Nonpathogenic CCR2-tropic SIVrcm after serial passage and its effect on SIVmac infection of Indian rhesus macaques

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Abstract

The natural host of SIVrcm is the red-capped mangabey (Cercocebus torquatus torquatus). Although this virus infects macaques and human PBMCs, its pathogenic potential is unknown. We serially passaged SIVrcm through 9 rhesus macaques to assess its potential for virulence. SIVrcm infected all macaques with peak viremia 2 weeks postinfection yet viral loads decreased to undetectable levels about one month after inoculation. Remarkably, SIVrcm replication and virulence did not increase following 7 serial passages. While CD4+ T cells in the gut were decreased in early infection, proportions of memory CD4+CCR5+ T cells were not affected. Three SIVrcm-infected macaques were subsequently challenged with SIVmac251 to assess the potential for superinfection. Interestingly, animals previously infected with SIVrcm had 100 fold lower levels of SIVmac251 in plasma compared to naive animals inoculated with SIVmac251. These results suggest that SIVrcm is nonpathogenic and may be useful for examining effective immune responses in SIV infection.

Introduction

Simian immunodeficiency virus (SIVrcm) was discovered in red-capped mangabeys (RCM) (Cercocebus torquatus torquatus) in Gabon and Nigeria, Africa (Beer et al., 2001; Chen et al., 1998; Georges-Courbot et al., 1998; Smith et al., 1998). Thus far, SIVrcm has only been partially characterized and has not been classified into any of the 6 major lineages of SIV by full-length genome phylogenetic analysis. SIVrcm has some specific characteristics distinguishing it from other SIVs. First, the genome of the virus, has a vpx gene similar to HIV-2/ SIVsm/SIVmac, whereas its pol region is genetically close to SIVcpz and gave rise to the theory that SIVcpz is a recombinant of SIVrcm and SIVgsn (a virus from greater spot-nosed monkey) (Bailes et al., 2003). SIVrcm or a close relative may be involved in SIVcpz evolution. Secondly, SIVrcm is the only known primate lentivirus that uses CCR2b as its major co-receptor for entry, rather than CCR5 or CXCR4 (Chen et al., 1998; Zhang et al., 2000).

The reasons that SIVrcm uses CCR2b as a coreceptor are not well understood. However, it has been hypothesized that this is an evolutionary adaptation in response to a high frequency of a 24 base pair (bp) deletion analogous to the delta 32 mutation in humans in the CCR5 gene. This results in lack of expression of CCR5 on cells, rendering them uninfected by viruses that rely on CCR5 for entry (Chen et al., 1998). With rare exceptions, SIVs do not cause AIDS in their natural hosts (Ling et al., 2004; Pandrea et al., 2001). However, cross-species transmission of SIV from a natural host (mangabey) to a naive host (macaque) resulted in the SIV-macaque model of AIDS primarily used today to examine the pathogenesis of HIV infection. Similarly, SIVrcm appears to be minimally pathogenic in its natural host, although studies are limited in this species (Georges-Courbot et al., 1998). SIVrcm experimentally infects other species such as cynomologus (Macaca fascicularis) and Indian-origin rhesus macaques (Macaca mulatta) (Georges-Courbot et al., 1998; Smith et al., 1998). However, whether serially passage SIVrcm has the potential to increase pathogenicity or cause an AIDS-like disease in Ind Rh macaques is unknown. We hypothesized that serial passage of this virus in a species that was naive to this virus would increase its potential for pathogenicity. The goal of this study was to determine whether serial passage of SIVrcm through rhesus macaques of Indian origin would increase its potential for pathogenicity. Serial passage of...
SIV is theorized to be a potential threat for adaptation of SIV to humans (Drucker et al., 2001). Since SIVrcm can replicate in rhesus and human lymphocytes, such studies could help us better understand the potential for cross species transmission of viruses.

Another goal was to determine if SIVrcm has a beneficial impact by preventing or alleviating “superinfection” by pathogenic strains of SIVmac. CCR5 is the primary coreceptor used by HIV and SIVmac. In fact, a number of CCR5 fusion inhibitors are now available or in development as therapies for HIV. It is also known that CXCR4 utilizing strains may emerge in HIV-infected patients in late infection, which sometimes are associated with increased viral loads and a more severe disease progression (Mild et al., 2007). However, it is still debated as to whether the pressure of CCR5 inhibitors or co-infection with other viral strains with different chemokine receptor usage may pressure a change in chemokine receptor usage of HIV, and HIV has the potential to use CCR2 as coreceptor (Frade et al., 1997). Therefore, blocking CCR2 expression by SIVrcm infection could affect the course or chemokine receptor expression of HIV infection. Thus, macaques previously infected with SIVrcm were subsequently challenged with pathogenic SIVmac251 and viral replication and pathogenicity were compared between dual infected and SIVmac251-only infected macaques.

In this study, 9 Ind Rh macaques were used for serial passage of SIVrcm. Three were superinfected with SIVmac251 after chronic SIVrcm infection. We found that the capacity of SIVrcm for replication in Ind Rh remained attenuated after serial passage. Although partial CD4+ T cell depletion occurred in the gut, mucosal memory CD4+CCR5+ T cells were not significantly reduced during SIVrcm infection. Serial passage of SIVrcm in Ind Rh did not significantly increase its virulence and no signs of AIDS were observed. We propose that SIVrcm infection mainly infects the monocyte/macrophage lineage on which CCR2 is expressed at high levels, and that memory CD4+ T Cells, the major target for HIV/SIVmac, are spared in SIVrcm infection. Furthermore, prior infection with SIVrcm resulted in significantly decreased viral loads and decreased the pathogenicity of SIVmac251 in “superinfected” animals.

Results

Viral replication of SIVrcm in Indian rhesus macaques by serial passage

A total of seven passages of SIVrcm were performed. All monkeys had a transient peak viremia from $10^4$ to $10^6$ copies/ml at day 11–14 after SIVrcm infection, which decreased rapidly to undetectable levels in all except the 5th passage animal, R265-p5, which had $10^3$ copies in
the 2nd month of infection (Fig. 1). Notably, the median levels of SIVrcm loads were relatively low compared to SIVmac infection of Ind Rh. Although most animals remained healthy, one animal (M854-p6) was euthanized due to severe pneumonia. However, plasma viral loads in this animal did not increase significantly over those of other animals in previous passages. Thus, to examine whether the development of disease was associated with a more pathogenic SIVrcm infection, a 7th passage of SIVrcm was performed in 3 Ind Rh (CF56-p7, CG29-p7 and G454-p7), which received plasma derived from monkey M854-p6 at the time of peak viremia. All 3 monkeys were infected with SIVrcm, but viral loads in plasma were not significantly increased, and no clinical signs or disease was noted in these animals. In summary, there was no significant increase in virus replication or virulence after 7 passages of the SIVrcm in Ind Rh.

**Dynamics of T cell subsets in the peripheral blood and gut**

Dynamics of CD4+, CD8+ T cells were monitored in the peripheral blood and gut throughout SIVrcm infection. There was no significant difference in peripheral blood between animals in different passages as shown in Fig. 2 panels a and b. Moreover, there were no significant changes in CD4+ T cell counts after SIVrcm infection compared with baseline CD4+ T cell counts before infection, although a transient decrease of CD4+ cells was seen at day 14 along with a slight increase in the CD8+ cells. However, no relationship between serial passage and changes in CD4+ T cells was detected.

Interestingly, the percentage of CD4+ T cells in the gut showed a significant decrease at day 28 (p<0.002) and day 90 (p<0.012), but not at day 14 (p=0.105). (Fig. 3a). However, there was no correlation between the time of serial passage and the degree of intestinal CD4 depletion between animals. Interestingly, the proportions of intestinal memory (CD45RA negative) CD4+CCR5+ T cells, the main targets of SIVmac and HIV (Brenchley et al., 2004; Mehandru et al., 2004; Veazey et al., 1998) remained relatively stable in the gut throughout infection (Fig. 3b), which is likely due to the fact that this virus does not use CCR5 as a coreceptor for infection and entry (Chen et al., 1998; Zhang et al., 2000).

**SIVrcm replication in vitro**

To test the capability of viral replication in vitro after serial passaging in animals, viruses were isolated from 4 animals AT74-1, AT83-p2, AT87-p3 and L836-p4. Viruses from AT74-p1 and L836-p4 had very similar low viral replications, virus from AT83-p2 started to grow slowly and had higher replication than AT74-1 and L836-p4 at day 21 of culture. Notably, AT87-p3 had a significantly higher level of viral replication and peaked at day 17 of culture (Fig. 4). This result was not consistent with the observation in vivo shown in Fig. 1, indicating in vitro replication does not reflect the ability of replication in vivo, in agreement with the observations in a report (Gautam et al., 2007). In addition, the SIVrcm was also able to replicate in human PBMCs as well as in human T cell line Molt4clone8 (data not shown).

**Expression of SIVrcm coreceptor CCR2 on monocytes**

To determine if CCR2 expression changed after SIVrcm infection, we tested the level of CCR2 on CD20+ B cells, CD4+ T cells and CD14+ monocytes of M854-p6 at multiple time points during SIVrcm infection and one time point in animal R265-p5. As shown in Fig. 5 panel a, CD4+ T lymphocytes and B lymphocytes had little or no expression of CCR2, but monocytes had high expression of CCR2. However, no changes in the expression of CCR2 were observed on monocytes after SIVrcm infection. Moreover, monocyte counts in blood were not significantly different throughout SIVrcm infection, although a minor increase was noted from day 7 through day 28, with highest levels observed at day 17 PI. (Fig. 5b).

**SIVmac251 superinfection in SIVrcm infected animals**

Since SIVrcm replication in infected animals decreased rapidly in most animals to undetectable levels (~10^2 copies/ml), we wanted to determine if prior infection a suppressive or enhancing effect after co-infection with a more pathogenic SIV. Thus, we challenged 3 Ind Rh that had been used in three different passages (AT87-p3, L836-p4 and R265-p5) with SIVmac251, one year after SIVrcm infection when SIVrcm viral loads were still undetectable. A cohort of 8 Indian rhesus macaques on an unrelated study that received the same dose of SIVmac251 intravenously were used for viral load comparison. In
infection with the viral loads stable at 10^3 copies/ml. Animal AT87-p3 is still healthy after 35 months of SIVmac251 challenged with SIVmac alone.

Superinfected with SIVmac251 following SIVrcm infection had lower CD4+CCR5+ T cells were not significantly impacted; and d) Macaques superinfected with SIVmac251 following SIVrcm infection had lower levels of viral replication and a better prognosis than animals challenged with SIVmac alone.

**Discussion**

Experimental infection of SIVrcm in Ind Rh has been previously reported (Smith et al., 1998). However, only 2 animals were studied and the methods for quantifying viral replication were relatively insensitive compared to current quantitative RT-PCR techniques.

In this study, we describe the following findings: a) serial passage of SIVrcm did not significantly increase its virulence for Ind Rh; b) SIVrcm replication was significantly lower than SIVmac infection alone in Ind Rh; c) intestinal mucosal CD4+ T cells decreased significantly after one month of infection, but intestinal memory CD4+CCR5+ T cells were not significantly impacted; and d) Macaques superinfected with SIVmac251 following SIVrcm infection had lower levels of viral replication and a better prognosis than animals challenged with SIVmac alone.

Our study of 7 serial passage in Ind Rh showed that although SIVrcm successfully infected this species, but viral virulence did not increase with passage. Serial passage of SIVs in one species or passage of virus in species other than its natural hosts may result in increasing virulence (Dewhurst et al., 1990; Fultz et al., 1989; Novembre et al., 1993; Tan et al., 1999). In contrast, serial passage may not increase viral virulence and not result in AIDS-like diseases in SHIV162p3 and p4 infected rhesus macaques (Teleshova et al., 2006a; Teleshova et al., 2006b). Moreover, serial passage of a virus could generate attenuated virus which may eventually be exploited for vaccine design or testing, such as in the development of the early rabies vaccines first developed by Pasteur over a century ago.

With regard to hosts, it appears that pig-tailed macaques (Macaca nemestrina) are more susceptible to disease from SIVs such as SIVagm (Hirsch et al., 1995), SIVsun, SIVhioest (Beer et al., 2005) and HIV-2 (Otten et al., 2000; Pullium et al., 2001). However, the above viruses have not been reported to be pathogenic for Ind Rh. The low pathogenicity of SIVrcm for Ind Rh may due to its unique usage of CCR2 as co-receptor.

It is noteworthy that although SIVrcm infection was not pathogenic in monkeys, a significant loss of gut CD4+ T cells occurred, and to a lesser extent in blood. These results suggest that some level of intestinal CD4+ T cell loss is common to all SIV infections, whether or not the infection is the result of an ultimately pathogenic SIVmac or nonpathogenic SIVsm, SIVagm or SIVrcm infection (Gordon et al., 2007; Ling et al., 2002, 2007; Pandrea et al., 2007; Veazey et al., 1998, 2000). Interestingly, however, the proportions of memory CD4+CCR5+ T cells in the gut of these animals did not show significant losses after SIVrcm infection, whereas memory CD4+CCR5+ T cells are depleted in the gut of SIV-infected macaques, mangabeys, and AGMs after various SIV infections and in HIV-infected humans (Brenchley et al., 2004; Gordon et al., 2007; Mattapallil et al., 2005; Mehandru et al., 2004 2354; Pandrea et al., 2007; Veazey et al., 1998; Veazey et al., 2000). This observation likely due to the unique co-receptor usage of this virus, as it utilizes CCR2 and not CCR5 for entry into cells. As a consequence of its unique CCR2 tropism, SIVrcm infection may use different replication strategies from other SIVs. This virus may target the monocyte–macrophage lineage rather than lymphocytes, since CCR2 is expressed mainly on monocytes. Development of a SIVrcm infection model in rhesus macaque could be useful in studying the role of macrophages in the establishment and control of infection. Monocytes and macrophages are considered to be crucial reservoirs of HIV-1 infection (Aquaro et al., 2006; Brown et al., 2006; Crowe et al., 2003; Zhu, 2002; Zhu et al., 2004), and serve as an important strategy for HIV-1 to evade and resist immune pressure (Stevenson, 2003). Furthermore, macrophages are less susceptible to viral cytopathic effects (Stevenson, 2003) which may explain why we did not detect significant changes in monocytes in this study. Understanding SIVrcm tropism and viral entry may help to determine the relationship between tropism and infectivity and provide insights into the development of rational therapeutic antiviral strategies in HIV infection.

SIVrcm interference of SIVmac251 infection may be via interaction or competition between CCR5 and CCR2. CCR2 has a strong linkage with CCR5 as they are located in the same region of human genome and in the same orientation (Samson et al., 1996). A recent study demonstrated that ongoing parallel gene conversion between CCR2 and CCR5 occurred and might be a potential mechanism for receptor dimerization (Vazquez-Salat et al., 2007). Another study has demonstrated that CCR2 has an amino-terminal domain that can bind to HIV-1, indicating a potential role of coreceptor for HIV-1 infection (Frade et al., 1997), thus there are reasons to presume that coreceptor usage switch could occur under the pressure of CCR5 inhibitor therapy in HIV-1 infection. It is also reported that MCP-1 binding to CCR2b efficiently prevents CCL4 (MIP-1β) and CCL5 (RANTES) binding to CCR5 on cells expressing both CCR2 and CCR5.

**Clinical outcome of SIVrcm after serial passage or superinfection with SIVmac251**

Seven of 9 SIVrcm-infected animals remained alive and had no signs of diseases throughout this study. One monkey (M854-p6) developed severe bacterial pneumonia and was humanely euthanized at day 42 after SIVrcm infection. Corynebacterium was isolated from the lungs. However, in the absence of low CD4 counts, recognized opportunistic infection, or giant cells in the lungs, the pneumonia could not be attributed to SIV infection or immunosuppression. Furthermore, we do not believe the pneumonia was related because the 3 macaques (CF56-p7, CG29-p7 and G454-p7) that received plasma from M854-p6 at peak viremia did not develop disease. Monkey L836-p4, which was in the 4th passage and subsequently challenged with SIVmac251, was euthanized due to non-AIDS disease with low SIVmac251 load of 3165 copies/ml. Animal R265-p5 was euthanized due to neoplasms/lymphoma with viral load at 10^5 copies/ml at 24 months after SIVmac251 infection. Animal AT87-p3 is still healthy after 35 months of SIVmac251 infection with the viral loads stable at 10^5 copies/ml.
receptors (El-Asmar et al., 2005) and HIV-1 fusion activity could be inhibited by MCP-1 (Alkhatib et al., 1997). SIVrcm binding to CCR2 may prevent MCP-1/CCR2 binding and lead to a higher affinity of CCR5 binding to its ligands, which might provide fewer opportunities for CCR5/SIVmac251 binding, which could reduce SIVmac251 entry. Although we have not yet shown the molecular mechanisms that underlie SIVrcm-associated reduction of SIVmac251 replication, our data may bear on vaccine development by showing that prior SIVrcm infection affects replication of a distantly related virus in vivo.

Another possibility is that immune responses may have protective effects. Some studies have demonstrated that prior SHIV or SIV infection control super-challenged virulent SIV or SHIV infection (Genesca et al., 2007; Tsukamoto et al., 2007) and Vezey—unpublished observation). The mechanisms may be due to enhanced CD8+ T cell function after the first infection (Tsukamoto et al., 2007) or due to polyfunctional virus-specific CD8+ T cells elicited by the “immunizing virus” as reported in associated with attenuated SHIV89.6 infection (Genesca et al., 2007). SIVrcm infection may also act as a virucide-enhanced attenuated virus that induces relatively effective immune responses against SIVmac251, but we did not sufficiently examine cellular immune responses in this study.

In summary, this study provides information on CCR2-tropic SIVrcm that could be valuable for studying infection of the monocyte/macrophage lineage. The fact that SIVrcm is nonpathogenic after 7 serial passages shows that serial passage of SIV does not result in increased virulence. Furthermore, SIVrcm infection suppresses subsequent SIVmac251 replication, and it may have therapeutic or vaccine-like effects via co-receptor interaction, immune protection or other mechanisms yet be shown important for decreasing SIVmac replication.

Materials and methods

Animals and inoculations

Nine Indian-origin rhesus macaques (Ind Rh) were used for seven serial passage of SIVrcm. One animal was used in each passage except in the 7th passage which had 3 animals. Animals and passage are described by animal number (e.g. AT74) followed by the passage sequence (p1=first passage) as AT74-p1, AT83-p2, AT87-p3, L836-p4, R265-p5, P648-p5, M854-p6, CF56-p7, CG29-p7 and G454-p7. Three macaques (AT87-p3, L836-p4, R265-p5) were subsequently intravenously challenged 100 TCID50 SIVmac251 after the level of SIVrcm load became undetectable. Another 8 naive Indian rhesus were intravenously infected with 100 TCID50 SIVmac251 alone for comparison with SIVrcm and SIVmac251 superinfected animals. All animals were housed at the Tulane National Primate Research Center (TNPRC) in accordance with the Guide for Care and Use of Laboratory Animals and all animal protocols were approved by the TNPRC Institutional Animal Care and Use Committee. Macaques were confirmed to be negative for SIV, simian D retrovirus and simian T-cell leukemia virus infection prior to use. The first animal (AT74-p1) was inoculated with 1000 TCID50 of cell-free SIVrcm. Thereafter, the 2nd to 7th passage were conducted by collecting 5 ml of bone marrow and 10 ml plasma at the peak viremia from the animal in each prior passage. Bone marrow was used as a source of virus in order to enhance potential pathogenicity of SIVrcm. All inoculations were performed by intravenous route.

Blood and tissue sample collection and lymphocytes isolation

Blood, lymph node and intestinal tissue samples were collected at various time points. EDTA-blood and plasma was used for viral load quantification. Peripheral blood mononuclear cells (PBMCs) were isolated by lymphocyte separation medium and used for further T cell subset immunophenotyping. Intestinal lymphocytes were isolated as previously described (Ling et al., 2002; Veazey et al., 1997, 1998). Briefly, macaques were anesthetized and 10-15 duodenal or upper jejunal 1 mm diameter pinch biopsies were collected using a flexible endoscope. Lymphocytes were collected by incubating biopsies in complete RPMI media containing 5 mM EDTA followed by 2-3 digestions with 60 U/ml collagenase (type II, Sigma, St. Louis, MO). To enrich lymphocytes, cells were layered on a discontinuous 35%/60% Percoll gradients (Sigma, St. Louis, MO) and centrifuged for 20 min at 1000 g, washed, and resuspended in RPMI containing 5% FCS.

Cell culture for viral replication

PBMCs were separated from heparinized blood of a SIV naive rhesus macaque using Lymphocyte Separation Medium (LSM). PBMCs were stimulated with ConA (5 mg/ml) in complete cell culture medium containing 10% fetal calf serum and antibiotics for 3 days, then washed. PBMCs isolated from each infected animal were cocultured with the same stimulated PBMCs from the SIV naive rhesus macaques for viral production. The positive results were determined by the production of SIV Gag p27 antigen in the culture supernatants by using SIV p27 Antigen ELISA Kit (ZeptoMetrix Co., Buffalo, NY) according to the manufacturer’s instructions.

Antibodies, immuno-fluorescent staining and flow cytometry

All antibodies (except anti-CCR2) were purchased from PharMin- gen or BD biosciences (BD biosciences, San Jose, CA). For phenotypic analysis, EDTA anticoagulated whole blood and purified intestinal lymphocytes were stained simultaneously with 4 different fluorescently labeled antibodies in each tube including CD3-FITC, CD8-PerCP, CD4-APC, CD45R-AFITC, CCR5-PE, CD3-PerCP, CCR2-PE (R and D Systems, Minneapolis, MN), CD20-PerCP and CD14-FITC. Samples were fixed overnight in 2% paraformaldehyde and analyzed on a FACS Calibur Flow Cytometer. At least 50,000 events were acquired for each sample. Data were analyzed using CellQuest Pro (BD Biosciences, San Jose, CA) or Flojo software (Tree Star Inc., San Carlos, CA). Absolute numbers of CD4+ and CD8+ T cells in peripheral blood were determined using the true-count method as previously described (Ling et al., 2002).

Quantification of SIVrcm plasma loads by real-time PCR (RT-PCR)

Plasma viral load was measured by real-time PCR using a 7700 ABI PRISM Sequence Detector (Applied Biosystems, Foster City, CA). Briefly, plasma viral RNA was used to generate cDNA using one-step RT-PCR Master Mix (Applied Biosystems). Primers and probe sequences were as follows: SIVrcm-F 5′ AGGGAGGAGGATCCCAGATTGTCATC-3′ (forward primer), SIVrcm-R 5′ GGGCTCTACAGCCGACGAC-3′ (reverse primer), SIVrcm-P 6FAM-TTACGTATCCATGATCTTTGAGCTTCAT-3′ (TaqMan probe). The following PCR conditions were used: Reverse-transcription at 48 °C for 30 min, denaturation at 95 °C for 10 min, followed by 40 cycles of heating at 95 °C for 15 s, annealing at 60 °C with a final elongation of 7 min at 72 °C. Absolute precision in the quantification of SIVrcm RNA in samples containing unknown quantities of viral RNA was assured by the parallel measurement of SIV RCM RNA standard. Standard templates were created as follows: complementary DNA was amplified by PCR in a nested assay using outer primers OF (5′-ATTCGCCCGCATTCCTT-3′), OR (5′-CCGGATTAGTCCGTAAC-3′) and inner primers IF (5′-TCTCCTCCGATCCTTCTA-3′) and IR (5′-GACGCTTCCGCTTAATATA-3′). The yield PCR product was inserted in a TOPO-TA Vector using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and the recombinant plasmid was transformed into E. coli. The plasmid was then extracted using Qiagen Plasmid Mini Kit (Qiagen) and linearized by restriction enzymes. The linearized plasmid was then

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used to perform an *in vitro* transcription (MEGAscriptTM High Yield Transcription kit, Ambion). Viral copy numbers in each sample were determined by plotting CT values against the standard curve representing known viral copy numbers. The efficiency of the RT-PCR reaction was >95%. The limit of detection was 100 copies/ml.

**Quantification of SIVmac plasma loads by branched DNA assay (bDNA assay)**

Plasma viral loads of three animals that were challenged with SIVmac were measured using the bDNA assay (Siemens Diagnostics, Berkeley, CA). This was because SIVmac251 and SIVrcm have a high disparity in sequence and the SIVmac specific probes only detect SIVmac group of strains including SIVmac239 and SIVmac251 (Greenier et al., 2001).

**Statistical analysis**

Nonparametric Mann–Whitney test was used for the percentage of mucosal CD4+ T cells and memory CD4+CCR5+ T cells between each time point. Values are expressed as median ± interquartile range. All reported p values were two-sided at the 0.05 significance level using Graph Pad Prism 4.0 software statistical program.

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