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Simian Immunodeficiency Virus-Specific Cytotoxic T Lymphocytes and Protection Against Challenge in Rhesus Macaques Immunized with a Live Attenuated Simian Immunodeficiency Virus Vaccine

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In this study, we examined the role of simian immunodeficiency virus (SIV)-specific cytotoxic T lymphocytes (CTLs) in macaques immunized with an attenuated strain of simian immunodeficiency virus (SIVmac239Δnef) in protection against pathogenic challenge with SIVmac251. Our results indicate that attenuated SIVmac239Δnef can elicit specific CTL precursor cells (CTLp), but no correlation was observed between breadth or strength of CTLp response to structural proteins SIV-Env, -Gamg or -Pol (as measured by limiting dilution assay) and protection against infection. In one animal, we longitudinally followed the SIV-Gag-specific response to an MHC class I Mamu-A*01-restricted epitope p11C, C-M using a tetrameric MHC/peptide complex reagent. A low frequency of SIV p11C, C-M peptide-specific tetramer-reactive cells was present at the time of challenge but could be expanded *in vitro*. Surprisingly, the low level of Mamu-A*01/p11C, C-M-specific CTLs induced through attenuated SIVmac239Δnef vaccination increased in the absence of detectable SIVmac251 or SIVmac239Δnef proviral DNA. Overall, our results suggest that protection against infection in this model can be achieved through more than one mechanism, with SIV-specific CTLs being important in controlling SIVmac239Δnef viral replication postchallenge. © 2000 Academic Press

INTRODUCTION

Nonhuman primate models have been extensively used to assess candidate human immunodeficiency virus (HIV) vaccines. The best protection against challenge with virulent virus has been observed after vaccination with live attenuated strains of simian immunodeficiency virus (SIV) (Beer *et al.,* 1997; Desrosiers *et al.,* 1998; Lohman *et al.,* 1994a; Nilsson *et al.,* 1998; Shibata *et al.,* 1997; Titti *et al.,* 1997). Animals vaccinated with SIVmac239 with a deletion in nef (SIVmac239Δnef) are protected against challenge with pathogenic strains of SIVmac239 and SIVmac251. The ability of live attenuated SIV to induce protection appears to vary with the degree of attenuation and length of time after vaccination (Connor *et al.,* 1998; Desrosiers, 1998; Johnson *et al.,* 1997, 1999; Langlois *et al.,* 1998; Norley *et al.,* 1996; Wyand *et al.,* 1997, 1999). Although the use of live attenuated viruses in humans remains controversial, particularly over concerns about safety of attenuated vaccines (Desrosiers, 1998, 1992; Farthing and Sullivan, 1998), an understanding of the immune correlates of protection in this model system would be of great value for vaccine development.

Several studies have evaluated the humoral and cellular immune responses in monkeys immunized with live attenuated SIV, but the correlates of protection have remained elusive (Almond *et al.,* 1997; Connor *et al.,* 1998; Dittmer *et al.,* 1995; Gauduin *et al.,* 1998; Gundlach *et al.,* 1998; Johnson *et al.,* 1997, 1999; Langlois *et al.,* 1998; Lohman *et al.,* 1994b; Norley *et al.,* 1996; Stebbings *et al.,* 1998). Initial studies suggested that maturation of protective responses took a prolonged period of time to develop, and the length of time required for protection increased with greater attenuation of the vaccine virus (Wyand *et al.,* 1997, 1999). We and others have shown that protection can be induced as early as 10 weeks after immunization with attenuated SIV, but a greater degree of protection can be achieved with a longer period before challenge (15–25 weeks) (Connor *et al.,* 1998; Norley *et al.,* 1996). We found that protection was achieved in the absence of detectable antibodies capable of neutralizing the SIVmac251 challenge virus *in vitro* (Connor *et al.,* 1998), suggesting that cellular immune responses may play a more critical role in mediating protection. Virus-specific cytotoxic T lymphocytes (CTLs) have been shown to suppress SIV replication (Schmitz *et al.,* 1999) and may have a role in protection against HIV-1 infection in some exposed but uninfected individuals (Fowke *et al.,* 1996; Rowland-Jones *et al.,* 1998, 1995). In this study, we

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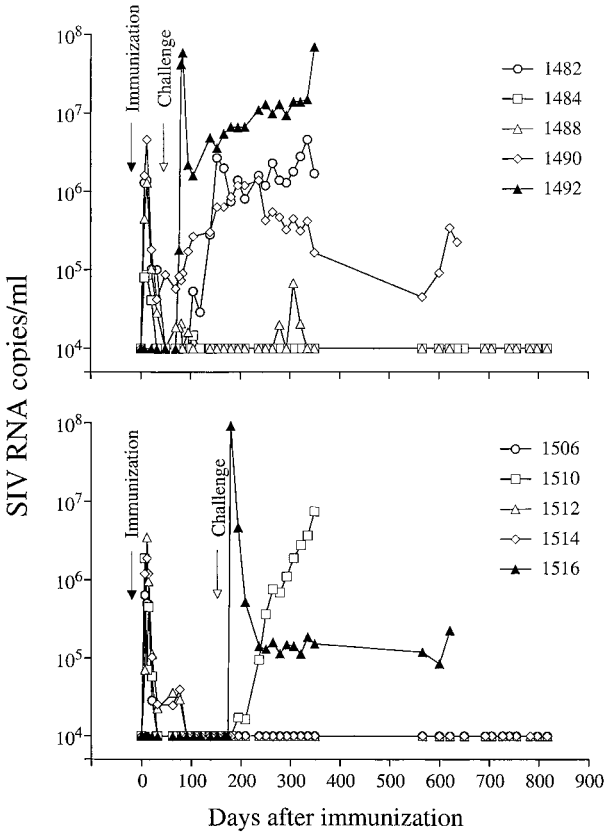

FIG. 1. SIV RNA in plasma after immunization with SIVmac239Δnef and challenge with SIV mac251. Viremia was determined by measuring total SIV RNA in plasma in a bDNA assay (sensitivity of 10^4 RNA copies/ml). Immunization with SIVmac239Δnef was performed on day 0. The animals were then challenged with SIVmac251 at either 10 (group B, top) or 25 (group D, bottom) weeks. Levels of SIV RNA for the non-immunized control animal in each challenge group are designated (▲) (Rh 1492 group B; Rh 1516 group D).

assessed SIV-specific CTL responses in rhesus macaques immunized with SIVmac239Δnef and challenged with pathogenic SIVmac251 at 10 or 25 weeks after vaccination (Connor *et al.,* 1998). We used limiting dilution analysis and soluble tetrameric MHC/peptide complexes to quantify SIV-specific T cells.

RESULTS

Longitudinal SIV-specific CTLp analysis

The immunological studies reported here were performed on monkeys included in the study by Connor *et al.* (1998). Figure 1 shows the changes in SIV plasma viremia over time (Connor *et al.,* 1998). Animals were infected intravenously with 2×10^4 $TCID_{50}$ SIVmac239Δnef and challenged with 10 animal infectious doses (AID) of uncloned SIVmac251 (kindly provided by Dr. R. Desrosiers) via the same route, either 10 (Fig. 1, top) or 25 (Fig. 1, bottom) weeks later. Nonvaccinated control animals in both groups (Rh 1492, 10 weeks; Rh 1516, 25 weeks) became infected with SIVmac251 and died from simian AIDS.

The CTL precursor frequencies to SIV-Env, -Gag, or -Pol antigens are shown in Table 1. Based on an earlier report of the induction of vigorous CTL responses by attenuated SIV (Johnson *et al.,* 1997), we had expected to find a high frequency of SIV-specific CTLp at the time of challenge in the SIVmac239Δnef-immunized macaques. Six of eight monkeys immunized with SIVmac239Δnef had SIV-specific CTLp above the level of detection after immunization and before the day of challenge, although the CTLp frequencies varied (Table 1). The 25-week challenge animals had a lower total mean of CTLp frequencies just before challenge compared with the 10-week group. However, we saw no significant difference between the frequency or breadth of SIV-specific CTLp frequencies after immunization with SIVmac239Δnef and protection against challenge with pathogenic SIV.

Longitudinal analysis of the Mamu-A*01/p11C, C-M peptide $CD8^+$ T cell response in animal Rh 1484

All animals were typed for the MHC class I Mamu-A*01 allele (D. Watkins, Wisconsin Primate Center) and one monkey, Rh 1484 was found positive. In this animal, we followed the SIV Gag-specific CTL response to the Mamu-A*01 Gag-derived p11C, C-M epitope longitudinally (Allen *et al.,* 1998; Miller *et al.,* 1991) using a tetrameric Mamu-A*01/p11C, C-M peptide complex (Altman *et al.,* 1996; Kuroda *et al.,* 1998). The tetramer was constructed as previously described (Hanke *et al.,* 1999) and was used to stain a known SIV Gag p11C, C-M CTL line as positive control. The negative control was peripheral blood mononuclear cells (PBMCs) from an SIV uninfected Mamu-A*01-positive animal (Hanke *et al.,* 1999). To our knowledge, this is the first report of SIV-specific $CD8^+$ T cell responses to attenuated SIV viruses quantified using the tetramer technology.

The initial measurements of Mamu-A*01/p11C, C-M-specific T cells after SIVmac239Δnef immunization showed that a low frequency of up to 0.1% of circulating $CD8^+$ PBMCs reacted with the tetramer (Fig. 2). This CTL response is about 10-fold lower than has been reported after infection with pathogenic SIV (Kuroda *et al.,* 1998, 1999a,b). There was no correlation between the number of tetramer positive T cells and the reduction in plasma viremia. Three weeks after challenge, plasma viremia was transiently detected at a low level and remained undetectable for the duration of the study; however, the frequency of SIV Gag p11C, C-M-specific T cells increased steadily (Fig. 2). This animal had >2% $CD8^+$ T cells specific for SIV Gag 830 days postchallenge and no detectable SIVmac251 or SIVmac239Δnef proviral DNA (K. Metzner, personal communication).

TABLE 1

SIV-Specific CTL Precursor Frequencies in Rhesus Macaques

Macaque	Protection[a]	Challenge[b]	Day	SIV CTLp[c]		
				env	gag	pol
1482	NP	10	49 pre	16	187	<15
1484	NP	10	49 pre	28	39	46
1488	P	10	49 pre	<15	<15	<15
1490	P[d]	10	49 pre	46	94	66
1492	C	10	49 pre	<15	<15	<15
1506	P	25	63 pre	<15	<15	<15
			139 pre	103	67	<15
			195 post	45	219	ND
1510	NP	25	63 pre	25	23	20
			139 pre	17	<15	<15
			180 post	350	<15	42
1512	P	25	63 pre	340	<15	36
			139 pre	<15	<15	<15
			195 post	403	<15	<15
1514	P	25	21 pre	<15	<15	<15
			63 pre	<15	<15	<15
			139 pre	<15	<15	<15
			195 post	<15	<15	<15
1516	C	25	63 pre	<15	<15	<15
			139 pre	<15	<15	<15
			195 post	<15	16	16

[a] Protection was defined as a failure to detect SIVmac251nef sequences by nested DNA PCR (Connor *et al.,* 1998). NP, not protected; P, protected; C, control.

[b] Macaques were challenged intravenously at either 10 or 25 weeks after immunization with SIVmac239Δnef using 10 AID SIVmac251 (Connor *et al.,* 1998). CTLp was measured before (pre) or after (post) challenge.

[c] SIV CTLp was measured using limiting dilution analysis for SIV env, gag, and pol antigens. Recombinant vaccinia viruses were vAbT252 (SIVmac251 gag), vAbT258 (SIVmac251 pol), and vAbT253 (SIVmac251 env). Results are expressed as CTLp/10^6 PBMCs. Background CTLp was <15/10^6 PBMCs. ND, not determined.

[d] Rh 1490 was unable to control replication of SIVmac239Δnef and died from simian AIDS. No evidence for infection with SIVmac251 was found (Connor *et al.,* 1998).

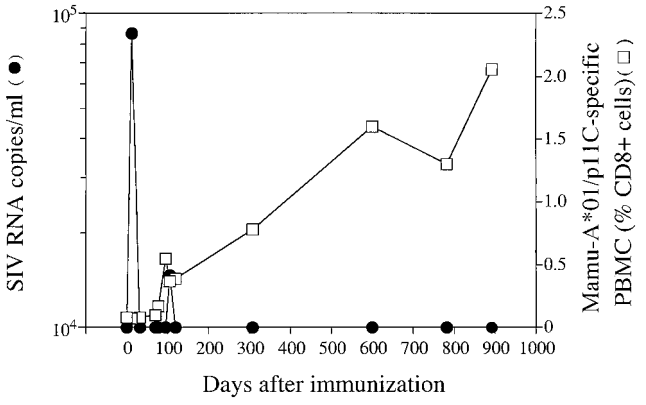

FIG. 2. Longitudinal analysis of SIV Gag p11, C-M-specific tetramer-positive CTLs in group B animal Rh 1484. This animal was immunized with SIVmac239Δnef and challenged with SIV mac251 10 weeks later. Plasma viremia is shown on the *y* axis (sensitivity of 10^4 RNA copies/ml), and percentage of $CD8^+$ T cells specific for the SIV Gag p11C, C-M tetramer is shown on the secondary *y* axis. Days after immunization is shown on the *x* axis.

Mamu-A*01/p11C, C-M peptide $CD8^+$ T cells killed peptide pulsed targets in a ^{51}Cr release assay

To assess the ability of tetramer-positive cells to mount effector functions *in vitro*, PBMCs at 14 and 781 days after SIVmac239Δnef immunization were expanded *in vitro* with the p11C, C-M peptide (1 μg/ml) and interleukin-2 (IL-2) and stained with tetramer after 7 days of *in vitro* culture (Fig. 3B). Even though there was less than 0.1% SIV Gag p11C, C-M-specific $CD8^+$ T cells in fresh blood at 14 days postimmunization, p11C, C-M tetramer-reactive T cells could be expanded (Fig. 3B). Functional CTLs were also expanded at the other time points tested (Fig. 3C).

DISCUSSION

Our results indicate that CTLs are induced in rhesus macaques after immunization with a live, attenuated SIV vaccine; however, we found no correlation between SIV-specific CTLp frequencies and protection from pathogenic SIV challenge. Relatively few studies have quantified the CTLp frequencies after vaccination of rhesus

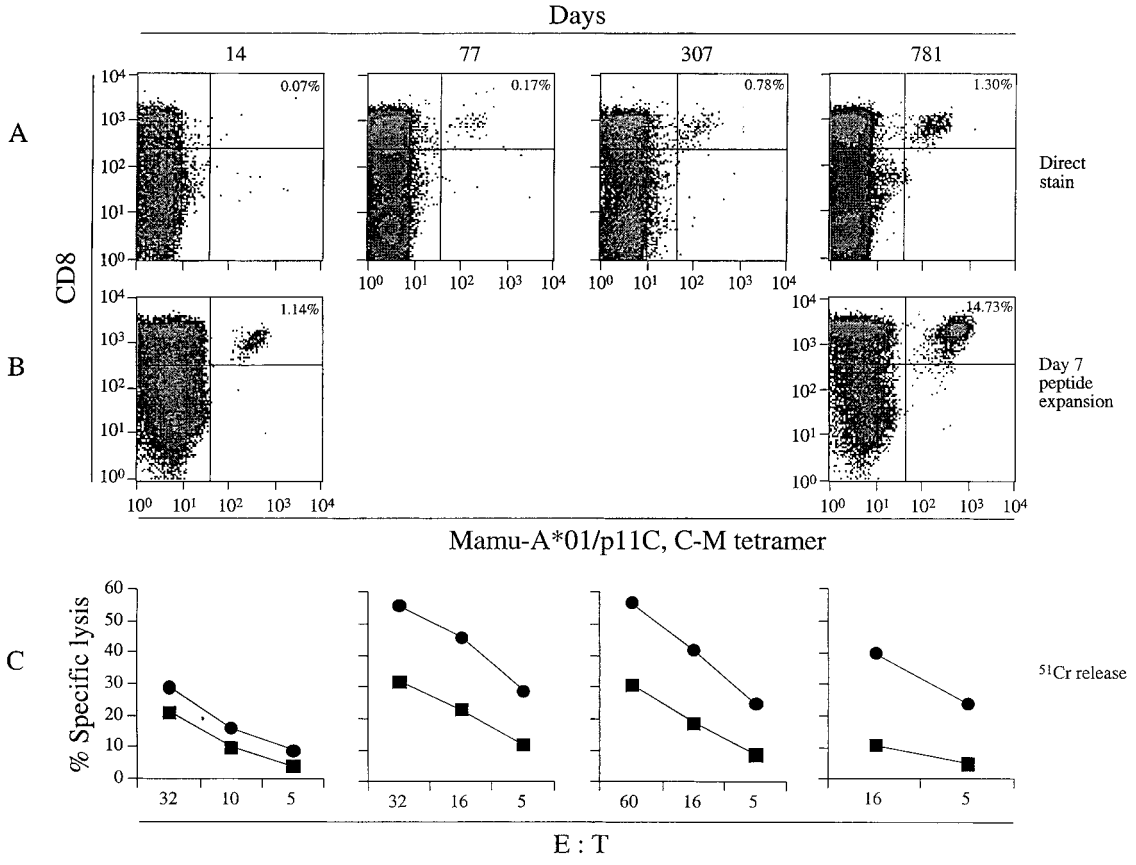

FIG. 3. Quantification of SIV Gag p11C, C-M-specific CD8$^+$ T cell responses. The percentage of SIV Gag p11C, C-M tetramer-positive CD8$^+$ T cell responses is shown in the top right corner of each FACS panel. Four representative time points are shown from days 14, 77, 307, and 781 after immunization. (A) Direct staining of PBMCs. (B) Expansion of SIV Gag p11C, C-M tetramer-positive CD8$^+$ T cells after a 7-day *in vitro* expansion with SIV Gag p11C, C-M peptide. (C) Effector CTLs lyse autologous target cells pulsed with the p11C, C-M peptide (●) but not control peptide (■). The effector/target (E/T) ratio is shown on the *x* axis.

macaques, and comparative analyses of the data are often complicated by differences in vaccine strategies and technical variations in the limiting dilution assay (Gallimore *et al.,* 1995; Hulskotte *et al.,* 1995; Johnson *et al.,* 1997; Kent *et al.,* 1996; Yasutomi, 1995). Of particular relevance to the current study, Johnson *et al.* (1997) reported vigorous CTL responses in rhesus macaques infected with live, attenuated strains of SIV. Their findings indicate that CTLs are maintained at relatively high levels throughout the course of infection, suggesting that CTLs play an important role in protection. It is worth noting, however, that CTLp frequency analysis was performed for only two animals, each of which was chronically infected with SIVmac239Δnef for more than 3 years (Johnson *et al.,* 1997). In contrast, we evaluated CTLp frequencies in 10 macaques during the first 6 months after immunization with SIVmac239Δnef, when protective responses are known to develop (Connor *et al.,* 1998; Norley *et al.,* 1996). Interestingly, the CTLp frequencies measured against SIV-specific proteins both in our study and that of Johnson *et al.* (1997) were similar in magnitude, indicating that CTL responses can be quantified both early and late in infection by these methods. Moreover, these results suggest that our inability to detect CTLp at certain time points cannot be attributed solely to technical differences in the limiting-dilution assay. One possible explanation for the low CTLp frequencies observed in some animals on day 139 after immunization is a decay in SIV-specific effector CTLs as a result of low or undetectable virus replication, similar to what has been observed in HIV-1-infected humans placed on highly-active antiretroviral therapy (Ogg *et al.,* 1999). In the latter case, HIV-1-specific CTL responses decayed exponentially after initiation of therapy, with a median half-life of 45 days, and continued to decline for as long as viremia remained undetectable.

Our results using tetrameric MHC/peptide staining of PBMCs from a Mamu-A*01 positive macaque indicate that immunization with SIVmac239Δnef elicits an SIV-specific CD8$^+$ T cell response at low frequency that consistently increases 10–15 weeks after immunization. The stimuli for these prolonged CD8$^+$ T-cell expansions are unknown but indicate a persistence of viral antigen. Using real-time PCR and a sensitive detection system capable of discriminating between SIVmac239Δnef and SIVmac251, we were unable to detect proviral DNA from

SIVmac251 in PBMCs from this animal for more than 2 years after challenge. Only low levels of SIVmac239Δnef proviral DNA were detected during this period, indicating significant inhibition of both the vaccine and challenge viruses (K. Metzner, personal communication). In lymph nodes from this animal, both SIVmac239Δnef and SIVmac251 were detected by DNA PCR (Sodora *et al.,* 1999), suggesting that low levels of virus replication in lymphoid tissues may be sufficient to stimulate cellular immune responses.

Because of the small number of animals studied and the absence of any data after pathogenic SIV challenge, it is not possible to know whether the CTLp frequencies reported by Johnson *et al.* (1997) are associated with protection from pathogenic SIV infection. Reports by other groups have noted the induction of SIV-specific CTL responses in rhesus macaques immunized with live, attenuated SIV (Dittmer *et al.,* 1995; Norley *et al.,* 1996) but have failed to find a correlation between the presence of CTLs and protection from challenge with SIV. An inverse correlation was found between vaccine-induced *nef*-specific CTLp frequencies and virus load after challenge with pathogenic SIVmac251 (32H) (Gallimore *et al.,* 1995), suggesting that both the quantitative and qualitative nature of the CTL response may play a critical role in preventing or containing SIV replication.

To address this issue, we quantified CTLp frequencies against a range of SIV-specific proteins, including Env, Gag, and Pol. Although CTLs were detected against one or more of the SIV structural proteins in virtually all macaques immunized with SIVmac239Δnef, we found no correlation between CTLp frequency and outcome after challenge with SIVmac251. This does not preclude the possibility that CTLs play an important role in mediating protection from pathogenic SIV-challenge; however, it does suggest that other mechanisms may contribute to blocking or inhibiting replication of the challenge virus. Indeed, recent work by Gauduin *et al.* (1998 has shown that SIV replication can be inhibited *in vitro* by soluble factors secreted by $CD8^+$ T lymphocytes from rhesus macaques immunized with live, attenuated SIV. In other studies, partial depletion of $CD8^+$ T cells *in vivo* failed to abrogate protection mediated by a live attenuated SIV vaccine (Stebbings *et al.,* 1998). However, interpretation of this result is complicated by the inability to effect complete and lasting $CD8^+$ T cell depletion in peripheral blood and lymph nodes.

The low frequency of tetramer-positive $CD8^+$ T cells seen in the first few weeks after immunization with SIVmac239Δnef contrasts with the early induction of virus-specific CTLs observed during primary infection with SIV (Kuroda *et al.,* 1999b; Yasutomi *et al.,* 1993) and HIV-1 (Koup *et al.,* 1994). The temporal discrepancy between the induction of CTL responses during acute SIV infection (typically 2–3 weeks) and the longer period required for protective immunity to develop (10–15 weeks) suggests that a distinct mechanism may contribute to containing virus replication in a vaccine setting. It is possible that the delay in developing protection may reflect a necessary maturation or broadening of the cellular immune response. Alternatively, recent data have highlighted protective mechanisms that do not apparently involve classic antigen-specific immune responses. For example, prior immunity to one virus can enhance clearance of a second, unrelated virus (Selin *et al.,* 1998), and noncytopathic antiviral mechanisms can contribute to viral clearance during acute hepatitis B infection (Guidotti *et al.,* 1999).

Taking these concepts into consideration, we suggest that innate and acquired cellular-based immunities may cooperate to prevent the establishment of long-term infection in animals immunized with an attenuated virus and "protected" against subsequent pathogenic infection. In this case, innate immunity may include such nonspecific effectors as NK cells or NK1.1T cells, which may be stimulated in response to vaccination. SIV-specific cellular immunity, including CTLs, may be considered as acquired immunity, developing during the course of infection. A humoral immunity model exists for such protection in influenza virus infection of mice, where "natural" immunoglobulins are thought to delay pathogenicity of infecting agents until antigen-induced high-affinity immunoglobulins are produced (Baumgarth *et al.,* 1999).

In macaques immunized with attenuated SIV, the cellular immune milieu is primed. On challenge with pathogenic SIV, innate mechanisms may initially suppress viral replication while the antigen-specific immune response develops. The SIV-specific CTL response, in concert with nonspecific effector mechanisms, may then become the primary control for residual virus replication. Alternatively, it is possible that infection with pathogenic SIV stimulates short-lived highly activated CTL effectors with low affinity for antigen, as has been seen in lymphocytic choriomeningitis virus infection (Gallimore *et al.,* 1998). SIVmac239Δnef immunization might stimulate T cells of higher affinity, which respond earlier and faster to infection. After infection with *L. monocytogens*, there is a selective expansion of high-affinity T cells, with consequent improvement in the quality of immunity (Busch and Pamer, 1999).

In summary, we quantified SIV-specific CTL responses in animals immunized with an attenuated virus and challenged with pathogenic SIV. The quantity of SIV-specific CTLs recognizing late structural proteins at time of challenge did not appear to correlate with protection against pathogenic SIV infection. However, innate and acquired cellular immune responses may synergize to prevent the establishment of infection with the challenge virus and control the attenuated vaccine virus. Our results suggest that greater emphasis should be placed on the induction of cellular immune responses for retroviral vaccination.

MATERIALS AND METHODS

Animals

Female rhesus macaques (*Macaca mulatta*) were housed either at the Laboratory for Experimental Medicine and Surgery in Primates (Tuxedo, NY) or at the Tulane Regional Primate Research Center (Covington, LA). Animals were maintained in accordance with the guidelines of the local institutional animal use committee. Eight animals were infected intravenously with 2×10^4 $TCID_{50}$ SIVmac239Δnef and challenged with 10 animal infectious doses (AID) of uncloned SIVmac251 (kindly provided by Dr. R. Desrosiers) using the same route either 10 weeks (Fig. 1, top) or 25 weeks (Fig. 1, bottom) later. Nonvaccinated control animals in both groups (Rh 1492, 10 weeks; Rh 1516, 25 weeks) became infected with SIVmac251 and died from simian AIDS. Two immunized macaques (Rh 1482, 10 weeks; Rh 1510, 25 weeks) were not protected from challenge and became infected with SIVmac251. The plasma viremia profiles for all macaques are shown in Fig. 1. Rhesus monkeys were screened for the presence of the Mamu-A*01 allele by a PCR-based technique (Dr. D. Watkins, Wisconsin Regional Primate Center) (Evans *et al.,* 1997).

Cell lines

Autologous B-lymphoblastoid cell lines (BCLs) from study animals were transformed by incubating PBMCs at 37°C with RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with R-15 (15% FCS, 10 mM HEPES, 2 mM L-glutamine, and 50 IU of penicillin/streptomycin) and *Herpesvirus papio* from the supernatant of S594 cell line (kindly provided by Dr. D. Watkins, Wisconsin Regional Primate Center, Madison, WI). PBMCs were isolated from heparinized or EDTA blood by centrifugation over a Ficoll-sodium diatrizoate gradient (Pharmacia, Uppsala, Sweden).

Limiting dilution analysis

Limiting dilution analysis was performed as previously described (Kakimoto *et al.,* 1999) In brief, cryopreserved PBMCs were thawed, washed twice in R-15, and resuspended at 2×10^6 cells/ml in R-15. PBMCs were incubated with Concanavalin A (Sigma) at 5 μg/ml for 3 days in RPMI 1640 (BioWhittaker) plus 15% FCS (R-15), washed, and seeded at 500–32,000 cells/100 μl in 24 replicates of a 96 U-bottom plate with 100 μl of R-15 plus 100 IU of human recombinant IL-2 in the presence of 2.5×10^4 γ-irradiated human PBMCs (Kakimoto *et al.,* 1999). CTL assays were performed 10–14 days after stimulation. SIV-specific cytolytic activity was measured using a standard ^{51}Cr release assay with cold target inhibition and autologous recombinant vaccinia-infected BCL as targets (Johnson *et al.,* 1997; Kakimoto *et al.,* 1999). Recombinant vaccinia viruses were rVV-Gag (vAbT252, encoding the SIVmac251 p55gag and protease proteins), rVV-Env (vAbT2531 encoding the SIVmac251 envelope) or control (vaccinia thymidine kinase negative) (Therion Biologics, Cambridge, MA). Wells with specific lysis equal to or greater than 10% were scored as positive. The fraction of nonresponding wells was determined for each dilution, and the CTL precursor cell frequency (CTLp) was calculated according to the maximum likelihood method (Kakimoto *et al.,* 1999). Limiting dilution analysis results are presented as $CTLp/10^6$ PBMCs with negative controls subtracted from experimental wells.

Peptide-expanded bulk culture cells were tested in a standard ^{51}Cr release assay on autologous BCL pulsed for 1 h with or without peptide p11C, C-M (10 μm) (Hanke *et al.,* 1999; Miller *et al.,* 1991).

Tetramer staining

The soluble Mamu-A*01 p11 C, C-M tetramer was made as previously described (Hanke *et al.,* 1999). To quantify tetramer-reactive cells, cryopreserved PBMCs were thawed and washed twice in R-15. Phycoerythrin-conjugated Mamu-A*01 p11C, C-M tetramer was added for 20 min at 37°C, and the suspension was then incubated with anti-CD8 TriColor (Caltag, clone 3B5) for 30 min at 4°C. Cells were washed twice and fixed with 2% paraformaldehyde in PBS. PBMCs were analyzed for the expression of cell surface markers and tetramer-positive cells using a fluorescent activated cell sorter (FACS) (Calibur; Becton-Dickinson, San Jose, CA) with CellQuest Software (Becton Dickinson). The mononuclear cell population was gated on the basis of forward and side scattering properties and subsequently evaluated for the fluorochromes conjugated to the tetramer and anti-CD8 antibody. Staining of PBMCs identified a distinct population of CD8/tetramer double-positive cells. The tetramer was used to stain a known SIV Gag p11C, C-M CTL line as positive control, and the negative control were PBMCs from an SIV-uninfected Mamu-A*01-positive animal.

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