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Vaccination with SIVΔnef activates CD4+ T cells in the absence of CD4+ T cell loss

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Abstract

Background—Pathogenic HIV and SIV infections characteristically deplete central memory $CD4^+$ T cells and induce chronic immune activation, but it is controversial whether this also occurs after vaccination with attenuated SIVs and whether depletion or activation of $CD4^+$ T cell play roles in protection against wild-type virus challenge.

Methods—Rhesus macaques were vaccinated with SIVΔnef and quantitative and phenotypic polychromatic flow cytometry analyses were performed on mononuclear cells from blood, lymph nodes and rectal biopsies.

Results—Animals vaccinated with SIVΔnef demonstrated no loss of $CD4^+$ T cells in any tissue, and in fact $CCR5^+$ and $CD28^+CD95^+$ central memory $CD4^+$ T cells were significantly increased. In contrast, $CD4^+$ T cell numbers and CCR5 expression significantly declined in unvaccinated controls challenged with SIVmac239. Also, intracellular Ki67 increased acutely as much as 3-fold over baseline in all tissues after SIVΔnef vaccination then declined following primary infection.

Conclusion—We demonstrated in this study that SIVΔnef vaccination did not deplete $CD4^+$ T cells but transiently activated and expanded the memory cell population. However, increases in numbers and activation of memory $CD4^+$ T cells did not appear to influence protective immunity.

Keywords

Simian immunodeficiency virus; live attenuated SIV; T cell activation; Protective immunity

Introduction

HIV infections and pathogenic SIV infections of rhesus macaques are typically characterized by a gradual depletion of $CD4^+$ T cells in the peripheral blood and acutely massive $CD4^+$ T cell loss in the gut mucosa [1–3]. More specifically, the predominant target cells for viral infection and subsequent loss are $CD28^+$ "central" memory $CD4^+$ T cells expressing the HIV/SIV coreceptor CCR5 [2,4–8]. $CD4^+$ T cell loss is driven, in part, by another feature of progressive HIV/SIV infections, chronic immune activation, which provides a pool of activated $CD4^+$ T cells to serve as substrates for viral replication [9–11].

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Currently, vaccinations with live attenuated SIV are the only effective means of inducing sterile protection against lentivirus infections [12–15], and although it is unlikely a live attenuated HIV vaccine will ever be used in practice, this model has provided fundamental insights into the necessary components for protective immunity. Various attenuated SIV strains have been shown to induce potent humoral and cellular immune responses, all of which may contribute to sterilizing immunity [13,16–20]. Moreover, live attenuated SIVs are replication-competent, and the *nef*-deficient virus, SIVmac239Δnef, has been shown to infect predominantly $CD4^+$ T cells *in vivo* [21]. However, it is controversial whether infections with live attenuated SIVs results in a net cellular loss of $CD4^+$ T cells. Veazey et al. [3] showed that while the wild-type virus SIVmac239, from which SIVmac239Δnef is derived, depleted $CD4^+$ T cells in gut mucosa, SIVmac239Δnef did not. Picker et al. [22] showed a similar lack of $CD4^+$ T cell depletion by SIVmac239Δnef in bronchoalveolar lavage. In contrast, some previous studies have demonstrated evidence of $CD4^+$ T cell loss and disease progression in a small number of macaques infected with another attenuated SIV, SIVmac239Δ3 [23–25]. However, cell loss and disease progression were most prominent in neonatal macaques where the immune system is less mature and in macaques immunosuppressed by steroid administration. Furthermore, those studies primarily focused on evaluation of the bulk $CD4^+$ T cell population and lacked the advantage of more modern methods of cellular quantification.

The objective of this study was to enumerate and characterize the activation states of both bulk and $CD28^+$ memory $CD4^+$ T cells during the primary phase of infection with the live attenuated virus, SIVmac239Δnef. We also evaluated what role(s) changes in quantitative and activation states might have on sterile protection of SIVmac239Δnef-vaccinated macaques against SIVmac239 challenge.

Material and methods

Animals and infections

A total of twelve male Indian rhesus macaques (*Macaca mulatta*) were used in this study. All animals were SIV-negative (naive) at the initiation of the study and were also free of simian retrovirus type D, SIV, simian T-lymphotrophic virus type 1, and herpes B virus. Ten animals were vaccinated intravenously with SIVmac239Δnef as previously described [14,15] and then intravenously challenged with 10 AID_{50} of SIVmac239 [26] at either 5 or 15 weeks post-vaccination (five vaccinated macaques and one unvaccinated control at each time point). Both control animals became infected with SIVmac239, while none of the SIVmac239Δnef-vaccinated animals showed any evidence of infection with SIVmac239, nor any evidence of anamnestic responses (R.K.R. and R.P.J., manuscript in preparation). The two vaccine groups (5 and 15 week challenges) are combined herein for selected evaluations of cellular dynamics. All of the macaques were housed at the New England Primate Research Center and maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the Guide for the Care and Use of Laboratory Animals.

Cell processing

Rhesus macaque PBMCs were isolated from EDTA-treated venous blood by LSM (MP Biomedicals, Solon, OH) density gradient centrifugation and tissue mononuclear cells were isolated from minced peripheral lymph node and rectal biopsies as described previously [27]. Cells were washed and resuspended in PBS supplemented with 2% FCS (Sigma-Aldrich, St. Louis, MO) for subsequent flow cytometric analysis.

Antibodies and flow cytometric analysis

Surface staining was carried out by standard procedures as described previously [28]. Except where noted, all mAb were obtained from BD Biosciences (San Diego, CA) and included: CD3

APC-Cy7 (clone SP34-2), CD4 AmCyan (clone L200, custom) or CD4 PerCP-Cy5.5 (SK3), CD8α Alexa700 (clone RPA-T8), CD28 Texas Red (clone CD28.2 from Beckman-Coulter, Fullerton, CA), CD95 APC (clone DX2), CCR5 PE (clone 3A9), and TCR γδ-FITC (clone B1). Intracellular staining for Ki67 (PE conjugate, clone B56) was performed on mononuclear cells using The Biolegend Fix/Perm kit (Biologend Inc., San Diego, CA) according to the manufacturer's suggested protocol. All acquisitions were made on an LSR II (BD Biosciences) and analyses were done using FlowJo software (Tree Star Inc., Ashland, OR).

Whole blood lymphocyte count assay

Fifty µl aliquots of whole blood were incubated with 50,050 fluorescent beads and a pool of lyophilized antibodies, or singularly for compensation tubes, for 20 minutes at room temperature and then lysed with FACSLyse (BD Biosciences) for 10 minutes. To preserve cell concentrations, no wash step was used. Acquisitions were performed on an LSR II and 5,000 beads were collected per sample. Analysis was performed using FlowJo software (Fig. 1A) and absolute cell numbers were calculated based on bead-to-cell frequencies (Fig. 1B) [29]. The following antibodies (all from BD) were used for compensation and sample tests: CD3 Alexa 700 (clone SP34-2), CD4 APC-Cy7 (clone L200), CD8 APC (clone SK1), CD14 PE-Cy7 (clone M5E2), CD20 PerCp-Cy5.5 (clone L27), CD45 PE (D058-1283), and HLA-DR FITC (clone L243). For some cell subsets (i.e., $CD28^+$ $CD4^+$ memory cells) absolute counts were determined using this assay in combination with cell frequency data derived from polychromatic flow cytometry.

Statistical analysis

All statistical and graphical analyses were done using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Nonparametric Wilcoxon and Mann-Whitney tests were used where indicated and $P < 0.05$ were assumed to be significant in all analyses.

Results

Identification and quantification of $CD4^+$ T cells in rhesus macaques

As described in the Material and Methods section and shown in Fig. 1, absolute numbers of $CD4^+$ T cells were enumerated using a whole-blood bead-based assay and cell frequencies were determined using polychromatic flow cytometry (Fig 2A). In normal rhesus macaques (day 0) the median number of total circulating $CD4^+$ T cells was 834 cells/µl of blood (range 345–1415 cells/µl; n = 12) and the median frequency of $CD4^+$ T cells among total T cells was 67% (range, 36–78%). $CD4^+$ T cells were further delineated into naïve, central memory, and effector populations using CD95 (FAS) and the costimulatory molecule CD28 as previously described [30]. Employing this gating strategy, we also defined $CD4^+$ T cell subsets as $CD28^{int}CD95^-$, $CD28^{bright}CD95^+$ and $CD28^-CD95^+$ corresponding to naïve, central memory and effector memory cells, respectively (Fig. 2B). The median frequencies of each of these subsets as fractions of the total $CD4^+$ T cell population in normal rhesus macaques were: naïve – 58%, central – 41%, and effector – 1.6% (n =12).

SIVmac239Δnef vaccination does not deplete $CD4^+$ T cells

Ten normal rhesus macaques were vaccinated with SIVΔnef. Subsequently, five macaques were challenged with SIVmac239 at 5 weeks and five at 15 weeks post-vaccination (Fig 3A). Neither animal group demonstrated any significant changes in $CD4^+$ T cell numbers or frequencies after vaccination or after challenge (Fig. 3A). Therefore, these ten animals were grouped collectively for further analyses (Fig. 3B, right panels). In contrast to SIVmac239Δnef-vaccinated animals, percentages of $CD4^+$ T cells declined in frequency by ~50% by week 5 post-challenge and remained reduced through 26 weeks post-challenge (Fig

3B, left panels). Similarly, the absolute numbers of $CD4^+$ T cells declined to 97 and 227 at weeks 25 (Mm # 353-99) and 26 (Mm # 313-97), respectively. Interestingly, $CD28^+$ memory $CD4^+$ T cells increased transiently in both frequency and absolute number over baseline levels (Fig. 4, right panels), reaching statistical significance at week one post-vaccination ($P = 0.002$ and $P = 0.0273$, respectively, Wilcoxon test). As expected, $CD28^+CD95^+$ memory $CD4^+$ T cells declined rapidly during acute SIVmac239 infection, followed by a rebound period, and then gradually declined longitudinally (Fig. 4, left panels). By comparison there was ~ 40% increase in the frequency of $CD28^+CD95^-$ naïve $CD4^+$ T cells two weeks after SIVmac239 infection of unvaccinated controls which was sustained throughout the observation period (Fig. 5A), likely due to decline in the frequency of central memory cells. This was not however observed after SIVmac239Δnef vaccination, where the naïve population was stable.

Since $CD4^+$ T cell depletion in HIV and SIV infections is most prominent in the gut mucosa, we also examined the frequency of $CD4^+$ T cells among the total T cell population in rectal biopsies (Fig. 6). When comparing pre-vaccination/infection biopsies to those taken 3 weeks post-SIVΔnef-vaccination, the median values (Pre – 58.5%, week 3 – 58.0%) were equivalent, while the frequency of $CD4^+$ T cells in rectal biopsies had declined in both unvaccinated controls.

Because the chemokine receptor CCR5 also delineates memory cells and is a necessary coreceptor for SIV infection, we also evaluated its expression on circulating $CD4^+$ T cells (Fig. 2C). Interestingly, while the frequency of CCR5 expressing $CD4^+$ T cells eventually declined in wild-type infection (Fig. 5B), after SIVmac239Δnef vaccination, CCR5 expression significantly increased at one week post-vaccination ($P = 0.0039$, Wilcoxon test; means, week 0 – 3.55%, week 1 – 6.6%). Although not statistically significant, CCR5 expression remained elevated until ~ 18 weeks post-vaccination (mean at 18 weeks, 3.08%).

SIVmac239Δnef activates $CD4^+$ T cells after vaccination

An acute increase in CCR5 expression on $CD4^+$ T cells suggested SIVΔnef vaccination could be activating these cells. To further evaluate this possibility we analyzed intracellular Ki67 expression in bulk $CD4^+$ T cells in PBMC, peripheral lymph nodes, and in rectal biopsies. Ki67 was upregulated acutely in all three tissues, but only significantly in PBMC and peripheral lymph nodes (Fig. 7). By 15 weeks post-vaccination, Ki67 expression had declined to near pre-vaccination levels, but in peripheral lymph nodes was still significantly higher than baseline. It is also interesting to note that basal levels of Ki67 expression were significantly lower in peripheral lymph nodes than in either PBMC ($P = 0.0022$, Mann Whitney test) or rectal biopsies ($P = 0.0050$).

Discussion

In this study we comprehensively examined the cellular dynamics of memory and bulk $CD4^+$ T cells after vaccination with the live attenuated SIV strain, SIVmac239Δnef. Unlike pathogenic lentivirus infections, SIVmac239Δnef-vaccinated animals demonstrated no loss of $CD4^+$ T cells either in the periphery or in gut mucosa. However, we did find a transient increase in $CD4^+$ T cell activation that declined coinciding with control of viremia, in contrast to the sustained increases in $CD4^+$ T cell activation observed in HIV-1 and pathogenic SIV infections [9–11,31]. Although the transient induction of $CD4^+$ T cell activation by SIVmac239Δnef did not appear to influence protection against wild-type virus challenge in this study, it could have implications for target cell availability, and ultimately vaccine efficacy.

Previous studies evaluating $CD4^+$ T cell loss in macaques infected with live attenuated SIVs have yielded conflicting results. Similar to our findings, Picker et al. [22] reported no evidence for loss of bulk or memory $CD4^+$ T cells in peripheral blood or bronchoalveolar lavage in

macaques vaccinated with SIVmac239Δnef. In contrast, naïve macaques vaccinated as infants with high doses of SIVmac239Δ3 almost uniformly progressed to simian AIDS [24], indicating that the infant immune system can be vulnerable to even attenuated lentivirus strains. However, vaccination of adult macaques with SIVmac239Δ3 was much less likely to cause disease – AIDS-like symptoms occurred in less than 20% of vaccinated macaques and only after a prolonged observation greater than 3 years or after steroid-induced immunosuppression [23–25]. Another *nef*-defective SIV, SIVmacC8, was also found to cause $CD4^+$ T cell loss as early as 12 weeks, but only in 1 animal [32,33]. Although the number of macaques in these studies progressing to disease is small, we cannot exclude the possibility that some fraction of SIVmac239Δnef-vaccinated animals could develop some symptoms of disease, particularly since our original observation period was concluded at 40 weeks and Picker et al. [22] only evaluated animals up to 200 days post-vaccination.

Although SIVmac239Δnef was derived over 15 years ago [14], the specific protective correlates remain poorly understood. Many immune components have been proposed to be involved in mediating protection – humoral immunity, cytoxic T lymphocytes, innate immune responses, and $CD4^+$ T cells [18,19]. Since SIVmac239Δnef primarily replicates in $CD4^+$ T cells in lymphoid tissue [21] a hypothesized mechanism of protection was that SIVmac239Δnef might actually deplete target cells, decreasing permissivity to superinfection with wild-type viruses. However, we demonstrate herein that in fact the opposite is true, at least acutely, – the frequency of memory $CD4^+$ T cells transiently increases following SIVmac239Δnef vaccination. Therefore, we must consider the opposite hypothesis – that increased numbers of target cells could exacerbate infection rates after wild-type virus challenge. In this study, when vaccinated macaques were challenged at 5 weeks post-vaccination (when levels of Ki67 and CCR5 expression were still elevated) all animals were sterilely protected, indicating that the increased target cell availability and activation did not results in superinfection with the SIVΔnef parental virus, SIVmac239. However, in a previous kinetic study, when SIVmac239Δnef-vaccinated macaques were challenged with the SIVmac239-related virus, SIVmac251 animals became infected at 5, but not 15 weeks vaccination [15]. Therefore, increases in activation or target cell availability might contribute to superinfection with viruses other than the parental strain, although the relative contribution of this effect is difficult to assess, since changes in SIV-specific immune responses also occur during this time frame.

While the question of whether live attenuated SIVs can induce AIDS-like disease in rhesus macaques remains controversial, our data show no evidence for this in primary infection or up to ~ 40 week post-vaccination. Also, since no loss of $CD4^+$ T cells occurred in peripheral blood or in the gut mucosa, this suggests that these macaques are less likely to progress to immunodeficiency, since early $CD4^+$ T cell loss often dictates disease course [1,2,22,34]. Furthermore, while our results indicate that acute increases in activated memory $CD4^+$ T cells do not influence infection rates upon challenge with SIVmac239, we cannot exclude the possibility that the transient increase in $CD4^+$ T cell activation may affect the susceptibility to challenge in other experimental settings. More detailed studies will be needed to assess the potential consequences of activation of the $CD4^+$ T cell memory pool to protective immunity induced by live attenuated SIV.

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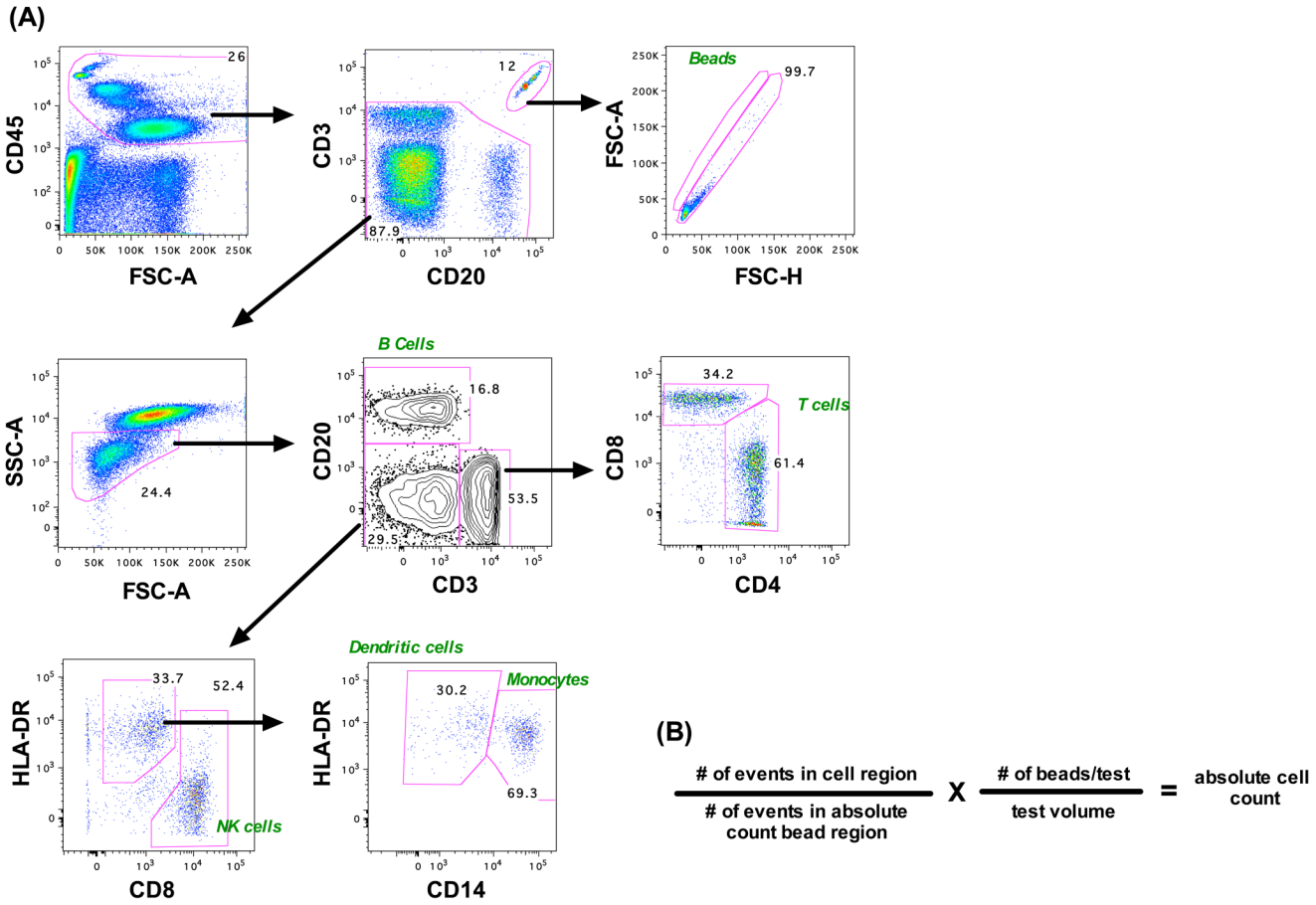

Figure 1.

Absolute lymphocyte determination by a whole-blood bead-based flow cytometry assay. (A) A representative gating strategy is shown identifying various lymphocyte subsets as well as fluorescent beads. (B) The absolute number of cells per volume of blood is calculated based on the number of events in each cell gate.

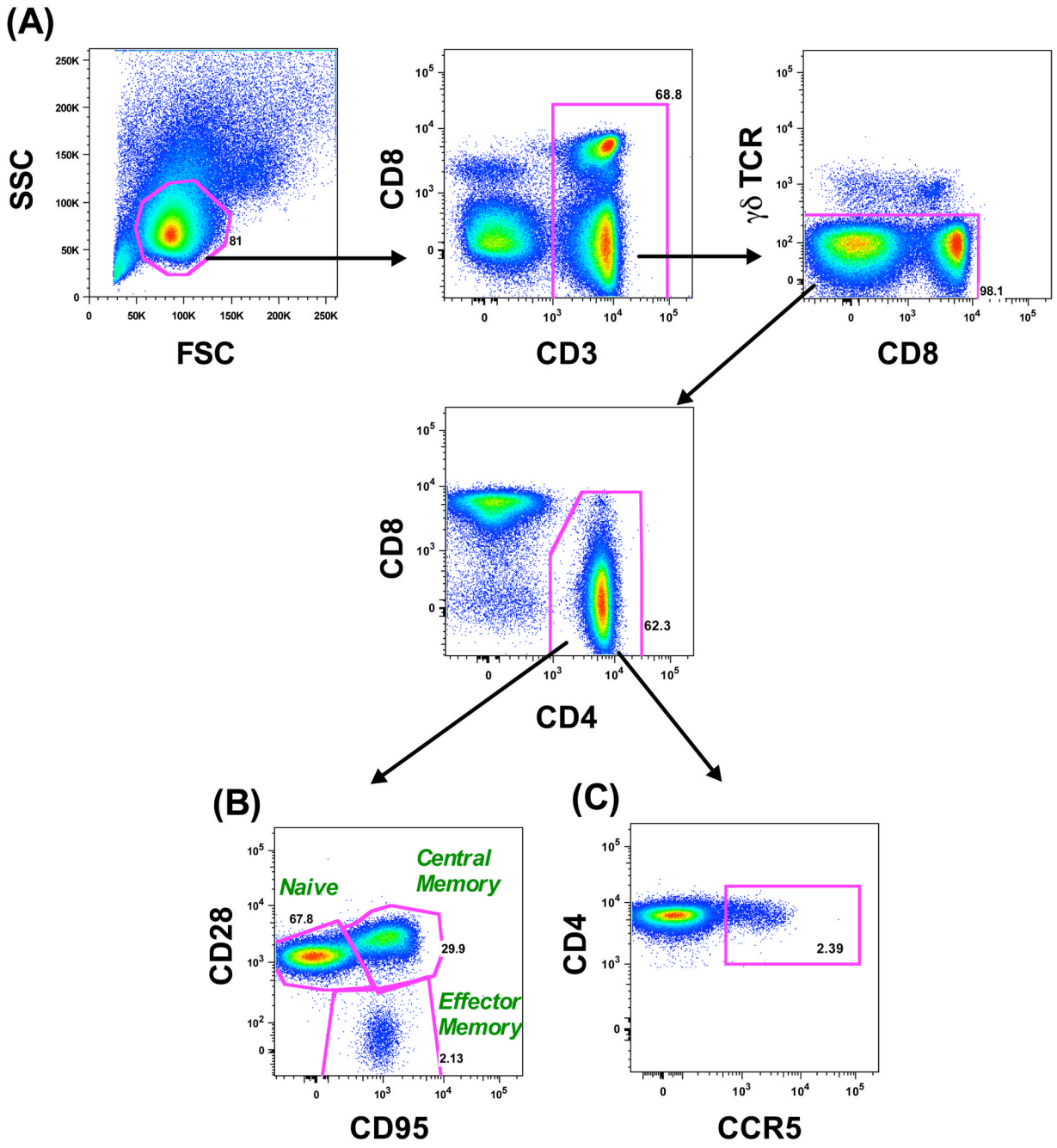

Figure 2.

Phenotypic identification of $CD4^+$ T lymphocyte subsets by polychromatic flow cytometry. Representative gating strategies are shown for identification of various subpopulations of $CD4^+$ T cells in whole PBMC. (A) First, lymphocytes were gated based on forward-versus-side-scatter characteristics and $CD4^+$ αβ T cells were identified as $CD4^+CD3^+CD8^-$ and negative for the γδ T cell receptor. Secondly, (B) naïve and central memory $CD4^+$ T cells were phenotyped as $CD28^+CD95^-$ and $CD28^+CD95^+$, respectively. (C) Frequency of CCR5 expression was quantified on the bulk $CD4^+$ T cell population.

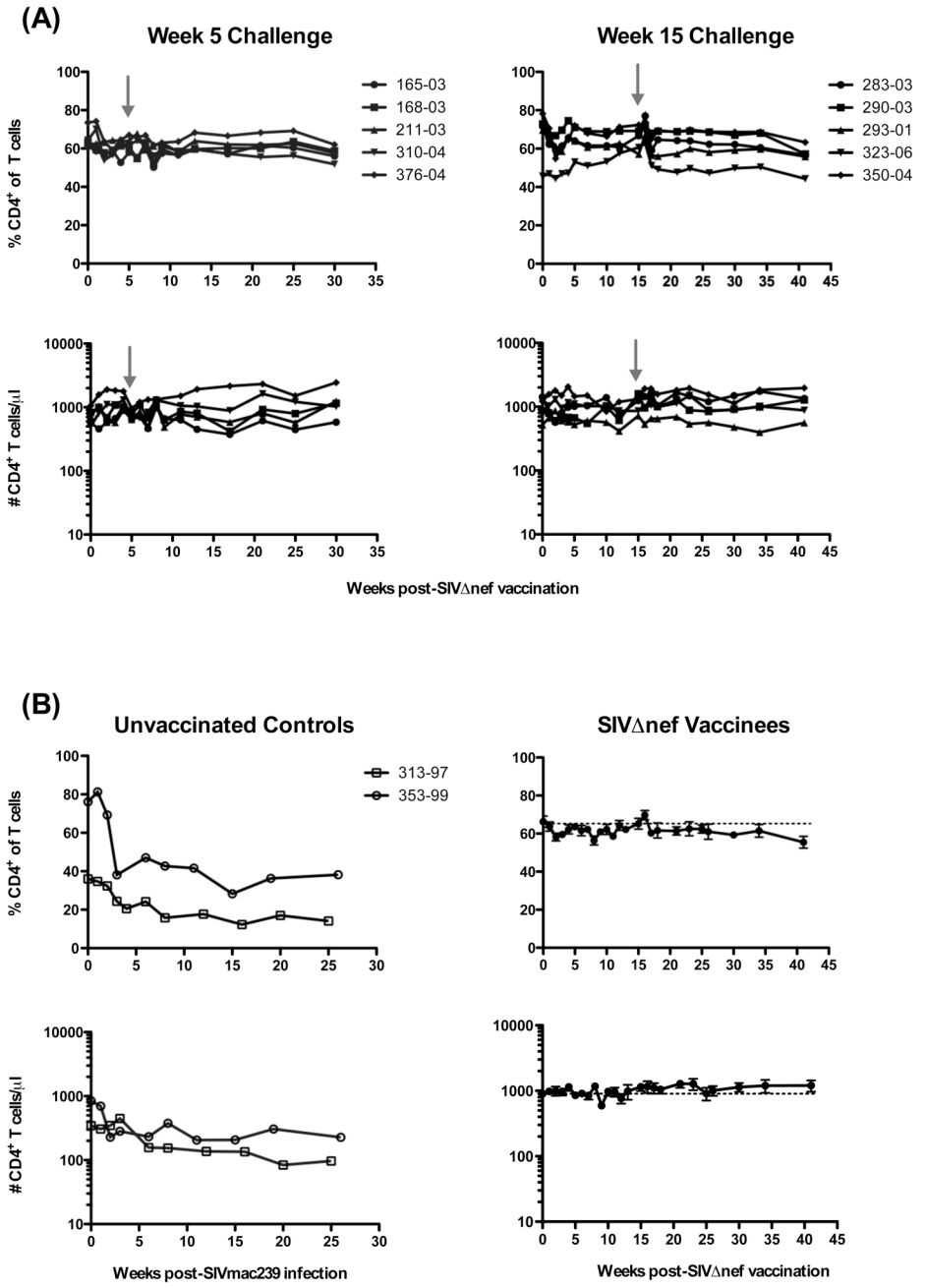

Figure 3.

Longitudinal analysis of circulating $CD4^+$ T cells in SIVΔnef-vaccinated and SIVmac239-infected rhesus macaques. (A) Frequencies (upper panels) and absolute numbers (lower panels) of circulating $CD4^+CD3^+$ lymphocytes are shown for SIVΔnef-vaccinated macaques challenged intravenously with SIVmac239 at either 5 or 15 weeks post-vaccination; grey arrows indicate 5 and 15 week challenges, respectively. (B) Frequencies and absolute numbers of $CD4^+CD3^+$ lymphocytes in two unvaccinated controls challenged with SIVmac239 (left panels) and means ± SEM of SIVΔnef-vaccinated macaques (right panels) combined from (A). Dashed horizontal lines indicate means at day of vaccination (day 0).

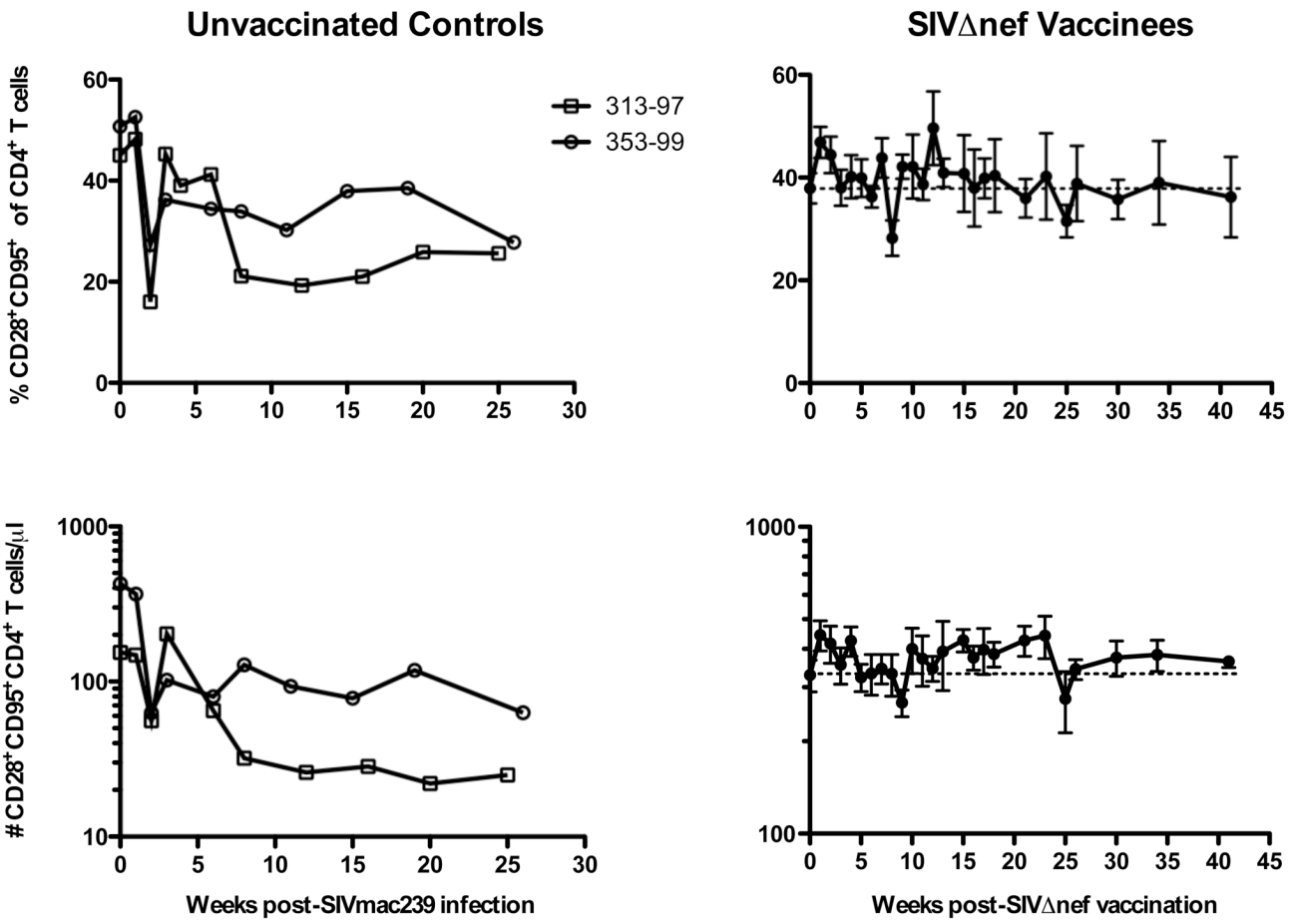

Figure 4.

Longitudinal analysis of central memory $CD4^+$ T cells. Frequencies (upper panels) and absolute numbers (lower panels) of $CD28^+CD95^+$ "central memory" $CD4^+$ T cells in two SIVmac239-infected macaques (left panels) and means ± SEM of ten SIVΔnef-vaccinated animals as shown in Figure 3.

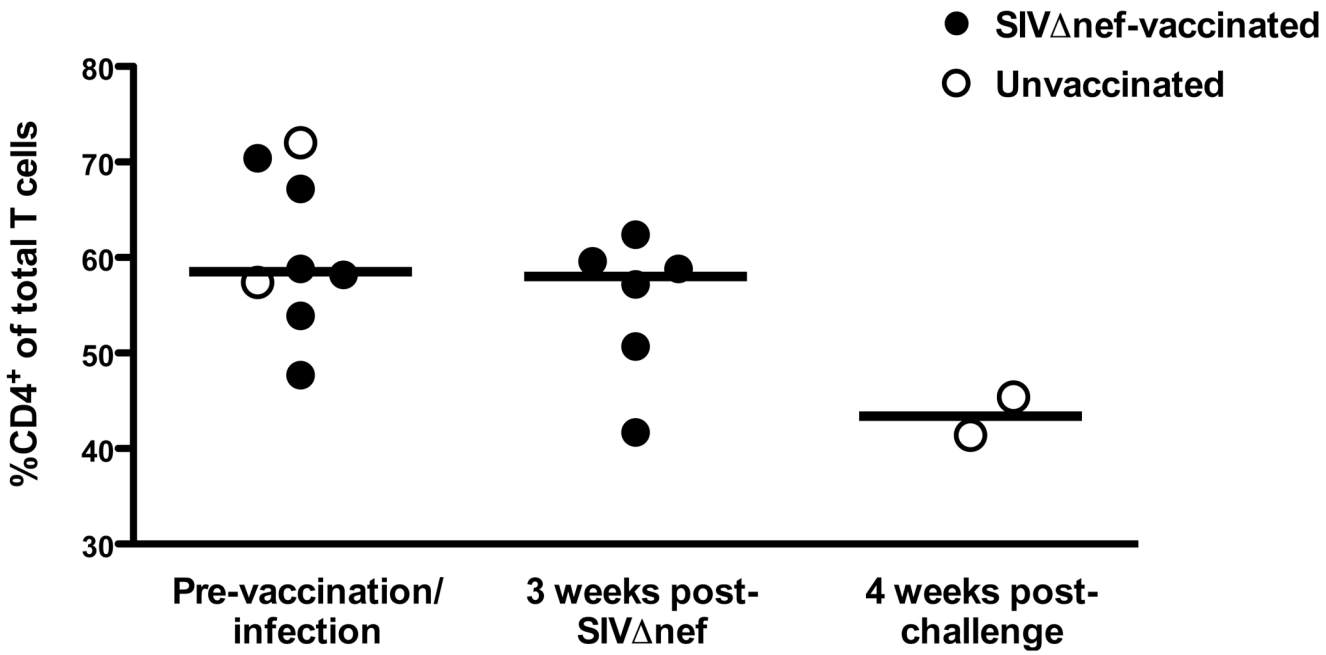

Figure 5.

$CD4^+$ T cell frequencies in the rectal mucosa of SIV-naïve, SIVΔnef-vaccinated, and SIVmac239-infected rhesus macaques. Percentages of $CD4^+$ T cells are shown as frequencies among total T cells in lymphocytes isolated from rectal biopsies performed at one week prior to and three weeks after SIVΔnef vaccination and four weeks after SIVmac239 challenge (weeks 5 and 15 challenge groups are combined). Bars indicate medians of two to eight animals.

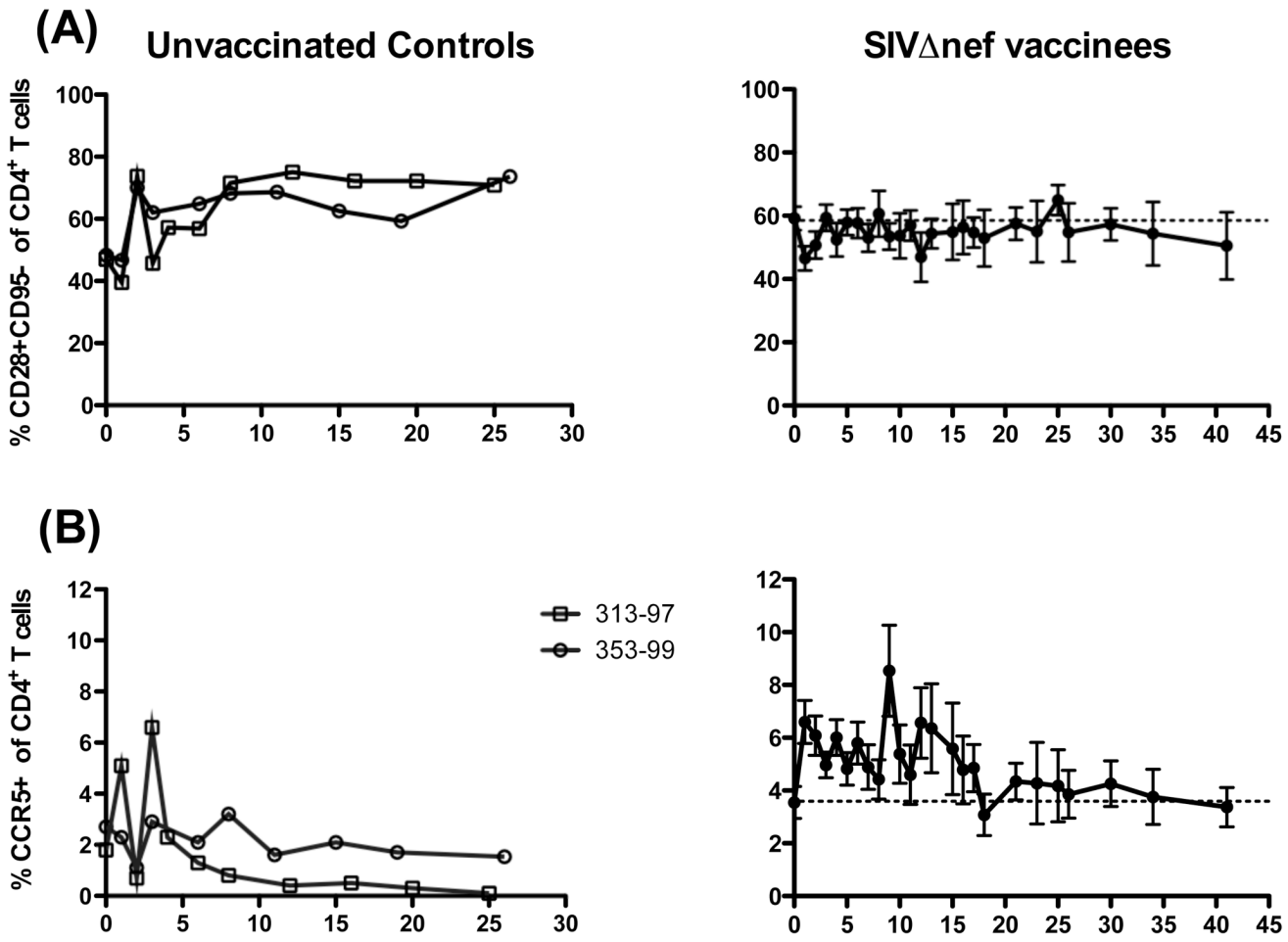

Figure 6.
Longitudinal analysis of $CD4^+$ T cell subsets. Frequencies of (A) $CCR5^+$ and (B) $CD28^+CD95^-$ naïve $CD4^+$ T cells in SIVmac239-infected and SIVΔnef-vaccinated macaques.

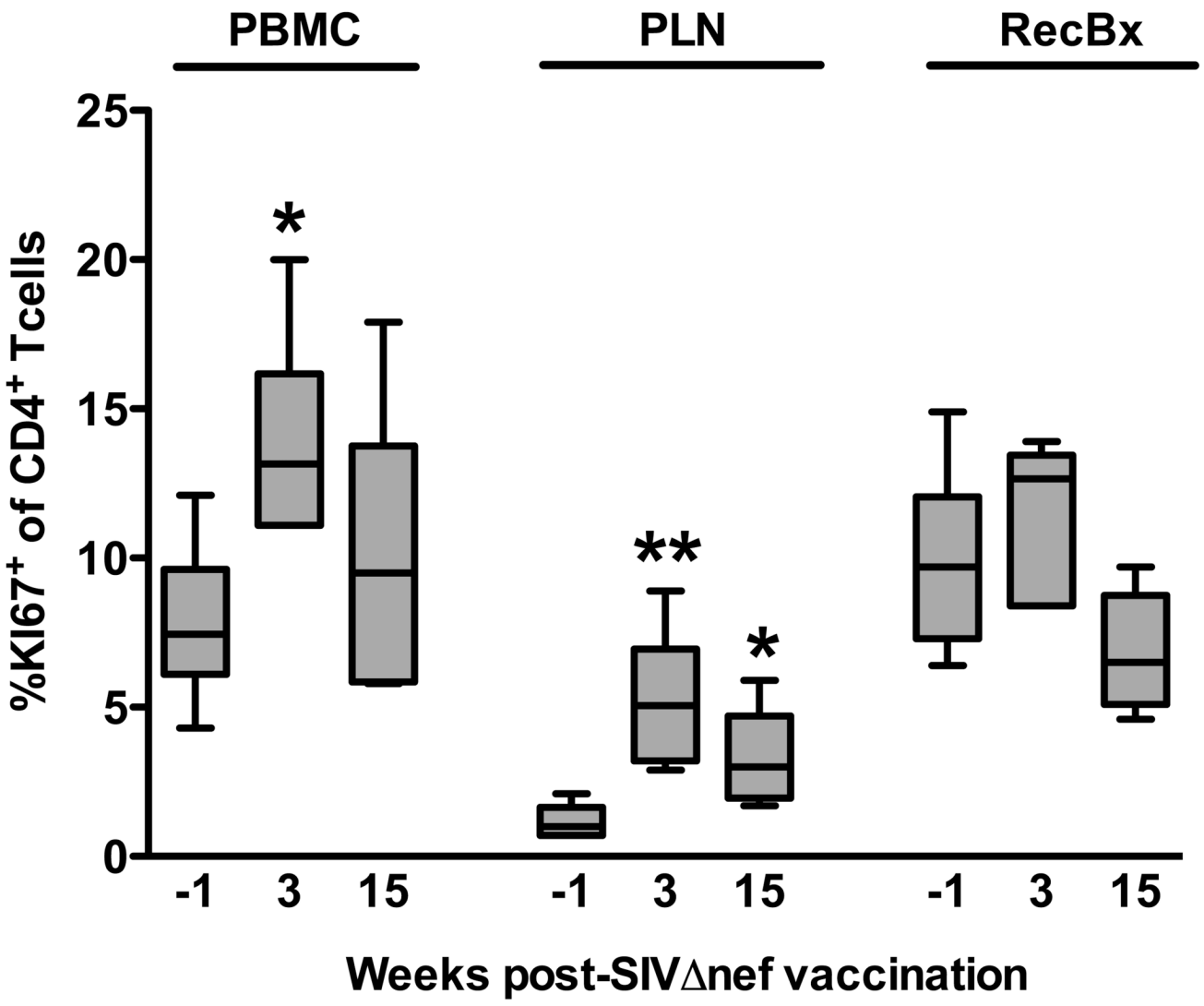

Figure 7.

Ki67 expression in $CD4^+$ T cells after SIVΔnef vaccination. Intracellular Ki67 expression was quantified in $CD4^+$ T cells by flow cytometry in lymphocytes isolated from blood (PBMC), peripheral lymph node biopsies (PLN), and rectal biopsies (RecBx) taken one week prior to and three weeks after SIVΔnef vaccination. Bars indicate medians of six animals; *, $P < 0.05$; **, $P < 0.01$; Mann Whitney test.