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α4+β7hiCD4+ Memory T cells Harbor Most Th-17 cells and are Preferentially Infected During Acute SIV Infection

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Abstract

HIV/SIV are thought to infect minimally activated $CD4^+$ T cells after viral entry. Not much is known about why SIV selectively targets these cells. Here we show that $CD4^+$ T cells that express high levels of the α4β7 heterodimer are preferentially infected very early during the course of SIV infection. At day 2–4 post infection, $\alpha4^+\beta7^{hi}CD4^+$ T cells had ~ 5x more SIV-gag DNA than $\beta7^-CD4^+$ T cells. $\alpha4^+\beta7^{hi}CD4^+$ T cells displayed a predominantly central memory ($CD45RA^-CD28^+CCR7^+$) and resting ($CD25^-CD69^-HLA\text{-}DR^-Ki\text{-}67^-$) phenotype. Though the expression of detectable CCR5 was variable on $\alpha4^+\beta7^{hi}$ and $\beta7^-CD4^+$ T cells, both $CCR5^+$ and $CCR5^-$ subsets of $\alpha4^+\beta7^{hi}$ and $\beta7^-CD4^+$ T cells were found to express sufficient levels of CCR5 mRNA suggesting that both these subsets could be efficiently infected by SIV. In line with this, we found similar levels of SIV infection in $\beta7^-CD4^+CCR5^+$ and $\beta7^-CD4^+CCR5-$ T cells. $\alpha4\beta7^{hi}CD4^+$ T cells were found to harbor most Th-17 cells that were significantly depleted during acute SIV infection. Taken together, our results show that resting memory $\alpha4^+\beta7^{hi}CD4^+$ T cells in blood are preferentially depleted during acute SIV infection, and the loss of these cells alters the balance between Th-17 and Th-1 responses thereby contributing to disease pathogenesis.

Keywords

HIV; SIV; simian; immunodeficiency; α4β7; Mucosa; CD4; Gut; Intestine; homing

Introduction

Acute human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections are characterized by a massive loss of memory $CD4^+$ T cells[1–7], a process that is most pronounced in mucosal tissues as these tissues are enriched for memory $CD4^+$ T cells.

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Author contributions

M.K processed the samples, performed all the experiments, analyzed and helped in the interpretation of data, and preparation of the manuscript. XW and RSV helped with samples and preparation of the manuscript. M.P and JL helped with plasma viral load assays, and reviewing the manuscript. M.R provided certain reagents and helped in reviewing the manuscript. J.J M designed, helped with analysis and interpretation of data, preparation of the manuscript, and supervised the study.

Competing Interests

The authors declare no competing financial interests.

Like in non-natural hosts, severe depletion of $CD4^+$ T cells have been reported in natural hosts such as sooty mangabeys and African green monkeys during acute SIV infection[8,9]. Surprisingly, the kinetics of infection in memory $CD4^+$ T cells that reach a peak ~ 10 days after SIV challenge, appear to be independent of the route of infection[4,5].

The factors determining which target cells are infected initially, and those responsible for the delay between initial infection and the explosive levels of viral replication observed 10 days later, remain unclear. Studies[5] have shown that prior to day 7 pi < 5–10% of $CD4^+$ T cells in either the mucosal tissues or periphery are infected, whereas by day 10 pi most memory $CD4^+$ T cells are infected and carry viral DNA. Li et al[4] demonstrated that a few founder population of $CD4^+$ T cells in the gut associated lymphoid tissue (GALT) are infected initially during SIV infection. Interestingly, these founder cells were minimally activated $CD4^+$ T cells ($CD25^-CD69^-Ki\text{-}67^-$) and had a predominantly resting phenotype. It is not clear why SIV targets these $CD4^+$ T cells during the early phase of viral infection.

Recent *in vitro* studies[10] have shown that $CD4^+$ T cells that express high levels of the α4β7 ($\alpha4^+\beta7^{hi}$) mucosal homing receptor can bind to HIV, and SIVsmm with high affinity making these cells more susceptible to infection. Arthos et al[10] suggested that expression of high levels of α4β7 on $CD4^+$ T cells likely offers a selective advantage to the virus, hence these cells may be the earliest cells targeted and infected by HIV. The loss of these mucosal homing T helper (Th) cells severely compromises the ability of the mucosal immune system. In line with this, recent studies have shown that mucosal Th–17 $CD4^+$ T cells play a critical role in maintaining the integrity of the mucosal barrier[11]. Others have shown that alterations in the Th-17 and Th-1 responses contribute to disease progression[12–14].

We hypothesized that if HIV selectively targeted the $\alpha4^+\beta7^{hi}CD4^+$ T cells during the early phase of infection, then these cells would be preferentially infected very early during the course of infection. We tested our hypothesis in the SIVmac251 infected rhesus macaque model by quantifying the cell-associated SIV DNA in $\alpha4^+\beta7^{hi}CD4^+$ T cells from peripheral blood during the early phase of SIV infection and compared them to SIV DNA levels in $CD4^+$ T cells that either lacked ($\beta7^-$) or expressed intermediate levels of α4β7 ($\alpha4^+\beta7^{int}$). Previous studies[15] have shown that HIV specific $CD4^+$ T cells that were preferentially infected carried 2–5x more viral DNA than EBV or CMV specific $CD4^+$ T cells. To determine if the differentiated and activated nature of $\alpha4^+\beta7^{hi}CD4^+$ T cells contributes to their susceptibility to SIV infection, we evaluated the expression of phenotypic (CD28, CD45RA and CCR7) and activation (CD25, CD69, HLA-DR, and Ki-67) markers on these cells. Additionally, to evaluate if preferential infection and depletion of $\alpha4^+\beta7^{hi}CD4^+$ T cells led to alterations in the balance between Th-17 and Th-1 type responses, we evaluated the expression of IL-17 and IFNγ in $\alpha4^+\beta7^{hi}CD4^+$ T cells and compared them to $\beta7^-CD4^+$ T cells.

Results

$\alpha4^+\beta7^{hi}CD4^+$ T cells are a minor population of peripheral $CD4^+$ T cells

We used an antibody against the β7 receptor to identify different subsets of $CD4^+$ T cells. Since β7 can form a heterodimer with α4 (CD49d) or αE (CD103)[16], we first evaluated the expression of these receptors on β7 subsets in peripheral blood of healthy rhesus macaques. Our results showed that ~50% of peripheral blood $CD4^+$ T cells expressed the β7 receptor, whereas the rest of the 50% lacked β7 expression. Approximately 20% of $CD4^+$ T cells that expressed the β7 receptor did so at high levels and had a $\beta7^{hi}$ phenotype, whereas ~ 30% of them expressed intermediate levels of β7 receptor and had a $\beta7^{int}$ phenotype (Fig. 1a). More than 95% of $\beta7^{hi}$, and ~65% of $\beta7^{int}$ $CD4^+$ T cells co-expressed the α4 receptor (Fig. 1b), and these are referred to as $\alpha4^+\beta7^{hi}$ and $\alpha4^+\beta7^{int}$ subsets in the text. Studies have shown that cells expressing high levels of the α4β7 efficiently traffic to mucosal tissues[16].

On the other hand, $CD4^+$ T cells that lacked β7 expression contained a mix of $\alpha4^+$ (~75%) and $\alpha4^-$ (~25%) cells (Fig. 1c), and these subsets together are referred to as $\beta7^-$ $CD4^+$ T cell subsets. α4 associates with β1 to form the α4β1 heterodimer, and binds to VCAM-1 (Vascular cell adhesion molecule-1) on activated endothelial cells which is involved in trafficking of T cells to peripheral lymphoid tissues[17–20]. Interestingly, $\beta7^-CD4^+$ T cell subsets expressed α4 integrin at a significantly higher density than $\alpha4\beta7^{hi}CD4^+$ T cell subsets (Fig. 1e). Our results suggest that most of the $\beta7^-CD4^+$ T cell subsets likely home to peripheral lymphoid tissues.

All of the $\alpha4^+\beta7^{hi}$ and $\beta7^-CD4^+$ T cell subsets expressed CD95 indicating that these were memory $CD4^+$ T cells. On the other hand, $\alpha4^+\beta7^{int}CD4^+$ T cells lacked CD95 expression indicative of a naïve phenotype. Previous studies have shown that rhesus macaque T cells expressing CD95 were memory T cells, whereas T cells that lacked CD95 were naïve T cells[21]. As our analysis was confined to peripheral blood, it is possible that the pattern of α4β7 expression may vary from that seen in GALT.

$\alpha4^+\beta7^{hi}CD4^+$ T cells express a central memory phenotype

Next we evaluated the phenotype of $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets based on the co-expression of α4 and β7 integrins. Memory $CD4^+$ T cells that express the α4β7 heterodimer appear on the diagonal as the antibodies to α4 and β7 bind to the same heterodimerized molecule, and co-labels with the Act-1 clone that recognizes an heterodimerized epitope of α4 and β7[22] (Suppl. Fig. 1a).

Three major subsets of memory $CD4^+$ T cells could be delineated based on the expression of these two integrin molecules namely, $\alpha4^+\beta7^{hi}$ subset and two subsets of $\beta7^-$ $CD4^+$ T cells namely, $\alpha4^+\beta7^-$ and $\alpha4^-\beta7^-$ subsets (Fig. 2a–b). Similar proportions of the 3 subsets are found within human memory CD4 T cells (personal communication; Mario Roederer, Suppl. Fig. 1e).

Majority of the $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets expressed a central memory phenotype ($C28^+CCR7^+CD45RA^-$), whereas most of the effector memory $CD4^+$ T cells ($CD28^-CCR7^-CD45RA^+$) are found within the $\alpha4^+\beta7^-$ subsets (Fig. 2 c–d).

Interestingly, most (>95%) of the $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets expressed detectable levels of CCR5 on their surface (Fig. 2e), whereas only a few (<20%) of the $\beta7^-$ $CD4^+$ T cell subsets expressed detectable levels of CCR5. To determine if these subsets expressed variable levels of CCR5 mRNA, we sorted $CCR5^+$ and $CCR5^-$ subsets of $\alpha4^+\beta7^{hi}$ and $\beta7^-$ $CD4^+$ T cells from uninfected animals (Suppl. Fig. 1b) and quantified the levels of CCR5 mRNA in these subsets using a relative qPCR assay (Fig. 2f). Our results show that though a majority of the $\beta7^-$ $CD4^+$ T cell subsets expressed little CCR5 on their surface they had significant levels of CCR5 mRNA that did not differ from that of the other memory CD4 T cell subsets.

$\alpha4^+\beta7^{hi}CD4^+$ T cells are preferentially infected during acute SIV infection

To determine if the differential expression of α4, and β7 integrin's on $CD4^+$ T cell subsets made them variably susceptible to infection and subsequent loss, we evaluated the dynamics of $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets in peripheral blood and compared them to $\alpha4^+\beta7^{int}$ and $\beta7^-CD4^+$ T cell subsets.

We observed a significant loss of both $\alpha4^+\beta7^{hi}$ and $\beta7^-CD4^+$ T cell subsets in peripheral blood at day 63 pi as compared to day 10 pi (Fig. 3a & b), whereas there was no major difference in either the frequency or absolute numbers of $\alpha4^+\beta7^{int}CD4^+$ T cell subsets or CD8 T cell subsets (Fig. 3a–d). The loss of both $\alpha4^+\beta7^{hi}$ and $\beta7^-CD4^+$ T cell subsets was not unexpected as previous studies[5] have reported a significant loss of memory $CD4^+$ T cells in peripheral blood during acute SIV infection; both $\alpha4^+\beta7^{hi}$ and $\beta7^-CD4^+$ T cell subsets have a memory

phenotype. However, we observed a significantly higher loss of $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets by day 63 pi as compared to the $\beta7^-CD4^+$ T cell subsets (Fig. 3e) suggesting that $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets were preferentially depleted during the course of acute SIV infection.

To determine if the preferential loss of $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets was due to a higher level of viral infection in these subsets, we measured the level of SIV-gag DNA in $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets and compared them to $\alpha4^+\beta7^{int}$, and $\beta7^-CD4^+$ T cell subsets. $\alpha4^+\beta7^{hi}$, $\alpha4^+\beta7^{int}$, and $\beta7^-CD4^+$ T cell subsets, discriminated based on the expression of β7 and CD95[21] (Supplementary Fig. 1c), were sorted from peripheral blood and used in a quantitative PCR assay to measure SIV-gag DNA. We have previously shown that this assay was highly sensitive for measuring viral infection in $CD4^+$ T cells[5]. Measuring the level of SIV DNA in target cells allows us to quantify both productively and non-productively infected cells, hence is a more sensitive assay for quantifying viral infection in cells than RNA based assays that measures only productively infected cells. Previous studies have shown that most of the memory $CD4^+$ T cells carried viral DNA during acute SIV infection, whereas only about 7–20% of them were productively infected[4,5].

We found ~5x more SIV-gag DNA in $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets at day 2–4 pi as compared to $\beta7^-CD4^+$ T cell subsets suggesting that $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets were preferentially infected very early during the course of infection (Fig. 4 b–c). By day 10 pi, there was a significant increase in the number of SIV-gag DNA copies in both subsets as compared to day 2–4 pi. However, like at day 2–4 pi, $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets harbored ~3x more SIV-gag DNA than $\beta7^-CD4^+$ T cell subsets (Fig. 4b–c).

By day 63 pi, however, the number of SIV-gag copies decreased significantly in both $\alpha4^+\beta7^{hi}$ and $\beta7^-CD4^+$ T cell subsets (Fig. 4b) indicating that most of the cells that were infected at day 10 pi, were destroyed by day 63 pi. Interestingly, unlike day 2–4 and 10 pi, both $\alpha4^+\beta7^{hi}$ and $\beta7^-CD4^+$ T cell subsets at day 63 pi had similar levels of SIV-gag DNA (Fig. 4c).

Few $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets expressed HLA-DR (Fig. 4d) that did not change after infection. Previous reports[23] have shown that there was no significant change in the expression of HLA-DR on memory $CD4^+$ T cells during the early phase of acute SIV infection. Like HLA-DR, few $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets expressed either CD25 (< 0.5%) or CD69 (< 0.5%) or Ki-67 (~ 3%) prior to infection that did not change significantly at day 4 pi (Fig. 4d–g). Previous studies have shown that there was little or no change in the expression of HLA-DR or Ki-67 on memory $CD4^+$ T cells at day 4, 7 and 10 pi as compared to preinfection values[23], whereas others have shown that very few peripheral blood $CD4^+$ T cells express CD25 or CD69 or Ki-67 during the acute phase of SIV infection[24].

Unlike $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets, higher proportions of $\beta7^-CD4^+$ T cell subsets expressed HLA-DR prior to infection, yet these cells were not preferentially infected. Likewise, $\beta7^-CD4^+$ T cell subsets were found to express significantly higher levels of Ki-67 at day 4 pi as compared to uninfected animals. Taken together, these findings indicate that preferential infection during the early phase of SIV infection is confined to $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets that display a predominantly resting phenotype. This is in line with previous reports by Li et al[4] who demonstrated that SIV primarily infects resting memory $CD4^+$ T cells in the GALT that express a $CD25^-CD69^-Ki\text{-}67^-$ phenotype. It is important to point out that our results only represent snap shots of the changes occurring *in vivo*. As such it is difficult to rule out if $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets were transiently activated at the time of infection.

To confirm if SIV preferentially targeted $CD4^+$ T cell subsets that expressed the α4β7 heterodimer during the early phase of SIV infection, we evaluated the levels of SIV-gag DNA

in $\alpha4^+\beta7^{hi}$, $\alpha4^+\beta7^-$, and $\alpha4^-\ \beta7^-$ subsets of memory $CD4^+$ T cells (Fig. 5a) using peripheral blood samples (day 2–3 pi) that were obtained from an unrelated study.

Our results showed that during the first few days after infection, the virus is largely confined to memory $CD4^+$ T cells that express the α4β7 heterodimer; $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets had significantly higher levels of SIV-gag DNA than either $\alpha4^+\beta7^-$ or $\alpha4^-\beta7^-CD4^+$ T cell subsets (Fig. 5a).

Taken together these results support the *in vitro* findings reported by Arthos et al[10], and shows that SIVmac251, selectively target $CD4^+$ T cells that express the α4β7 heterodimer.

Both $\beta7^-CD4^+CCR5^+$ and $\beta7^-CD4^+CCR5^-$ T cell subsets are equally infected during acute SIV infection

To determine if detectable surface expression of CCR5 makes $CD4^+$ T cells subsets more susceptible to SIV infection, we evaluated the levels of SIV infection in $\beta7^-CD4^+CCR5^+$ T cell subsets and compared them to $\beta7^-CD4^+CCR5^-$ T cell subsets. Krzysiek at al[25] demonstrated that $CCR5^+CD4^+$ T helper cells with non-lymphoid homing potential are preferentially depleted during HIV infection despite early treatment. Likewise, a number of studies have shown that $CCR5^+CD4^+$ T helper cells were preferentially depleted during HIV and SIV infection, whereas others have shown that both $CCR5^+$ and $CCR5^-CD4^+$T cell subsets were infected during acute SIV infection[1,5,26,27].

Since most (>95%) of the $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets express detectable levels of CCR5 on their surface, we did not have sufficient samples to sort enough $\alpha4^+\beta7^{hi}CD4^+CCR5^-$ T cell subsets for the qPCR analysis. Hence we restricted our analysis to $\beta7^-CD4^+$ T cell subsets. We sorted $\beta7^-CD4^+CCR5^+$ and $\beta7^-CD4^+CCR5^-$ T cell subsets from peripheral blood of SIV infected animals (day 7 &14 pi) obtained from an unrelated study, and quantified the levels of SIV-gag DNA by qPCR.

Our results (Fig. 5b) show that both $\beta7^-CD4^+CCR5^+$ and $\beta7^-CD4^+CCR5^-$ T cell subsets had similar levels of SIV-gag DNA which did not differ significantly suggesting that preferential infection was not directly dependent on the surface level of CCR5 expression. As shown in Fig. 2f, $CCR5^-$ T cell subsets express sufficient levels of CCR5 message that likely enough to make these cells susceptible to SIV infection. These results support previous studies[5] showing that there was no difference in the level of SIV infection in memory $CD4^+CCR5^+$ and memory $CD4^+CCR5^-$ T cell subsets at day 3, 7, 10 and 14 pi.

$\alpha4^+\beta7^hiCD4^+$ T cell subsets harbor most Th-17 cells and are lost during acute SIV infection

Previous studies have shown that mucosal $CD4^+$ T cells are major producers of IL-17, and HIV infection was associated with alterations in the balance between Th-17 and Th-1 type responses[11–14]. To determine if the preferential loss of $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets was accompanied by changes in the profile of Th-17 vs Th-1 type responses, we evaluated the production of IL-17 and IFNγ by $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets and compared them to $\beta7^-CD4^+$ T cell subsets.

Our results showed that $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets harbored significantly higher frequencies of Th-17 type cells whereas $\beta7^-$ $CD4^+$ T cell subsets harbored higher proportions of IFNγ producing Th-1 type cells (Fig. 6a–b). Most of the Th-17 cells were found to be $CD28^+$ memory $CD4^+$ T cells (Suppl. Fig. 1d). The ratio of IFNγ: IL-17 producing cells was ~ 2 within the $\alpha4\beta7^{hi}CD4^+$ T cell subsets (Fig. 6c), whereas it was >10 within the $\beta7^-$ $CD4^+$ T cell subsets indicating that $\alpha4\beta7^{hi}CD4^+$ T cell subsets were the primary source of Th-17 responses. SIV infection was found to significantly skew the ratio to a predominantly Th-1 phenotype, as there was a significant depletion of Th-17 cells during early SIV infection.

Discussion

Early SIV infection have been shown to target founder populations of $CD4^+$ T cells[4]. These cells are minimally activated, and play a central role in early viral replication and dissemination. Not much is known about why SIV specifically targets these founder populations of $CD4^+$ T cells. Given the highly activated microenvironment in mucosal tissues, and the enrichment of target cells in these tissues one would expect massive infection and replication to occur after viral entry. However, there appears to be a substantial delay of about 7–8 days before infection explodes out of the early founder population of cells to infect and destroy most of the memory $CD4^+$ T cell compartment[4,5]. Arthos et al[10] using *in vitro* studies demonstrated that HIV, and SIVsmm selectively targeted $CD4^+$ T cells that expressed the α4β7 heterodimer suggesting that these cells could be earliest targets for viral infection *in vivo*. It is not known if HIV preferentially targets memory $CD4^+$ T cells that express the α4β7 receptor *in vivo*.

Using the SIVmac251 infection model, we show that memory $CD4^+$ T cells that expressed high levels of the α4β7 heterodimer are preferentially infected during the early phase of viral infection; $\alpha4^+\beta7^{hi}CD4^+$ T cells harbored ~5x more SIV-gag DNA as compared to $\beta7^-CD4^+$ T cells. Surprisingly, $\alpha4^+\beta7^{hi}CD4^+$ T cells were found to be predominantly resting T cells ($CD25^-CD69^-HLA\text{-}DR^-Ki67^-$) with a central memory ($CD28^+CD45RA^-CCR7^+$) phenotype that did not significantly change during the early phase of viral infection. Li et al[4] reported that during the early phase of infection, SIV infection in the GALT is confined to resting $CD4^+$ T cells that were $CD25^-CD69^-Ki67^-$.

Both $CCR5^+$ and CCR5– subsets of $\alpha4^+\beta7^{hi}CD4^+$ and (37–$CD4^+CCR5^+$T cells were found to have significant levels of CCR5 mRNA suggesting that all the memory $CD4^+$ T cells subsets have sufficient CCR5 for infection by SIV. In line with this, both $\beta7^-$ $CD4^+CCR5^+$ and $\beta7^-CD4^+CCR5^-$ T cells had similar levels of SIV infection. It is likely that once SIV binds to the cells, the high levels of CCR5 expression enables SIV to more efficiently infect $\alpha4^+\beta7^{hi}CD4^+$ T cells, especially during the early phase of infection, when viral loads are very low, as seen at day 2–4 pi.

By day 7–10 pi, there was a significant increase in the extent of infection in both $\alpha4^+\beta7^{hi}$ and $\beta7^-CD4^+$ T cells, most likely as a consequence of the massive viral replication occurring during this period. However, $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets were found to harbor ~3x more SIV DNA than $\beta7^-CD4^+$ T cell subsets.

Interestingly, $\alpha4^+\beta7^{hi}CD4^+$ T cells were found to contain ~3.5 to 4 copies of SIV / cell at day 10 pi suggesting that there were likely more than 1 virion / cell. The assay used to quantify SIV infection in cells is stringently standardized using a non-expressing SIV infected cell line that carries only a single copy for SIV DNA[5]. As such we do not think that these high copy numbers are assay related. It is more likely that the expression of the α4β7 receptor along with CD4 and high levels of CCR5 makes $\alpha4^+\beta7^{hi}CD4^+$ T cells more susceptible to super infection with SIV. Previous studies have reported multiply infected $CD4^+$ T cells in HIV infected subjects. Jung et al[28] using fluorescent in situ hybridization assays showed that $CD4^+$ T cells in the spleen of HIV infected subjects carried 4–6 proviruses per infected cell. Future studies will attempt to address this question in more detail.

By day 63 pi, the extent of infection had equalized between the two subsets suggesting that there was a significantly higher loss of infected $\alpha4^+\beta7^{hi}CD4^+$ T cells following peak viral infection. In line with this conclusion, a significantly greater number of $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets were lost between day 0 and 63 pi as compared to the $\beta7^-CD4^+$ T cell subsets.

A number of factors likely contribute to the amplification of infection in $\beta7^-CD4^+$ T cell subsets. Acute SIV infection has been shown to be associated with acute immune

activation[23,24] leading to the production of numerous pro-inflammatory mediators such as IL-15[23]. Likewise, acute HIV infection is associated with an increase in numerous pro-inflammatory cytokines such as TNFα, IFNγ, IL-6, IL8, MCP-1, IFNα etc[29]. These factors likely play a role in making $CD4^+$ T cells more susceptible to infection. Eberly et al[23] showed that there was a significant increase in the production of IL-15 between day 7–14 pi that was found to correlate with infection in memory $CD4^+$ T cells during the early stages of infection. Others have shown that efficiency of infection was greatly influenced by the density of CD4, but not CCR5 expression on the surface of target cells[30–33].

It is also possible that during periods of high viremia, as seen during the ramp-up phase of acute viral infection, the threshold for efficient infection is lower than during the pre-ramp phase, when viral loads are very low. Thus, even though $\beta7^-CD4^+$ T cell subsets inherently expressed low levels of CCR5, it was likely enough for SIV to efficiently infect these cells between day 7–14 pi when plasma viral loads are as high as 7–8 logs / ml.

Previous studies[4,5] have shown that <5% of memory CD4 T cells were infected prior to day 7 pi, whereas by day 10–14 pi there was a significant increase in infection of these cells. Though these memory $CD4^+$ T cells lacked detectable CCR5 on their surface, they were found to have 20x more CCR5 mRNA as compared to naïve $CD4^+$ T cells that was sufficient for them to be infected by SIV. In line with these studies, we found significant levels of CCR5 mRNA in all the subsets of $\alpha4^+\beta7^{hi}$ and $\beta7^-CD4^+$ T cells. Others have shown that, minimal expression of CCR5 was sufficient for infection of $CD4^+$ T cells[30,32,33]. Eberly et al[23] demonstrated that acute SIV infection was associated with little or no upregulation of CCR5 expression on memory $CD4^+$ T cells during the first 10 days of infection, yet there was a significant increase in the level of SIV infection in these cells between day 7 and 10 pi. Taken together, these studies indicate that when viral loads are high, minimal levels of CCR5 is likely sufficient to support efficient infection of memory $CD4^+$ T cell subsets.

Interestingly, $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets harbored a significantly higher potential to produce IL-17 suggesting that most of the T helper-17 (Th-17) cells are found within these mucosal homing T cell subsets. The preferential loss of these subsets during acute SIV infection likely cripples the ability of the immune system to maintain homeostasis in mucosal tissues. On the other hand, most of the IFNγ producing T helper (Th-1) cells were found to be $\beta7^-CD4^+$ T cell subsets, and fewer of these cells were lost suggesting to a skewing of the T helper response to a Th-1 phenotype. Recent studies[11] have shown that Th-17 responses play a central role in protecting the mucosal barrier function, and the loss of Th-17 cells was associated with systemic dissemination of Salmonella in SIV infected rhesus macaques. Cecchinato et al[13] showed that altered balance between Th-17 and Th-1 cells in mucosal sites predicted AIDS progression in SIV infected rhesus macaques. On the other hand, Favre et al[14] showed that pathogenic SIV infection was associated with altered balance between Th-17 and T regulatory populations. Likewise, Brenchley et al[12] demonstrated that Th-17 cells were differentially depleted in pathogenic vs non-pathogenic lentiviral infections. Macal et al[34] showed that restoration of mucosal CD4 T cells was associated with enhanced Th-17 responses in HIV infected patients. Taken together, these studies suggest that preferential infection and depletion of $\alpha4\beta7^{hi}CD4^+$ T cell subsets significantly skews T helper responses towards a Th-1 phenotype thereby contributing to disease progression.

In conclusion, our results provide *in vivo* evidence that, during the early phase of SIV infection, SIVmac251 selectively targets $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets. These cells are predominantly central memory T cells with a resting phenotype and resemble the founder populations of $CD4^+$ T cells described by Li et al[4]. The inherently high level of CCR5 expression likely allows SIV to more efficiently infect the $\alpha4^+\beta7^{hi}CD4^+$ T cell subsets very early during the course of viral infection. Development of therapeutic strategies targeted at preventing the early infection

of memory $CD4^+$ T cell subsets that express high levels of α4β7 heterodimer may significantly aid in the early control of viral infection and contribute to the maintenance of homeostasis in mucosal tissues.

Materials and Methods

Animals & infection

Mamu*A01^{neg} rhesus macaques (Macaca mulatta) of Indian origin (n = 8) housed at Advanced Bioscience Laboratories Inc., MD were used in this study. Animals were housed in accordance with American Association for Accreditation of Laboratory Animal Care guidelines and were sero-negative for SIV, simian retrovirus and simian T-cell leukemia virus type-1. All the animals were infected with 100 animal-infectious doses of uncloned pathogenic SIVmac251 intravenously; Peripheral blood was collected at different time points (day 0, 2, 4, 10 and 63) after challenge, and Complete blood counts (CBC) data was obtained at day 0 and 63 pi. No CBC data was collected at other time points, hence absolute counts for various subsets could only be determined at day 0 and 63 pi. Additionally, peripheral blood samples were also obtained from 14 SIVmac251 infected rhesus macaques at day 2–3 (n = 4) and 7–14 pi (n = 10), 77 (n = 8) and from uninfected animals (n = 6 – 8).

Tissue samples

PBMC were isolated by density gradient centrifugation as previously described[5]. Plasma viral loads were determined by real-time PCR (ABI Prism 7700 sequence detection system, Applied Biosystems) using reverse-transcribed viral RNA as templates using methods previously described[35] (limit of detection < 30 copies / ml of plasma).

Antibodies and flow cytometry

All antibodies, except for CD103, used in this study were obtained from BD Biosciences (San Diego, CA), and titrated using rhesus macaque PBMC. For phenotypic analysis and sorting of $CD4^+$ T cell subsets cells were labeled simultaneously with the following combinations of antibodies: CD3-Cy7APC, CD8-Alexa-700, CD4-APC, CD95-FITC, CCR5-PE, HLA-DR-TexasRed-PE and Integrin β7 (FIB504 clone)-Cy5-PE. FIB504 clone has been shown to cross react with rhesus macaques[10,21]. β7 integrin forms a heterodimer with α4 (CD49d) or αE (CD103) integrins, and plays a central role in homing of T cells to mucosal tissues[36–39]. To delineate $\alpha4^+\beta7^+CD4^+$ T cell subsets from $\alpha E\beta7^+$ $CD4^+$ T cell subsets, cells were labeled with CD3-Cy7APC, CD8-Alexa-700, CD4-APC, CD95-FITC, CD49d–PE, CD103-FITC (clone 2G5 from Beckman Coulter) and Integrin β7-Cy5-PE. Clone 2G5 has been shown to cross react with rhesus macaque mucosal homing T cells, and expressed by intraepithelial lymphocytes[40,41]. To evaluate the phenotype of α4β7 subsets of $CD4^+$ T cells, cells were labeled with a panel of CD3-Cy7APC, CD8-Qdot-605, CD4-APC, CD95-FITC, CD49d–PE and β7-Cy5-PE, CD45RA-TR-PE, CD28-Cy7-PE and CCR7-Alexa-680. The expression of activation markers was determined by using a panel of CD3-Cy7APC, CD8-Alexa-700, CD4-TR-PE, CD95-FITC, CD49d–PE, β7-Cy5-PE, CD69 or CD25-APC.

To determine which α4β7 subsets were preferentially infected, α4β7 subsets of $CD4^+$ T cells (discriminated based on the expression of β7 and CD95[21]) were sorted, and subjected to qPCR assay for measuring SIV-gag DNA as described previously[5,15]. To determine if $CD4^+$ T cells that expressed the α4β7 heterodimer were preferentially infected, PBMC were labeled with: CD3-Cy7APC, CD8-Alexa-700, CD4-APC, CD95-FITC, CD49d–PE and β7-Cy5-PE. Additionally, KI-67 expression was evaluated using a panel of CD3-Cy7APC, CD8-Alexa-700, CD4-APC, CD95-PE and β7-Cy5-PE. After surface labeling, cells were fixed / permeabilized using the Cytofix/perm kit (BD Biosciences, San Diego, CA) and labeled with Ki-67-FITC.

To evaluate the ability of α4β7 subsets of $CD4^+$ T cells to produce IL-17 and IFNγ, PBMC were stimulated with Phorbol myristate acetate (PMA) at 10 ng / ml and ionomycin at 500 ng / ml in the presence of Brefeldin-A (1 uM) for 4 hours. Cells were harvested and labeled with CD3-Cy7APC, CD8-Alexa-700, CD4-Qdot-605, CD95-APC, and β7-Cy5-PE. After surface labeling, cells were fixed / permeabilized using the Cytofix/perm kit and labeled with IL-17-PE (e-Biosciences) and IFNγ-FITC (BD Biosciences).

Labeled cells were fixed in 0.5% paraformaldehyde, and analyzed using a modified Becton Dickinson Aria sorter.

qPCR assay

T-cell-associated viral DNA was measured by a quantitative PCR assay for SIV *gag* using a Perkin-Elmer ABI 7700 instrument as previously described[5,15] using SIV *gag* primers and probe as described by Lifson et al[42]. Cell numbers were quantified simultaneously using rhesus macaque Albumin specific primers and probe using previously described protocols[5].The assay was calibrated using a cell line that carried a single copy of proviral SIV DNA as shown previously[5]. Sorted samples were lysed in Proteinase-K and used for PCR analysis. We have previously shown that this assay was highly sensitive, and can be used to successfully measure SIV-DNA in single cells[5].

CCR5 mRNA levels were determined using primer/probes as described previously[5], and normalized to β2M levels in sorted subsets. Data are shown relative to $\alpha4^+\beta7^{int}CD4^+$ (naïve) T cells.

Data analysis

Flow cytometric data was analyzed using FlowJo version 8.6 (Tree Star, Inc., Ashland, OR). Statistical analysis was performed with GraphPad Prism Version 4.0 software (GraphPad Prism Software, Inc. San Diego, CA) using non-parametric tests. As the data did not exhibit normal distribution due to small sample size, comparisons between groups were performed using one-tailed *Mann-Whitney U* test, and multiple comparisons between groups was performed using the *Kruskal-Wallis One Way Analysis of Variance (ANOVA). $P < 0.05$* was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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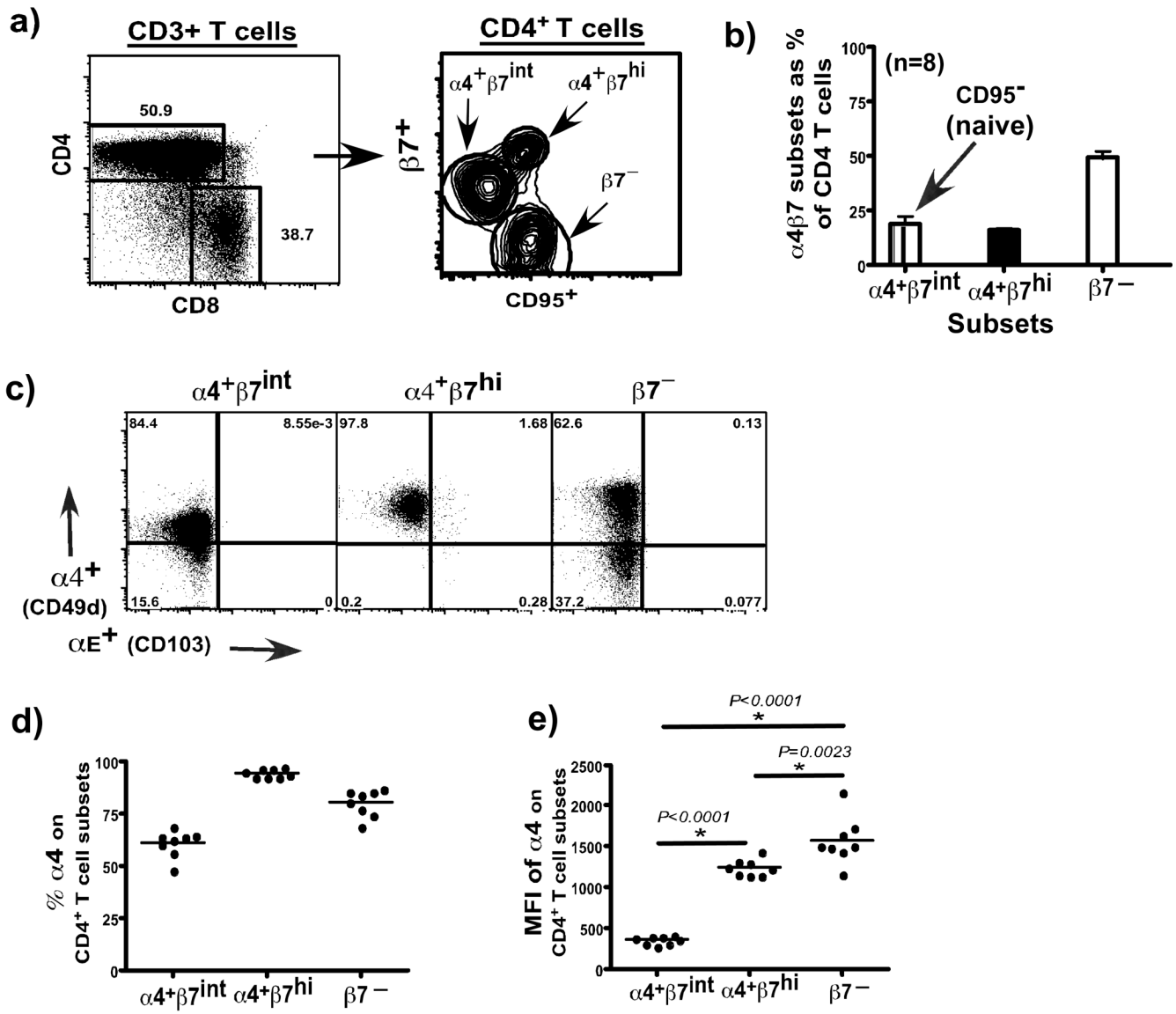

Figure 1. Phenotypic analysis of $CD3^+CD4^+$ T cells in peripheral blood from healthy rhesus macaques (n = 8)

(a) Representative contour plots and (b) relative proportions of $CD95^+\beta7^{hi}$ ($\alpha4^+\beta7^{hi}$), $CD95^-\beta7^{int}$ ($\alpha4^+\beta7^{int}$), and $CD95^+\beta7^-$ ($\beta7^-$) subsets of $CD4^+$ T cells. (c) Expression of α4 and αE integrins on $\alpha4^+\beta7^{hi}$, $\alpha4^+\beta7i^{nt}$, $\beta7^-CD4^+$ T cell subsets. (d) Relative proportions and (e) mean fluorescence intensity (MFI) of α4 expression on $\alpha4^+\beta7^{hi}$, $\alpha4^+\beta7^{int}$, $\beta7^-CD4^+$ T cell subsets. MFI was determined using Flowjo 8.6 software after gating on each subset.

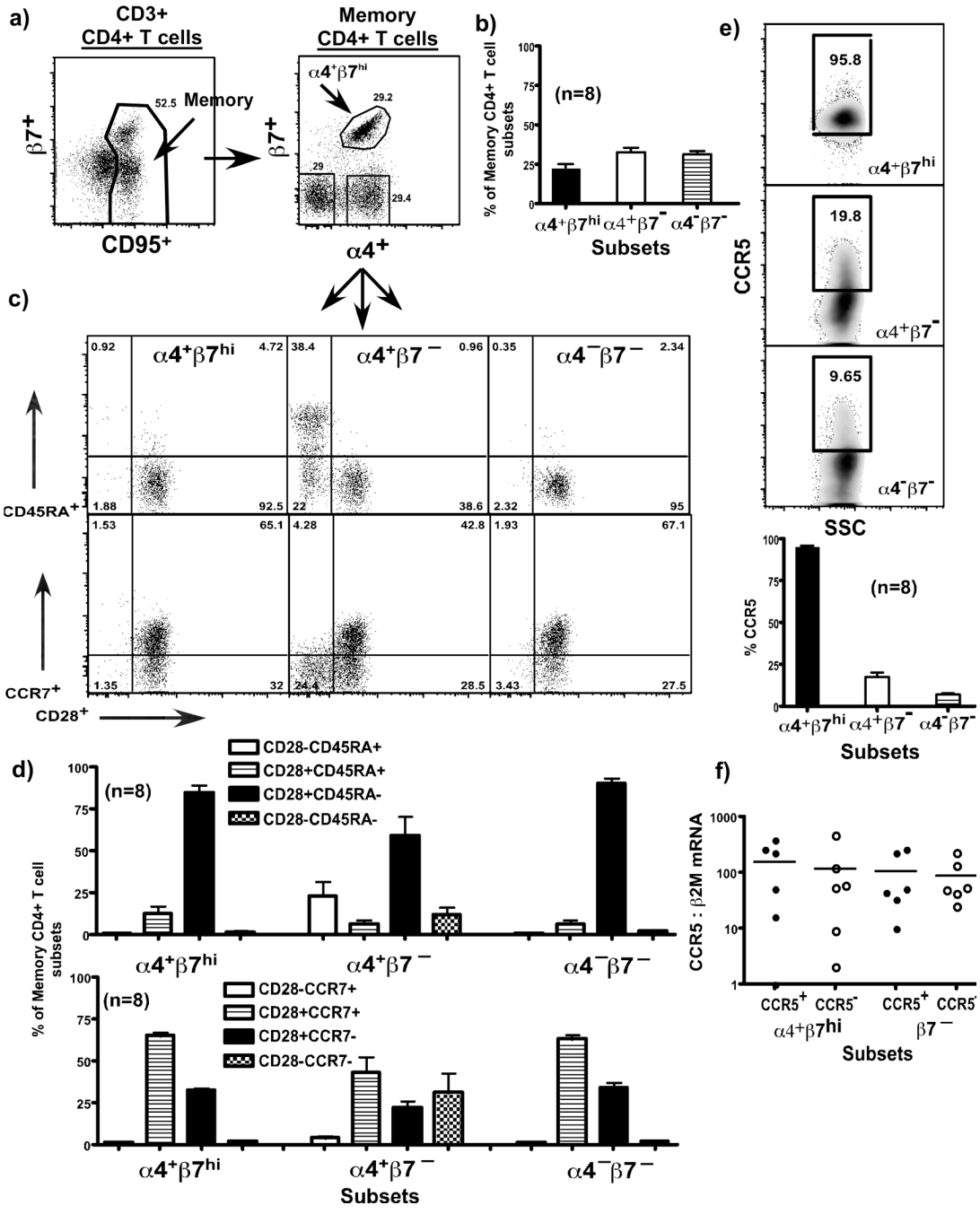

Figure 2. $\alpha4^{+}\beta7^{hi}$iCD4^{+} T cell subsets display a predominantly central memory phenotype (n = 8)

Costaining for α4 and β7 identifies memory CD4 T cells that express the α4β7 heterodimer along a diagonal. (a) Representative dot plots and (b) relative proportions of $\alpha4^{+}\beta7^{hi}$, $\alpha4^{+}\beta7^{-}$ and $\alpha4^{-}\beta7^{-}$ subsets of CD4^{+} T cells. (c) Representative dot plots and (d) relative proportions of $\alpha4^{+}\beta7^{hi}$, $\alpha4^{+}\beta7^{-}$ and $\alpha4^{-}\beta7^{-}$ subsets of CD4^{+} T cells expressing CD45RA, CD28 and CCR7. (e) Representative dot plots and relative proportions of $\alpha4^{+}\beta7^{hi}$, $\alpha4^{+}\beta7^{-}$ and $\alpha4^{-}\beta7^{-}$ subsets of CD4^{+} T cells expressing CCR5. Most $\alpha4^{+}\beta7^{hi}$ subsets express detectable levels of surface CCR5 (f) Relative expression of CCR5 mRNA in CCR5^{+} and CCR5^{-} subsets of $\alpha4^{+}\beta7^{hi}$ and $\beta7^{-}$ CD4^{+} T cells (n = 6).

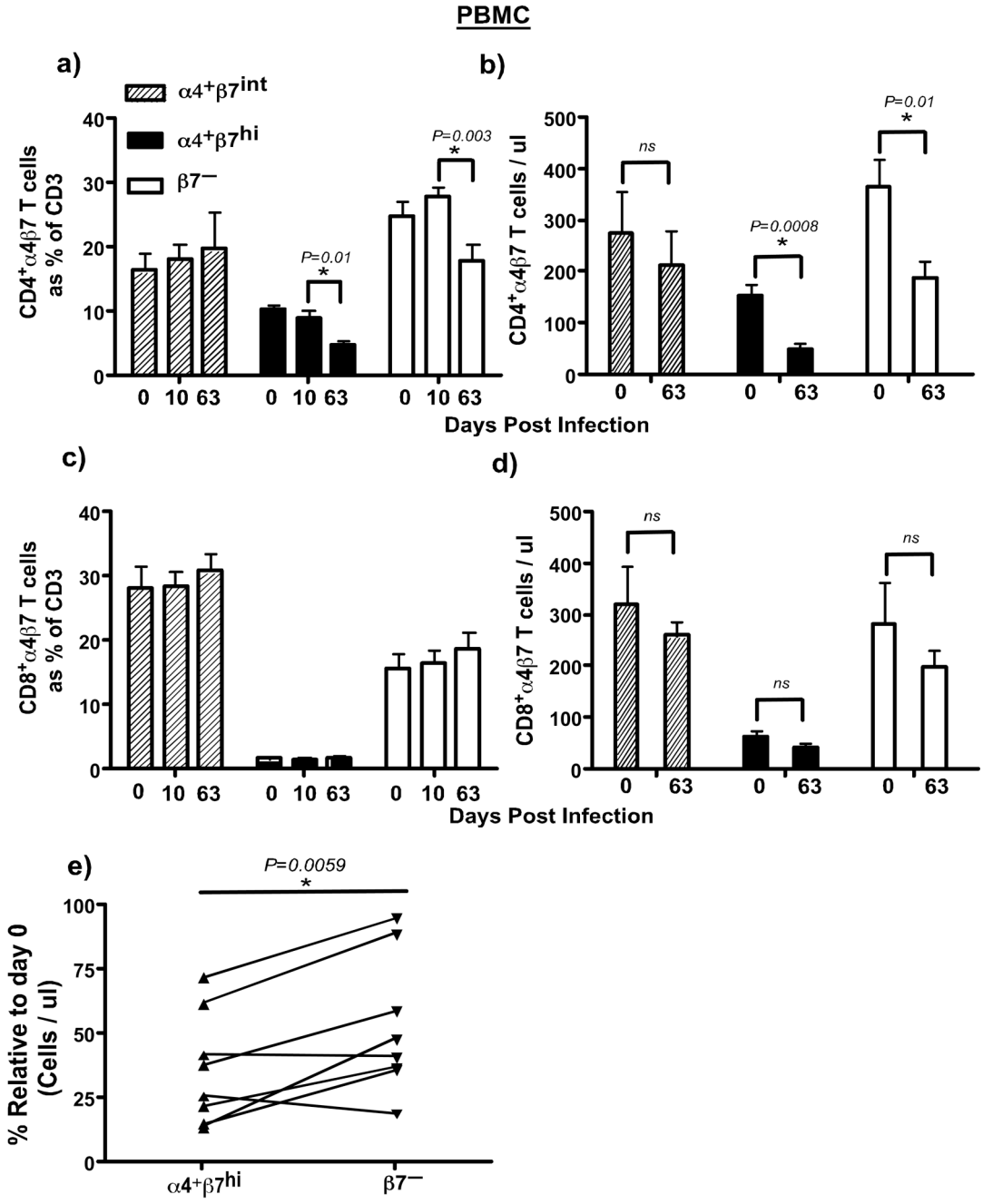

Figure 3. Dynamics of α4 β 7 subsets of CD4$^+$ T cells in peripheral blood during acute SIV infection

Significant loss of α4β7^{hi} and β7$^-$ CD4$^+$ T cells is observed during acute SIV infection. Both frequency and absolute numbers are shown for (a–b) CD4$^+$ and (c–d) CD8$^+$ T cells (n = 8). (e) Higher numbers of α4$^+$β7^{hi}CD4$^+$ T cells were destroyed by day 63 pi as compared to β7$^-$ CD4$^+$ T cells. The % change in absolute numbers of α4$^+$β7^{hi} and β7$^-$CD4$^+$ T cells was calculated using each animals day 63 values relative to its day 0 values using day 0 values as 100%.

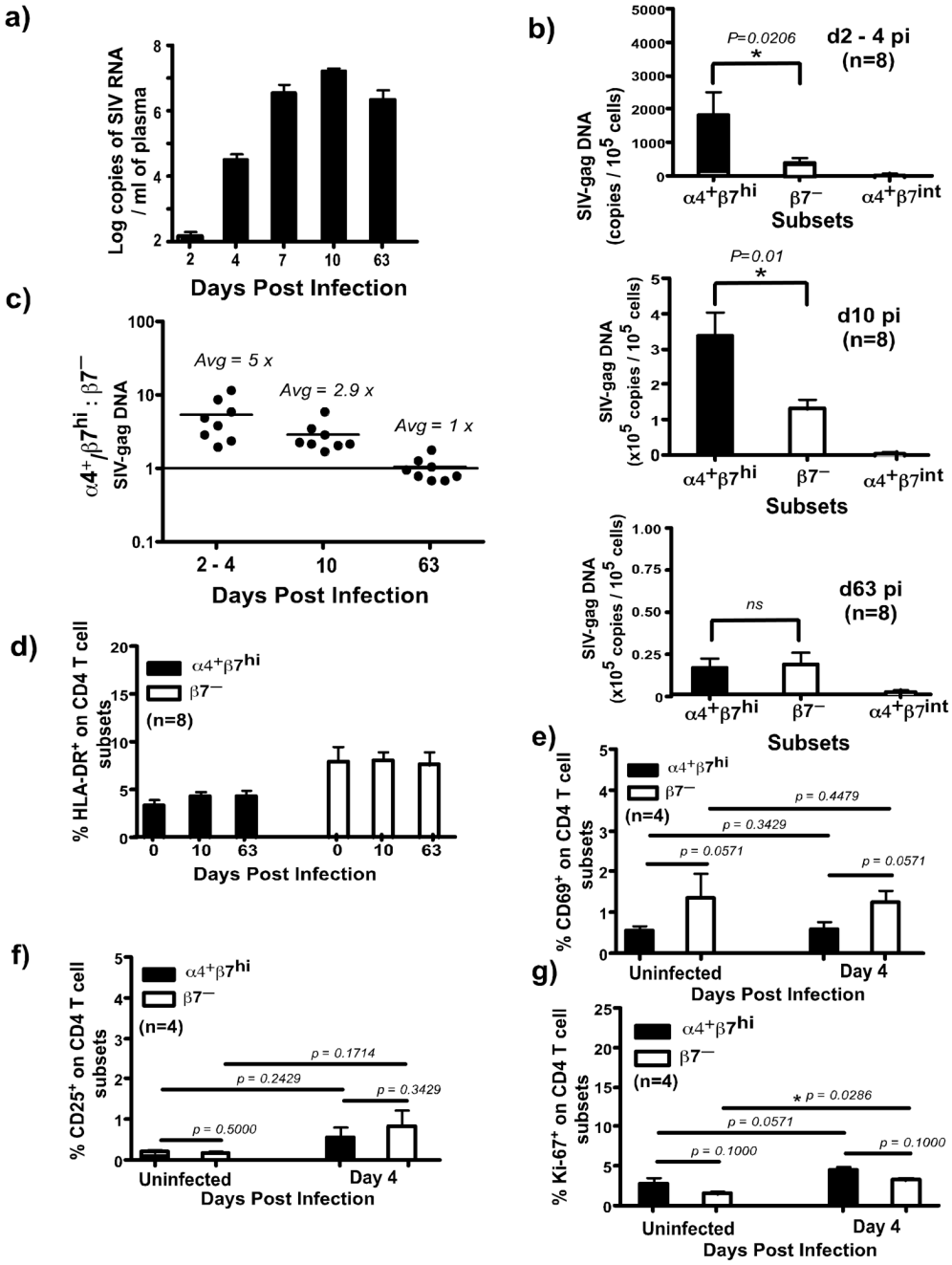

Figure 4. $\alpha4^+\beta7^{hi}CD4^+$ T cells are preferentially infected during acute SIV infection

(a) Plasma viral loads. Limit of detection is <30 copies /ml of plasma. (b) Levels of SIV-gag DNA were determined using qPCR assay for SIV-gag in sorted subsets of $\alpha4^+\beta7^{hi}$, $\alpha4^+\beta7i^{nt}$ and $\beta7^-$ $CD4^+$ T cells. $\alpha4^+\beta7^{hi}CD4^+$ T cells are preferentially infected at day 2–4 (n = 8) and day 10 pi (n = 8). (c) Ratio of SIV-gag DNA levels in $\alpha4^+\beta7^{hi}$: $\beta7^-CD4^+$ T cells at day 2–4 (n = 8), 10 (n = 8) and 63 (n = 8) pi. The expression of (d) HLA-DR (n = 8) (e) CD69 (n = 4) (f) CD25 (n = 4) and (g) Ki-67 (n = 4) on $\alpha4^+\beta7^{hi}$ and $\beta7^-CD4^+$ T cells during the early phase infection.

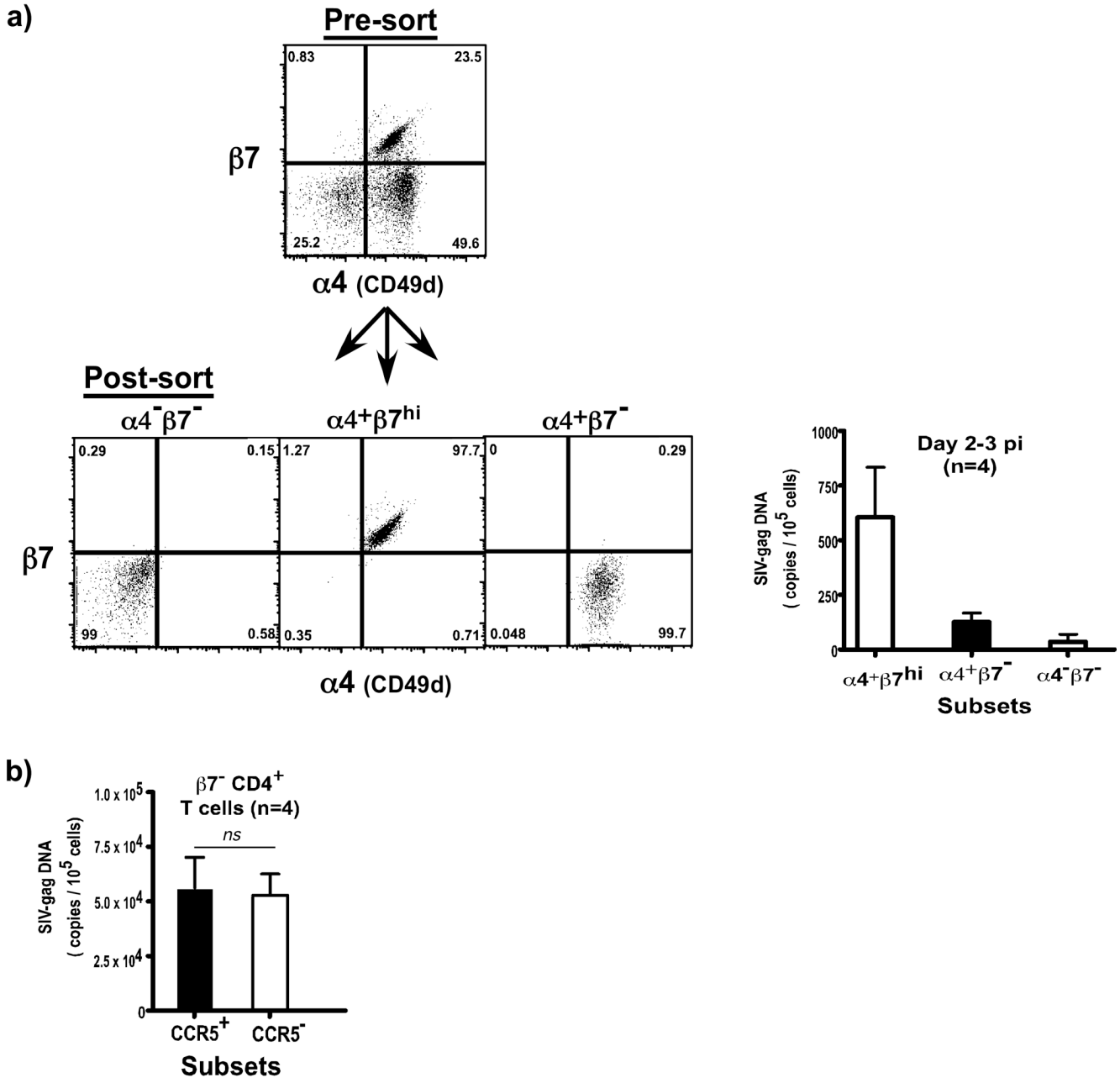

Figure 5. CD4$^+$ T cells that express the α4β7 hetrodimer are preferentially infected *in vivo*

Memory CD4$^+$ T cells were sorted based on the co-expression of α4 and β7 integrins by flow cytometry, and used in the qPCR assay for SIV-gag DNA. (a) Sorting strategy and purity of sorted subsets are shown along with the level of SIV infection in α4$^+$β7^{hi}, α4$^+$β7$^-$ and α4$^-$β7$^-$ subsets at day 2–3 pi (n = 4). α4$^+$β7^{hi} subsets harbor significantly higher levels of SIV DNA at day 2–3 pi (n = 4; *p = 0.0244*) as compared to α4$^+$β7$^-$ and α4$^-$β7$^-$ subsets. Statistical analysis was performed using *Kruskal-Wallis One Way Analysis of Variance (ANOVA)* (b) There is no significant difference in the level of SIV-gag DNA between β7$^-$CD4$^+$CCR5$^+$ and β7$^-$CD4$^+$CCR5$^-$ T cell subsets of memory CD4$^+$ T cells from peripheral blood at day 7 &14

pi (day 7: n = 2, day 14: n = 2). $\beta7^{-}$ subsets were gated based on the lack of β7 expression and CD95, followed by sorting of CCR5+ and CCR5- subsets.

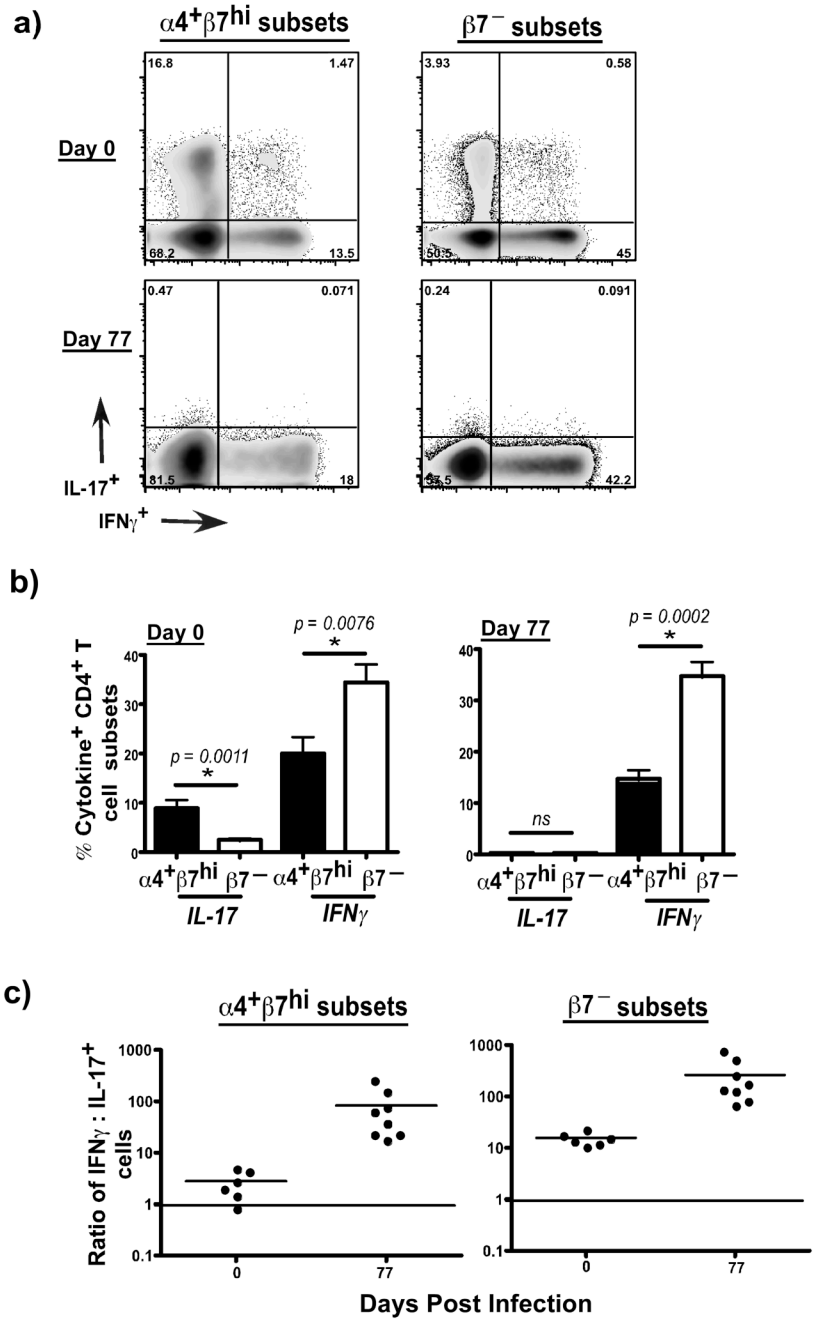

Figure 6. α4$^+$β7^{hi}CD4$^+$ T cells harbor significantly higher frequencies of Th-17 cells that are lost after SIV infection

α4$^+$β7^{hi}CD4$^+$ T cell subsets have a significantly higher capacity to produce IL-17 as compared to β7$^-$CD4$^+$ T cell subsets. Expression of IL-17 and IFNγ was determined by flow cytometry using PBMC from uninfected (n = 6) and SIV infected animals (n = 8) following stimulation with PMA/Ionomycin for 4 hours. (a) Representative dotplots showing IL-17 and IFNγ production α4$^+$β7^{hi} and β7$^-$CD4$^+$ T cell subsets at day 0 and day 77 pi. (b) IL-17 and IFNγ production α4$^+$β7^{hi} and β7$^-$CD4$^+$ T cell subsets at day 0 (n = 6) and day 77 pi (n = 8). (c) Ratio of IFNγ: IL-17 producing α4$^+$β7^{hi} and β7$^-$CD4$^+$ T cell subsets at day 0 and day 77 pi.