lowest viral loads were both vaccinated prior to SIV challenge. Animal 95061 was vaccinated with DNA and MVA encoding the nonameric Mamu-A*01-restricted CTPYDINQM peptide, whereas animal 95096 was vaccinated with the same epitope delivered as a lipopeptide. None of the other animals that received these minimal vaccines differed clinically from the vaccine-naive controls. However, our observed Mamu-A*01, -B*17, and -B*29 effect may have been amplified by vaccination. Vaccination alone, however, cannot account for the improved outcome of Mamu-A*01, -B*17, and -B*29 animals, as the other four animals that expressed these two alleles were vaccine naive.

Understanding the precise role of Mamu-A*01, -B*17, and -B*29 in SIV control will require studies of additional Mamu-A*01-, -B*17-, and -B*29-positive animals infected with SIV$_{mac}$239 and other SIV strains. It is presently unclear whether the observed protective effect of Mamu-A*01, -B*17, and -B*29 positivity confers any benefit against closely related viruses such as SIV$_{mac}$251 or SIV-HIV chimeras such as SHIV89.6P. Interestingly, we previously studied a Mamu-A*01-negative, Mamu-B*17-, and -B*29-positive macaque infected with a rhesus-passaged derivative of SIV$_{mac}$239, SIVppm. Though we lacked the technology to fully evaluate the immune response in this animal at the time of the study, she lived for 127 weeks postinfection and was the longest lived animal in that five-animal cohort (9).

If these results are generalizable to other virus strains besides SIV$_{mac}$239, they may have important implications for preclinical vaccine testing. Because many CTL epitopes bound by Mamu-A∗01 have been previously identified, Mamu-A*01 animals are frequently selected for trials of vaccine efficacy. While the Mamu-A∗01 allele by itself offers only modest protection from disease progression, the selection of Mamu-A∗01-positive animals into vaccine cohorts will occasionally result in the inclusion of Mamu-A*01-, -B*17-, and -B*29-positive animals. If these animals are assigned to vaccine-naive control groups, a vaccine effect could be masked by uncharacteristically low viral loads in the controls. Perhaps more troubling, the inclusion of Mamu-A*01-, -B*17-, and -B*29-positive animals in groups of vaccinated animals could confuse vaccine-mediated viral control with genotype-mediated viral control.

One of the more interesting findings in this study is the dominance of cellular immune responses against targets bound by the alleles associated with slow progression, namely, Mamu-A*01, -B*17, and -B*29. Our association of dominance with Mamu-A*01 and Mamu-B*17 relied on our precise knowledge of strong responses directed against regions of Gag, Tat, and Nef. The three dominant responses restricted by Mamu-A*01 and Mamu-B*17 remained strong irrespective of the other MHC-I alleles present in these animals. Moreover, additional subdominant responses restricted by Mamu-A*01 and Mamu-B*17 may have further magnified the contribution of these alleles to the cellular immune response during acute infection.

Our results show, for the first time, that MHC-I alleles associated with slow disease progression bind epitopes recognized by dominant acute-phase CTL responses. In the two animals with the most effective containment of viremia, wild-type sequences persist in two CTL epitopes that normally escape CTL recognition during early infection. Thus, our study provides the first link between host immunogenetics, acute-phase CTL responses, reduction in CTL escape, and improved disease outcome following infection with a highly pathogenic immunodeficiency virus.

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