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Abstract

Variety of conventional vaccine strategies tested against HIV-1 have failed to induce protection against HIV acquisition or durable control of viremia. Therefore, innovative strategies that can induce long lasting protective immunity against HIV chronic infection are needed. Recently, we developed an integration-defective HIV lentiDNA vaccine that undergoes a single cycle of replication in target cells in which most viral antigens are produced. A single immunization with such lentiDNA induced long-lasting T-cell and modest antibody responses in cynomolgus macaques. Here eighteen months after this single immunization, all animals were subjected to repeated low dose intra-rectal challenges with a heterologous pathogenic SIVmac251 isolate. Although the viral set point in SIVmac-infected cynomolgus is commonly lower than that seen in Indian rhesus macaques, the vaccinated group of macaques displayed a two log reduction of peak of viremia followed by a progressive and sustained control of virus replication relative to control animals. This antiviral control correlated with antigen-specific CD4+ and CD8+ T cells with high capacity of recall responses comprising effector and central memory T cells but also memory T cell precursors. This is the first description of SIV control in NHP model infected at 18 months following a single immunization with a non-integrative single cycle lentiDNA HIV vaccine. While not delivering sterilizing immunity, our single immunization strategy with a single-cycle lentivector DNA vaccine appears to provide an interesting and safe vaccine platform that warrants further exploration.

Keywords: HIV vaccine, CD8+ T cells, TSCM, SIVmac251, Challenge,

Running title: Vaccine-induced SIVmac251 control in cynomolgus macaques

1. INTRODUCTION

The development of a safe and efficacious protective vaccine against HIV/AIDS remains therefore an unfulfilled priority. However, classical vectors and strategies for vaccine development, while inducing protective responses against numerous infectious diseases, have failed to prevent HIV acquisition or control viral replication. This suggests that novel vectors/strategies are needed to induce efficient immunity against this persistent infection. One significant hurdle to this progress is the fact that correlates of protection are not fully elucidated [1]. Among HIV-1 infected patients, rare individuals termed Long-Term Non-progressors (LTNP) and Elite suppressors (ES) have shown successful spontaneous control of HIV replication [2-4]. In some of these patients, HIV-1 variants naturally attenuated by mutations in the *nef* gene were isolated [5-8]. This observation provided a rationale for testing live-attenuated SIV and SHIV vaccines (LAV) in non-human primates (NHP). LAV prototypes remain the "vaccines" found to achieve reproducible protection in macaques challenged with highly pathogenic viruses [9-12]. However, these "vaccines" are fraught with unacceptable risks, retaining pathogenicity especially for infants born from vaccinated mothers [13, 14]. Moreover, integration of the provirus into the host's genome lead to persistent infection associated with mutations and gain of virulence over time [13-16]. Nevertheless, the protective responses afforded by LAV provide valuable leads for correlates and mechanisms of protection [17].

We and others [18-20] have focused on the use of plasmid DNA alone as a vaccine because of the multiple associated advantages. Our strategy focuses on the development of lentivector-based HIV DNA vaccines to better mimic the natural production of HIV proteins and their antigenic exposure. In early studies, deletions of *vpu,* and *nef* were tested in the context of the pathogenic SHIV-KU2 infectious virus as LAV vaccines. NHP inoculated with this LAV were protected from challenge with pathogenic viruses [12] but this protection had a finite duration [21]. Additional

truncations of *reverse transcriptase, integrase* and *vif* genes along with the replacement of the 3' LTR with the SV40 poly A sequences in the SHIV-KU2 genome yielded Δ4-SHIV-KU2 [22-24]. This lentivector was a replication- and integration-defective DNA vaccine produces VLPs, which along with the non-assembled proteins served as potent antigens to prime T cell responses. NHP vaccinated repeatedly with this DNA vaccine alone had no detectable humoral and only modest levels of T cell-mediated responses, but they were protected from a challenge with SHIV89.6p virus [22, 24, 25]. Improved responses were achieved using a single administration of a high dose of Δ4-SHIV-KU2 DNA in mice and macaques [26, 27]. These studies demonstrated that the vaccine elicits life-long SHIV-specific T cells against Gag and other HIV antigens in the mouse and macaque models. These cells displayed polyfunctional properties (proliferation, lytic content, but limited effector functions) upon *in vitro* restimulation. In macaques, persistent low levels of long lasting vaccine-specific T cells were induced, which were expanded further *via* the administration of IL-15 *in vivo* [27]. Further we substituted the SIV LTR with that of a naturally attenuated lentivirus, caprine arthritis encephalitis virus (CAEV), which promotes a constitutive expression of viral proteins [28, 29]. Of importance, this LTR substitution maintained the immunogenicity of the SHIV-KU2-based vaccine after a single dose in mice and macaques [30]. In this study, the SIV *integrase* (IN) gene was deleted in the SHIV-KU2 genome and both SIV LTRs were substituted with those from CAEV to generate CAL-SHIV-IN$^-$. This novel lentiDNA vaccine is designed to be administered as plasmid DNA leading to the production of viral antigens that will assemble into VLPs and pseudo-infectious particles that have the potential to cause one cycle of infection in target cells without integration of the viral genome. To augment the responses we applied intradermal (ID) injections plus electroporation (EP) of the CAL-SHIV-IN$^-$ DNA vaccine during the single immunization. In this report, after 18 months of the initial single immunization we performed repeated low dose intrarectal challenge in these macaques to test the efficacy of the immune responses elicited against vaccine expressed antigens. We additionally performed longitudinal phenotypic and functional analyses of cellular virus specific responses in macaque

samples up to a year post-challenge. Data presented here are from the first evaluation of control of a late SIV challenge in macaques immunized only once with a lentiDNA vaccine alone showing a durable control of heterologous SIV challenge. Immunization conditions were in basic conditions in which there was no codon optimization in the lentiDNA, no boost, no adjuvant, no evaluation of different doses,… which may improve further the control of the challenge virus.

2. MATERIALS AND METHODS

2.1 SIV and HIV peptides

Overlapping 15-mer peptides, with 11-aa overlaps, spanning the entire SIV Gag, Pol, Env and Nef and HIV Env, Tat, and Rev proteins were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (catalog # 6204, 8762, 6443, 6451, 5138, 6445 and 6883 respectively). HIV Env, Tat and Rev peptides are based on the consensus sequences from clade B HIV genomes.

2.2 Vaccine CAL-SHIV-IN$^-$ plasmid DNA

Our SHIV-based DNA vaccine encodes clade B Env from the HXB2 and Tat and Rev from the SF2 HIV strains. The CAL-SHIV-IN$^-$DNA vaccine has been described earlier [31] (S1 Fig).

2.3 Ethic Statements

All macaques were used in accordance with the guidelines of the EU Directive 86/609/EEC as published in the French Official Journal of February 13th 2001 for experiments using non-human primates before January 1st 2013 and in accordance with the guidelines of the EU Directive 2010/63/UE as published in the French Official Journal of February 1st 2013. The immunization conditions were described in a previous report [32]. For the challenges, animals were housed in the facilities of the CEA, Fontenay-aux-Roses, France. Macaques were under the supervision of national veterinary inspectors (CEA permit number A 92-032-02). The CEA complies with the Standards for Human Care and Use of Laboratory Animals of the Office for Laboratory Animal Welfare (OLAW) (USA) under OLAW Insurance number A5826-01. The challenge experiment was approved by the ethics committee under statement #A14-047, and registered under number 44 by the French Ministry of Research. Animals were housed in adjoining individual cages allowing social interactions, under controlled conditions of humidity, temperature and light (12-hour light/12-hour dark cycles). Water was available *ad libitum*. Animals were monitored and fed 1-2 times daily with commercial monkey chow and fruits by trained personnel. Macaques were provided with environmental enrichment including toys, novel food items and music under the supervision of the CEA Animal Welfare Body. Experimental procedures (animal handling, intrarectal inoculations, and blood samplings) were conducted after animal sedation with ketamine chlorhydrate (Rhône-Mérieux, Lyon, France, 10 mg/kg). At the completion of the study, animals were sedated with ketamine chlorhydrate, and then humanely euthanized by intravenous injection of 180 mg/kg sodium pentobarbital and tissues were collected during necropsies.

2.4 Macaque immunization

Macaques and immunization procedure were described earlier [32]. Briefly, each of the six vaccinees (BX72, BX73, BX78, BX80, BX83 and BX84) received a single immunization with a total of 5 mg of CAL-SHIV-IN$^-$ DNA into 5 ml of PBS. Four mg (2 mg/site) were injected intramuscularly in the rear legs and 1 mg was delivered subcutaneously in 10 sites (0.1mg/site) of the back followed by electroporation (six successive 10-msec square-wave pulses, output current 300–600 mA, with 90-msec intervals between pulses) using a portable pulse generator (CUY21 EDIT; Nepa gene, Ichikawa, Chiba, Japan) and Tweezer electrodes. A second group of six naive macaques (BL632, BL667, BL716, BL767, BR740 and BR741) of matched MHC haplotypes (H2, H6 and H4) [33] was included as control at the time of the challenge.

2.5 Challenge of vaccine and control animals

For the purpose of this study all 12 animals were exposed to repeated low doses of SIVmac251 pathogenic virus [34] once a week starting at week 82 post immunization (PIm) for a maximum of 10 weeks using 0.5 animal infectious dose 50% (AID_{50}). Rectal exposures were interrupted for animals confirmed infected after two consecutive positive detections of viral RNA in plasma. Two animals, one vaccinated and one control, who failed to become infected after 10 rectal applications with 0.5 AID_{50}, resisted another four consecutive doses of one AID_{50} and two doses of two AID_{50}. These two macaques were then challenged at week 100 by the intravenous route (IV) with a 50 AID_{50} of SIVmac251 and became infected.

2.6 Sample collection

Blood samples were collected weekly for virus detection during weeks 1-7 post infection (PI), and further collections were obtained every 4 weeks till week 51 PI. Processing of blood for PBMC and plasma and serum isolations has been described earlier [32].

2.7 Quantification of SIV RNA in plasma and proviral DNA in PBMCs

SIV RNA in the plasma was quantified as previously described [35, 36]. The lower limit of detection (LOD) of this assay was12 copies of viral RNA/ml. Proviral DNA in total DNA of PBMCs was measured by quantitative nested-PCR, using sets of primers amplifying the gag region of SIV [36]. Nested-PCR generated a 311bp product from the 745bp product of the first PCR reaction [34, 36].

2.8 Detection of IFN-γ producing cells by ELISPOT assay

We used a commercial non-human primate ELISPOT kit (Mabtech, France) to evaluate IFN-γ-producing cells in response to pools of overlapping peptides in PBMC samples of macaques as previously described [37-38]. Pools of peptides for Gag, Env, Tat+Rev+Nef (TRN) and Pol (1mg of each peptide pool/ml), medium alone (used as negative control), medium containing anti-CD3 mAb (used as positive control) or individual Gag and Nef peptide (5mM/ml) were used for cell activation.

2.9 Detection of antigen-specific Precursors with High Proliferation Capacity T cells

We performed the PHPC assay to detect highly proliferative antigen-specific T cell precursors as described earlier [37]. Briefly, PBMCs were seeded at a density of $2x10^6$ per well into a 24-well tissue culture plate and cultured for 11 days at 37°C in 2 ml of serum-free medium (AIM-V, Invitrogen) with or without stimulating peptides. On day 3, cultures were supplemented with simian IL-2 (10 U/ml) and on day 7 with a cocktail containing simian IL-2, IL-15 (10 U/ml each, from the Resource for NHP immune Reagents) and IL-7 (500 ng/ml, gift from Cytheris, Issy-les-Moulineaux, France). On day 11, expanded cells were counted and 3.5 x 10^4 PBMCs per well were used for IFN-γ-ELISPOT assay and for polychromatic flow cytometry analysis.

2.10 Flow cytometry assays for antigen specific T cells

Polychromatic flow cytometry analysis was performed using a three-laser BD LSRII instrument and data files were collected and analyzed using the FACS Diva software (version 6.1.3; BD Biosciences) and Flowjo 10.5.3 as described earlier [27, 30, 32].

2.10.1 Blood cell counts (CBC) and lymphocyte activation

Whole blood samples from each of the animals were stained with anti-CD45, CD3, CD4, CD8, HLA-DR, CD69 monoclonal antibodies cross-reactive with macaque cells (S1 Table) as previously described [38, 39] to follow lymphocyte subsets and T cell activation.

2.10.2 Characterization of antigen-specific T cells

Freshly thawed PBMCs or 11-days culture of cells for antigen stimulation, as described in the PHPC assay, were used to assess the memory phenotypes and effector functions of SHIV-specific T cells. Cells were stimulated or re-stimulated with the antigen for 16 h then washed with PBS with 2% FBS and then cell pellets were re-suspended into 50 µl of APC-labeled 9AA tetramers (Gag GW9 or Nef RM9) and incubated 10 min at 37°C before staining with FITC-labeled anti CCR7 mAb (5 µl) 30 min at room temperature. Cells were then surface stained with a cocktail containing anti-CD3 BD V500, -CD4 Alexa 700, -CD8 APCH7, -CD28 PercpCy5.5, -CD95 PE and-CD45RA- V450 (S2 Table) in the presence of EMA (1 µl) for 15min in dark and 15 min under light at room temperature. Stained cells were then washed with PBS, fixed with 1% PFA in 1xPBS and acquired in a BD LSRII flow cytometer using the gating strategy presented in Figure S3.

2.11 Evaluation of lymphocyte counts, T cell activation and preservation

Whole blood samples from each of the animals were stained with anti-CD45, CD3, CD4, CD8, CD95, CD28, HLA-DR CD69, CD20, CD27 and IgD antibodies cross-reactive with macaque cells (S1 Table) as previously described [38, 39] to characterize the white blood cell subtypes and T-cell activation status.

2.12 Necropsy and tissue harvest

At W51-PI, each animal was humanely euthanized and submitted to necropsy with collection of multiple lymphoid organs for analyses. None of the animals presented any gross pathological findings.

2.13 Immunohistochemistry

Immunohistochemical (IHC) staining on sections of lymph nodes from vaccinated and control animals were performed using a biotin-free polymer system as described earlier [40]. Goat anti-human PD-1 (polyclonal Ab, R&D Systems) and rabbit anti-human Ki67 (clone SP6, Abcam), rabbit anti-human CD4 (clone EPR6855; Abcam) and mouse anti-human CD8 antibodies (clone LT8; GeneTex) were used in this study. Digital images of lymphoid follicles were randomly captures at 200 x 400, magnification with an Olympus BX43 microscope equipped with a digital camera (DP26, Olympus). For quantification, the PD-1 labeled cells within LN germinal centers (GC) defined by Ki67 staining were manually counted using Cellsens® digital imaging software 1.11 (Olympus). The GC size analysis was not performed due to sample size variation. Blind scoring was performed by three independent observers.

2.14 Statistical analysis

Statistical analyses of acquired data (standard deviation, Mann-Whitney tests) were performed using Graph Pad Prism 5.0 software.

3. RESULTS

3.1 Challenge of control and vaccinated macaques

Macaques immunized only once with the CAL-SHIV-IN$^-$ DNA vaccine as described in the method section, and naïve control macaques were challenged by repeated low dose intrarectal exposures to 0.5 AID_{50} of SIVmac251 starting at 82 weeks post-immunization (PIm) except for macaque BX73 which was challenged starting at W70-PIm (Fig 1A). Five out of six control animals became infected after 1-7 inoculations (BL767 & BR716 after a single inoculation; BL632 & BR740 after 2 inoculations and BL667 after 7 inoculations) (Fig 1B). The last animal (BR471) resisted 16 rectal inoculations including a dose escalation for challenges 11-16 (4 at 1AID_{50} followed by 2 at 2 AID_{50}). This animal was infected after a single IV injection with a 50 AID_{50} high dose at W100 PIm (Fig 1A and 1B). Similarly, five of six vaccinated animals became infected after 2-4 inoculations (BX72 after 2 inoculations, BX83 after 3 inoculations and BX73, BX78 & BX84 after 4 inoculations). The last animal (BX80) behaved like the control BR471 and became infected only after the single IV injection with 50 AID_{50} of the virus at W100 PIm (Fig 1A and 1B).

3.2 Vaccinated animals exhibit a reduction of peak viremia

Viremia of SIVmac251 infected animals was quantified longitudinally until W51 post infection (PI) when the macaques were euthanized (Fig 2A). Peaks of viremia were observed at W1 or W2 PI, followed by a gradual decrease, reaching a stable plateau ranging from 10^2 to 10^4 copies/ml of

viral RNA in four out of the six controls. The two other controls (BR716 and BR740) spontaneously controlled their viremia around the level of detection (LOD) (Fig 2A). In contrast, among vaccinated macaques, there was a rapid and more pronounced drop of viral loads in all six animals (Fig 2A and 2B), reaching values below 250 copies/ml of plasma by W11-PI. Interestingly, this control of virus replication was not restricted to mucosally acquired virus but also seen in vaccinated animal BX80 but not the control BR471 that acquired infection after a last challenge by the IV route with a high dose (Fig 2B). While differences of viremia were not significant between the two groups of animals at W1-PI [p=0.387] (Fig 2C), at W2-PI, viral loads showed significant differences between the two groups [p=0.043], with highest viremia values in the control group ($4x10^4$ to $3.5x10^6$ copies/ml) compared to the vaccinated group ($0.8x10^3$ to $4.7x10^4$ copies/ml). The median value of the control group viremia ($1.2x10^6$ copies/ml) was 100 times higher than that of the vaccinated group ($0.9x10^4$ copies/ml) [p=0.026] at W2-PI. Statistical differences were maintained at W7-, W11-, W15- and W19-PI (Fig 2C). Due to spontaneous virus control in 2 out of the 6 control animals, the statistical significance was lost from W23-PI till the end of the experiment at W51-PI (Fig 2C), reflecting some of the limit of pathogenicity in this model. The early control of viremia was also evident by comparing areas under the curve plasma viral loads in the phase of infection (Fig 2D, p=0.0152). These results clearly show that while the vaccine-induced immune responses did not prevent acquisition of infection, they were efficient at inhibiting SIVmac251 replication during early and potentially late chronic infection. We did not expect a prevention of virus acquisition given the wide differences in envelops between vaccine (HIV-1 HXB2) and challenge (SIVmac251). Interestingly, evaluation of DNA proviral loads in PBMC samples before challenge, and at 2, 7 and 47 weeks post challenge showed consistently lower proviral loads post challenge in vaccinated animals (S2 Fig.). These lower proviral loads indicated that the lower viral loads seen in vaccinees correlate with lower proportion of cells harboring the provirus.

3.3 Immunological characteristics post-challenge with SIVmac251

3.3.1 Dynamics of immune cell subsets post-challenge with SIVmac251

The longitudinal evaluation of blood lymphocyte cell counts performed as described in S3 Fig in samples from all animals is summarized in S4 Fig**.** A decrease of lymphocytes was observed in both groups at W1-PI, which was maintained at W2-PI in the controls but not in the vaccinees which recovered their count to the level of the pre-infection. Thereafter, lymphocyte counts remained higher over the 23 weeks of follow-up in vaccinated group with statistical significance at W4-, W7- and W11-PI [p=0.0022, p=0.0130, p=0.022 respectively] (S4A Fig). This profile was similarly seen for CD3+, CD4+ and CD8+ lymphocyte populations (S4B-D Fig). There were statistical significance at W2-7-PI and W23-PI [p=0.0260, p=0.0303, p=0.026, p=0.0411 and p=0.0411 respectively] for the CD3+ counts (S5B Fig), and at W2-, W4- and W11- [p=0.0260 for all 3 week points] and W23-PI [p=0.0411] for the CD3+CD8+ T cell counts (S4D Fig). Despite the trend of higher proportion of CD3+CD4+ T cell counts in the vaccines starting at W2- to W23-PI, there was no statistical difference at any of these time points (S4C Fig).

One important hallmark of SIV and HIV infection is the presence of persistent T cell activation that is not fully resolved even during ART. We therefore monitored the expression of the early and late cell activation markers CD69+ and HLA-DR+ respectively, on circulating CD4+ and CD8+ T cells.

In the acute phase of infection (W2-4-PI) the proportions of CD4+CD69+ and CD4+HLA-DR+ T cells were both higher in the control than the vaccine groups (Fig 3A). Similarly, there was higher CD8+CD69+ T cell proportion in the control group than the vaccinees. In contrast, there was a lower proportion of CD8+HLA-DR+ in the controls compared to the vaccinees (Fig 3B). In the

chronic phase of infection (W23-PI) the proportion of CD4+CD69+, CD4+HLA-DR+, CD8+CD69+ and CD8+HLA-DR+ T cells were all higher in the control group (Fig 3C&D).

Altogether, these data showing persistent lower T cell activation in the vaccinees compared to the control animals correlate with a controlled SIVmac251 viremia in the vaccinees.

3.3.2 Evaluation of antigen-specific T cell responses

3.3.2.1 Evaluation of antigen-specific T cells with immediate effector functions

PBMC samples collected at W-1, W7 and W19-31-PI were stimulated overnight with Gag, Nef and Env pools of peptides or with the immunodominant SIV Gag GW9 and Nef RM9 epitope peptides, and used for the standard Elispot. Data are summarized in Fig 4. Before challenge, there was no response that can be detected against the tested antigens in any control macaques (Fig 4A). In contrast, detectable responses against SIV Gag, Nef, HIV Env, GW9 and RM9 were observed in PBMC from all vaccinated animals (Fig 4A). While the responses against the immunodominant GW9 and RM9 were high in the vaccinees, weaker responses were observed against HIV and SIV Env antigens. These results observed 18 months PI demonstrate that the vaccine induced T cell responses were long-lasting in all vaccinated animals. During the early stage of SIVmac251 infection (W7-PI), the control animals showed detectable responses against the viral antigens (mainly Gag and Nef). In contrast, the vaccinated animals showed a stronger anamnestic increase in the proportion of IFN-γ-producing cells against all viral antigens. At this early time point, the responses in spot forming units (SFU) were over three fold higher in the vaccinees, compared to the controls (means of 850 and 240 SFU/10^6 PBMCs for Gag and Nef, respectively) (Fig 4B). Interestingly, in the vaccinees, the increase of anti-Env responses was higher against HIV-1 than

SIV. This may suggest cross-reactive recall of vaccine specific cells by the SIVmac251 challenge antigens. Responses to the immunodominant Gag (GW9) and Nef (RM9) peptides were one log higher in vaccinees relative to controls (p=0.0281 and p=0.0079 respectively). However, in the chronic phase of infection within W19-31-PI, overall responses seemed equivalent in both groups except those against the immunodominant peptides showing significant increase in the vaccinees (Fig 5C). There was also a correlation between the increase of T cell responses and reduction of viremia in the control animals at the chronic phase of infection.

3.3.2.2 Detection of persistent antigen-specific T cells precursors with high proliferation capacity (PHPC)

We used the PHPC assay as previously described [32, 37] to detect the presence of antigen-specific precursor memory T cells able to expand. At one week before infection, Nef-specific (RM9) PHPC cells were detected in the vaccine group but only at low levels in the control group (Fig 4D). Interestingly, at late phase of infection (W19-31-PI), these responses augmented in the vaccinees while only marginally in the control group (Fig 4D). These data demonstrate that our single-immunization strategy induced antigen-specific precursor memory T cells that were maintained till 81 weeks PIm, and these cells were able to rapidly expand in response to antigenic re-stimulation following SIVmac251infection.

Using the PHPC assay we previously demonstrated that the expanded IFN-γ producing cells were mainly composed of central memory (CM) CD4+ and CD8+, and effector memory (EM) CD8+ T cells. To better characterize the lineages of cells delivering PHPC responses post infection, we analyzed the cells with memory markers and found a correlation of central memory CD8+ T cell responses with protection in vaccinated animals. Using three markers, we found that vaccine-

specific T-cells vigorously expanded in response to antigen, IL-15 and IL-7 and mostly regenerated CM (CD28+ CD95+) and EM (CD25- CD95+) T cells.

Flow cytometry characterization of cells derived from PHPC at day 11 in samples of vaccinees demonstrated that IFN-γ producing cells are mainly CM CD4+ and EM and CM CD8+ T cells. We further used flow cytometry to detect whether these precursors contain a particular subset of CD8+ stem cell like memory T cells (TSCM). These cells could be detected using a panel of mAbs against surface markers including CCR7, CD45RA, CD95 and CD28. In addition we used the mafa-A1*063-restricted Nef 103-111 RM9 tetramer to detect antigen specific T cells (S5 Fig). Interestingly, the frequency of Nef RM9-specific CD8+ TSCM was higher in vaccinees compared to controls at W81 PIm prior to infection (Fig 4E). Post infection the proportions of these cells increased in both groups with a higher frequency in the vaccinees compared to control although with no statistical significance (Fig 4E). These data demonstrate that our vaccination strategy induced long living precursors associated with enhanced proliferative and polyfunctional properties [41-43]

3.4 Evaluation of lymph node architecture at W51-PI

Immunohistochemistry staining of lymph nodes collected at necropsy did not reveal marked differences in CD4 density or architecture between samples from vaccines and controls but demonstrated a significant up regulation of PD-1 labeled cells within germinal centers of vaccinated animals relative to controls ($p=0.0321$, $n=3$). This may illustrate robust lymphoid responses in these animals, even though the significance of this data remains to be fully elucidated (Fig 5A-G). Interestingly, the PD-1 overexpression in vaccinee samples was associated with the CD4+T cells but not with the CD8+ cells (S6 Fig).This excludes the possibility of PD-1-

405 associated exhaustion of CD8+ T cells, but rather suggests increased presence of follicular helper
406 T cells in vaccinated animals [44-46]

407

4. DISCUSSION

Classical vaccine strategies that elicit neutralizing antibodies failed against HIV mainly because of the high variability of the envelope glycoproteins and their heavy glycosylation hiding the key neutralization epitopes. However, evidence of efficient antibody mediated prevention of lentiviral infection has been demonstrated by the use of passive administration of broadly neutralizing antibodies [47, 48]. The limitation of use of this strategy against HIV is due to the inability to induce broadly neutralizing antibodies with classically used immunogens [49]. We focused on the development of T-cell based lentiDNA vaccines that mimic the early HIV control by the CD8+ T cells and improve their function to reach a persistent and durable control of HIV. The main limitation of development of HIV DNA vaccines is the insufficient immunogenicity of the formulations in primates. Therefore, most current experimental DNA vaccine approaches against HIV use prime-boost combinations of antigen deliveries in order to increase DNA vaccine immunogenicity [50-55]. Immunizations with LAV have unequivocally shown that maturation of induced immune response is a critical component of protective efficacy [12, 56-59] with durable responses able to contain subsequent challenges [60]. While integrating live attenuated lentivirus presents unacceptable risks in humans, the concept of a replication-limited lentivirus was hypothesized to be a viable option to attempt to develop potent antiviral responses. Our group developed a series of such non-integrative lentivector DNA constructs as a vaccine platform [22-24, 27, 31]. Moreover, we limited the immunization procedure to a single time point to immunize humanized SCID mice first [63] but harnessed the potency of electroporation to immunize cynomolgus macaques which developed promising anti-SHIV immune responses *in vivo* using the current CAL-SHIV-IN$^-$ vector [32]. Vaccine specific effector and memory T cells induced by this immunization were found to be durable in the peripheral blood of vaccinated animals [32]. Since our lentivector vaccine induced only modest antibody responses, but durable memory and effector T-cell responses, prevention of virus acquisition was not expected. However, we hypothesized that

the responses would be able to control the replication of the virus post acquisition. Challenge of animals using a stringent repeated low dose rectal inoculation of the pathogenic SIVmac251 showed that the rate of virus acquisition did not differ between immunized and control animals but interestingly, as early as one week post infection, all vaccinated animals appeared to exert better control of their viremia compared to control animals (Fig 2). This control was durable leading to undetectable or barely detectable chronic plasma viral loads in all vaccinated animals while four out the six control animals showed a persistent chronic viremia with viral loads ranging from 10^{2-5}copies/ml, though clearly the SIV infected cynomolgus macaque model is not the most pathogenic. In addition, there was at least one log reduction of the peak viremia in the vaccine group compared to control (Fig 2A). While not absolute, these data provide the proof-of-concept that a single immunization with our vaccine lentivector prototype was sufficient to limit viral replication *in vivo*. Vaccine studies using LAV/SIV followed by challenge with pathogenic SIV often show some vaccinated animals that develop high viremia [61-63]. None of our vaccinated cynomolgus macaques developed high viremia in the chronic phase following challenge. The low viremia in vaccinated animals correlated with lower proviral loads in PBMCs both in the acute and chronic phages of infection. This might indicate either there was a mechanism that limits infection of target cells early in vaccinated animals or there is rapid killing of infected cells limiting the proportion of PBMCs harboring the provirus. The data is also reminiscent of a recent and surprising anti-α4β7/ART mediated immunotherapy leading to the control of an otherwise highly virulent NHP lentivirus [64]. While our protocol did not allow for more detailed *in vivo* evaluation of protective mechanisms, control of viral replication in our model was associated with pre-challenge virus specific polyfunctional CD4+ and CD8+ T cell responses [32]. Moreover, these CD4+ and CD8+ T-cell responses were directed against all vaccine-expressed antigens with high proportions of cells responding to Gag and Nef antigens [32]. Of note, we did not detect any specific difference in the immune responses of monkey BX80 which resisted all mucosal challenges from the other vaccinees. These responses were rapidly boosted upon infection with

SIVmac251 against all viral antigens tested early PI and remained high even after quasi-resolution of the plasma viremia (Fig 4B and C). In contrast, infected control animals required about 19-31 weeks to develop SIV-specific T cells with similar magnitudes to those of the vaccinees, in spite of markedly higher levels of virus replication and antigenic stimulation (Fig 4C). Interestingly, the vigorous homeostatic proliferation of antigen specific T cells that has been reported previously while monitoring responses to the single immunization demonstrated outstanding durability of long lived antigen specific PHPC from which effector T cells are rapidly mobilized. These cells appeared not only well maintained but also expanded post infection as seen in samples collected at W19-31-PI (Fig 4D and E). There are growing data in both chronic infectious and non-infectious diseases as well as autoimmunity showing the importance of TSCM in the modulation of disease progression. In HIV infection, while subsets of CD4+ TSCM are activated upon antigen exposure and may serve as key cells for persistence of virus in tissues [65], others contribute to restriction of virus replication [66]. In contrast, CD8+ TSCM are only associated with control of virus replication since they are involved in long lasting virus specific T cell responses [67, 68]. In the SIV model of macaque infection the percentage of CD4+CCR5+ TSCM is significantly decreased [69], but initiation of ART restores their homeostasis [70]. *In vitro* prepared human alloantigen-specific CD8+ TSCM were shown to be highly efficient at eradicating allogenic targets in humanized NOD-SCID mice [71]. It is well accepted now that these long-lived precursors T cells play a determinant role in the control of virus replication. This is due to their capacity of expansion upon antigen resolution [41], and their capacity to generate central and effector memory cells [72, 73]. Interestingly, memory T cells elicited by another live attenuated immunizing vector, namely the RhCMV-based SIV vaccine [74, 75], even though the latter vaccine appeared able to eradicate SIV from about 50% of immunized NHP, provided another proof of validity of T cell-based vaccine strategy.

Previous vaccine studies using vectors that induce potent cellular immune responses have shown that the elicitation of potent CD8+ T cell mediated antiviral responses but, can also be associated

with increased levels of activated CD4+ T cells at mucosal sites, providing additional targets and thereby facilitating virus acquisition [56, 76-79]. While current efforts by our group strive to augment the elicitation of cell mediated and humoral responses, in this preliminary study at least, despite the long lasting antigen-specific T-cell responses observed in vaccinated macaques, none showed early or late activated markers on CD4+ T cells even post infection, which contrasted with observations in the control infected macaques (Fig 3). CD8+ T cells on the other hand showed higher levels of HLA-DR expression in vaccinated macaques, a finding that has been associated with slower disease progression in HIV-infected individuals [80-86]. The lack of CD4+ T cell activation in the vaccinated animals (Fig 4A&C) was also associated with a significant maintenance of CD4+ T cells compared to control animals (S4D Fig).

An unexpected result was the finding of enlarged germinal centers and presence of follicular helper T cells in the lymph nodes of the vaccinees relative to the control animals, at necropsy corresponding with potent control of the virus replication. Our finding of increased TfH and germinal centers in the context of controlled viral replication in vaccinees may represent a better ability to respond to novel immunizations. However, addressing the kinetics of such finding will require sequential collections of lymphoid tissues in future experimental immunizations with lentiviral vector. In contrast, observations of early lymphoid changes in SIV infected macaques, were found to be associated with marked expansion of germinal centers, immunoglobulin production and increased TfH cells [44, 45, 87].

In conclusion, our prototypic study provides the proof of concept that a single immunization with a lentivector DNA-based antigen delivery that mimics viral infection with the majority of viral antigens in absence of persistent antigen expression is able to exert control of virus replication *in vivo*. It remains to be seen whether the level of protection reported herein may be reproduced in the Indian Rhesus macaque model challenged with SIV Mac239 as used in US. We will also examine whether further increase of the pools of antigen-specific memory T cells and/or generation of

humoral responses without affecting what might be a delicate balance between the generation of protective surveillance without activation of potential viral targets will help to increase the protection.

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Figure Legends

Fig 1. Diagram of vaccination and SIVmac251 multiple low-dose challenges

(A). Six cynomolgus macaques immunized with a 5 mg single dose of the CAL-SHIV-IN$^-$ lentivector DNA as previously reported [32] and six control macaques were challenged at week W82-PIm. Animals were exposed to repeated low intrarectal doses (0.5 AID_{50}) of SIVmac251, delivered once a week (light grey arrows). Exposures were interrupted once two successive positive viral RNAs results were detected by RT-PCR in the plasma. Most animals became infected following 2 to 7 exposures (B). Out of the 12 animals, Two animals, one vaccinated (BX80) and one control (BR471), failed to be infected mucosally, after over 16 rectal applications (10 at 0.5 AID_{50} (light grey arrows), 4 at 1 AID_{50} (dark grey arrows) and 2 at 2 AID_{50} (black arrows). These 2 animals were then injected with 50 AID_{50} of SIVmac251 by the IV route (Green arrow). Both became immediately infected.

(B). Kaplan Meier depiction of SIVmac251 acquisition over time. Vaccinated (VAC in red) or control (CTR in blue) animal infections are shown. Virus was detected at W1 post challenge (W1-PC) in the plasma of BL767 and BL716 control animals, W2-PC in BL632 and BR740 control and BX72 vaccinated animals, W3-PC in BX83 vaccinated, W4-PC in BX73, BX78 and BX84 vaccinated animals, and at W7-PC in BL667 control animal. The two animals (BR471 (control) and BX80 (vaccinated) which resisted the escalated doses of mucosal challenge became immediately infected after the IV injection of 50 AID_{50} of SIVmac251 at W18-PC (corresponding to W100-PIm).

Fig 2. Plasma viral load in control and vaccinated macaques.

(A). Kinetics of viremia in vaccinated and control animals. Individual values are plotted; Level of detection (LOD) was 12 copies/ml; **(B).** Kinetics of viremia in control BR471 and vaccinated BX80 animals that have been challenged IV with 50 AID_{50} high dose ; **(C).** Distribution of the viral loads in plasma samples at various times post-infection for all 6 vaccinated and 6 control animals. **(D)**. Distribution of the area under the curve (AUC) between week 1 and week 7 post infection. All p values were calculated using the non-parametric Mann-Whitney test at 95% confidence interval (* $p<0.05$, ** $p<0.005$).

Fig 3. Activation of CD4+ and CD8+ T cells post infection.

PBMC samples taken at W2-4- (A and B) or W23-PI (C and D) were stained with a mix of surface mAbs as described in Methods and S1 Table. The early activation was detected using the CD69 and the late activation HLA-DR markers in both CD4+ and CD8+ populations of T cells. Stained cells from vaccinated (red) and control (blue) animals were examined by flow cytometry and data summarized in A-D. Individual data and mean±SD were plotted. Statistical analysis was performed using the non-parametric Mann-Whitney test at 95% confidence interval. Statistically significant p values are indicated (* $p=0.0312$).

Fig 4. Evaluation of effector T cell and PHPC responses post SIVmac251 infection. Frozen PBMC samples taken at the indicated weeks pre- and post-infection (A–C) were used for the classical overnight ELISPOT assay. This was to evaluate IFN-γ producing cells in response to SIV Gag, SIV Nef and HIV-1 Env peptides as well as to SIV Gag GW9 and Nef RM9 immunodominant peptides. A fraction of PBMCs was also used for the PHPC assay to detect Nef RM9 specific cells and obtained values were used to plot the graph in (D). Furthermore cells were stained as described in Methods to evaluate the T_{SCM} specific to the Nef RM9 epitope and values

were used to plot the graph in (E). Vaccinated animals are in red and controls in blue. SIV Gag W-1 p=0.0173, SIV Nef W-1 p=0.0152, SIV GW9 W7 p=0.0281, SIV RM9 W19-31 p=0.0079.

Fig 5: Immunopathological staining of lymph node sections.

Sections of inguinal lymph nodes taken after necropsy from both control and vaccinated animals were double-stained with anti-Ki67 and anti-PD-1 mAbs *via* immunohistochemistry (IHC). Representative PD-1 (black) and Ki67 (Fuchsin-red) stainings (20 × and 200×) in one vaccinated (A, B, C; top panel) and one control (D, E, F; bottom panel) cynomolgus macaques are shown. Random 200 × and 400 × images of lymph nodes were taken and the PD-1-labeled cells were counted in vaccinated and control cynomolgus macaques. Values were used to plot the graph (G) showing a statistical significant difference (p=0.0321).

Supporting information

S1 Table. Panel antibodies used for blood lymphocyte count and T cell activation

Specificity	Clone	Fluorochrome	Company
CD45	D058-1283	PerCP	BD Biosciences
CD3	SP34-2	V500	BD Biosciences
CD4	L200	FITC	BD Biosciences
CD8	BW135/80	VioBlue	Miltenyi Biotec
CD95	DX2	APC	BD Biosciences
CD28	CD28.2	ECD	Beckman Coulter

CD69	FN50	PE-Cy7	Ozyme
HLA-DR	L243 (G46-6)	APC-H7	BD Biosciences
CD20	2H7	A700	eBioscience
CD27	M-T271	PE	BD Biosciences
IgD	Polyclonal	FITC	BD Biosciences

831

832

833 **S2 Table.** Panel mAbs for TSCM

Specificity	Clone	Fluorochrome	Company
CD3	SP34-2	V500	BD Biosciences
CD4	SK3/L200	PE cy7/V500	BD Biosciences
CD8	SK1	APC-H7	BD Biosciences
CD95	3193519	PE	BD Biosciences
CD45 RA	5H9	V450	BD Biosciences
CD197 (CCR7)	150503	FITC	BD Biosciences
19852Mafa-A1-063	19852	APC	NIH tetramer facility
19851Mafa-A1-063	19851	APC	NIH tetramer Facility

840

841 **S1 Fig. Physical map of CAL-SHIV-IN$^-$ lentivector DNA construct.**

842 Details of the construct were previously reported [31]. SIV coding sequences are in green. Del. int
843 indicates the deletion of the SIV coding sequences for the integrase. HIV-1 coding sequences are
844 in dark orange. The dashed arrows at both ends of the viral constructs are the sequences of CAEV
845 LTRs. Kan^R coding sequences (blue) is in within the pET9 plasmid DNA.

846

847 **S2 Fig. Sequential Proviral loads in PBMC DNA.**

Total DNAs isolated from frozen PBMCs of control and vaccinated animals were used for quantitative nested-PCR detection of SIV *gag* sequences using specific sets of primers as previously described [34, 36]. Copy numbers were used to plot the graph for vaccinated (red squares) and control (red circles) at one week before (-1) and 2, 7, and 47 weeks post challenge. *p=0.0238.

S3 Fig. Lymphocyte gating strategy for lymphocyte counts and activation.

Lymphocyte population was gated using side scatter parameters and CD45, after doublet exclusion. B lymphocytes and NK cells were identified as CD20+HLA-DR+ and CD3-CD8+, respectively. For T cell activation gating, B lymphocytes and NK cells were excluded. CD4+ and CD8+ T cells were then gated in CD3+ cells as CD4+CD8- and CD4-CD8+, respectively. Boolean gates were then used to identify early activated CD4+ and CD8+ T cells as CD69+ and HLA-DR+, respectively.

S4 Fig. Longitudinal evaluation of lymphocyte cell counts.

Blood samples were stained with a panel of surface mAbs against macaque cells as described in Materials and Methods, in supplemental Table 1 and previously [38, 39]. Absolute cell counts were determined at 2 weeks before challenge and W1- to W23-PI post infection by flow cytometry using a LSRII cytometer (BD Biosciences). Cell counts were used to plot (**A**) total lymphocyte counts, with statistical significance at W4- p=0.0022, W7- p=0.0145 and W11-PI p=0.0022; (**B**) T cell counts including CD3+, with statistical significance at W2- (p=0.0260), W3- (p=0.0303), W4- (p=0.026), W7- (p=0.0411) and W23-PI (p=0.0411); (**C**) CD3+CD8+T cell counts, with statistical significance at W2- (p= 0.0260), W4- (p=0.0260), W11- (p=0.0260) and W23-PI (p=0.0043) and

(**D**) CD3+CD4+, with no statistical significance. Blue dots are for control and red for vaccinated animals. Individual data and mean±SD were plotted. Statistical analysis was performed using the non-parametric Mann-Whitney test at 95% confidence interval.

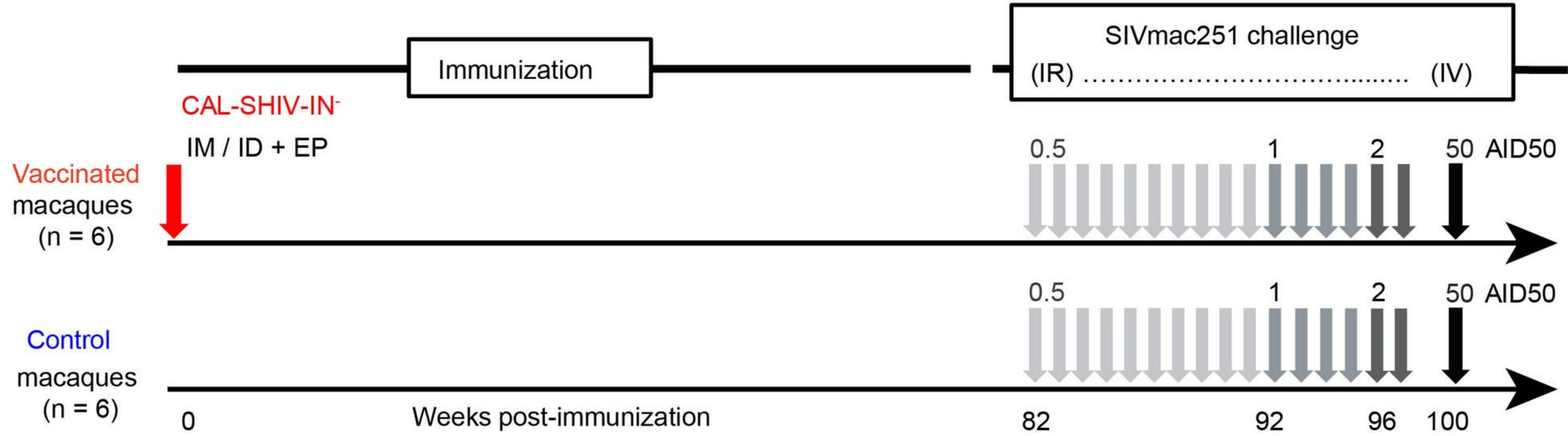
S5 Fig. CD8+ TSCM gating strategy of labeled peripheral mononuclear cells.

Lymphocyte population was gated using forward and side scatter parameters, prior to doublets and EMA^+ dead cells exclusion. $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were then selected based on markers expressed on their surface. Total $CD8^+$ T cells were further divided into four subsets based on expression of surface CCR7 and CD45RA markers: $CCR7^+CD45RA^+$ (naive: N), $CCR7^+CD45RA^-$ (central memory: CM), $CCR7^-CD45RA^-$ (effector memory: EM) and $CCR7^-CD45RA^+$ (terminal effector memory: TEM). Subsequently, TSCM subset was investigated in the $CCR7^+CD45RA^+$ population using CD95 and CD28 marker expression. The straight red arrows indicate the sequential forward gating. The curved red arrow indicates the gate copied from the gating of total CD3+CD8+ T cells using CD95 and CD28 and used for TSCM identification. The same gating strategy was used on tetramer $RM9^+$ $CD3^+CD8^+$ T cells to identify antigen specific TSCM cells.

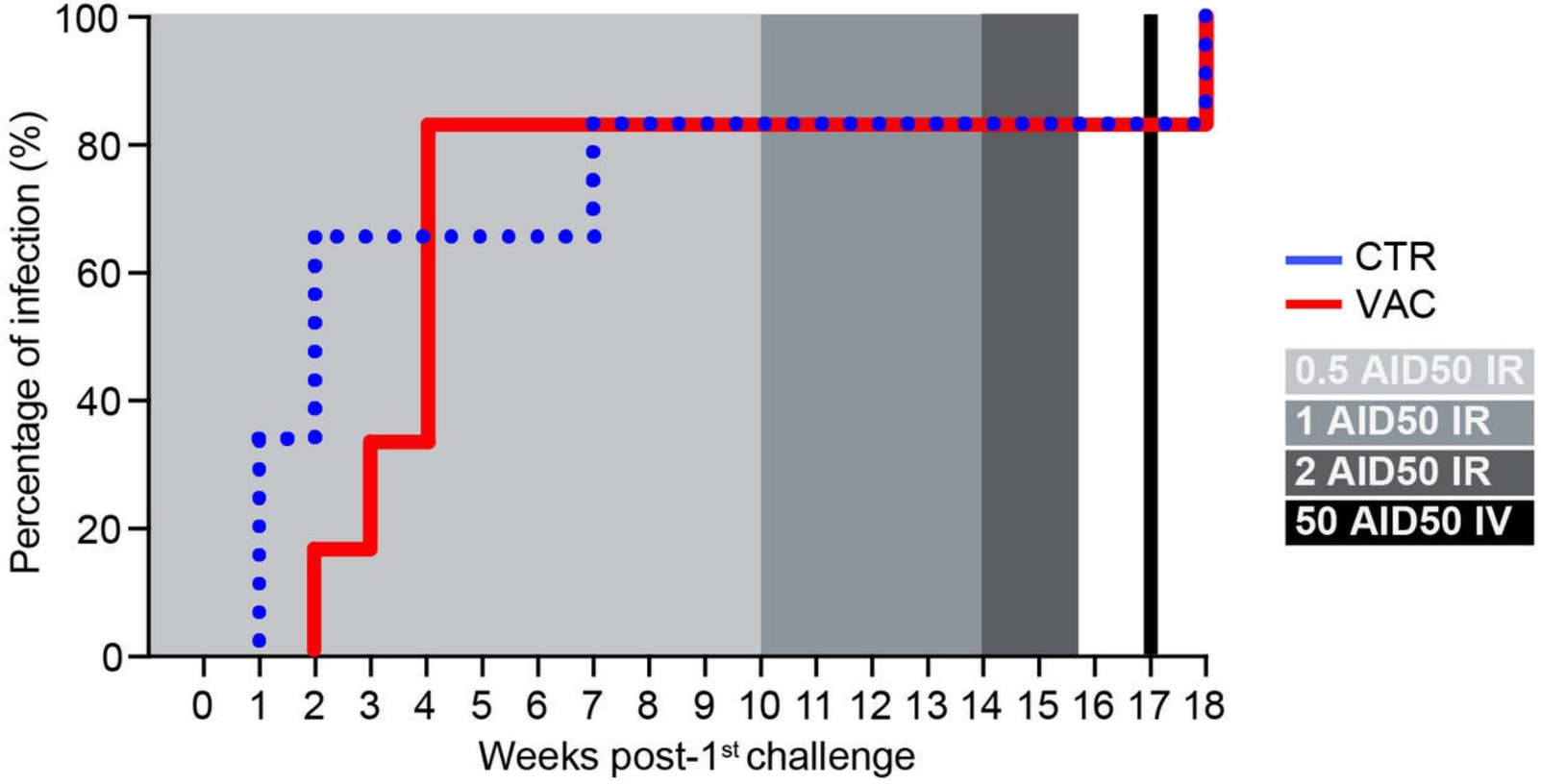
S6 Fig. Immunopathological staining of lymph node sections.

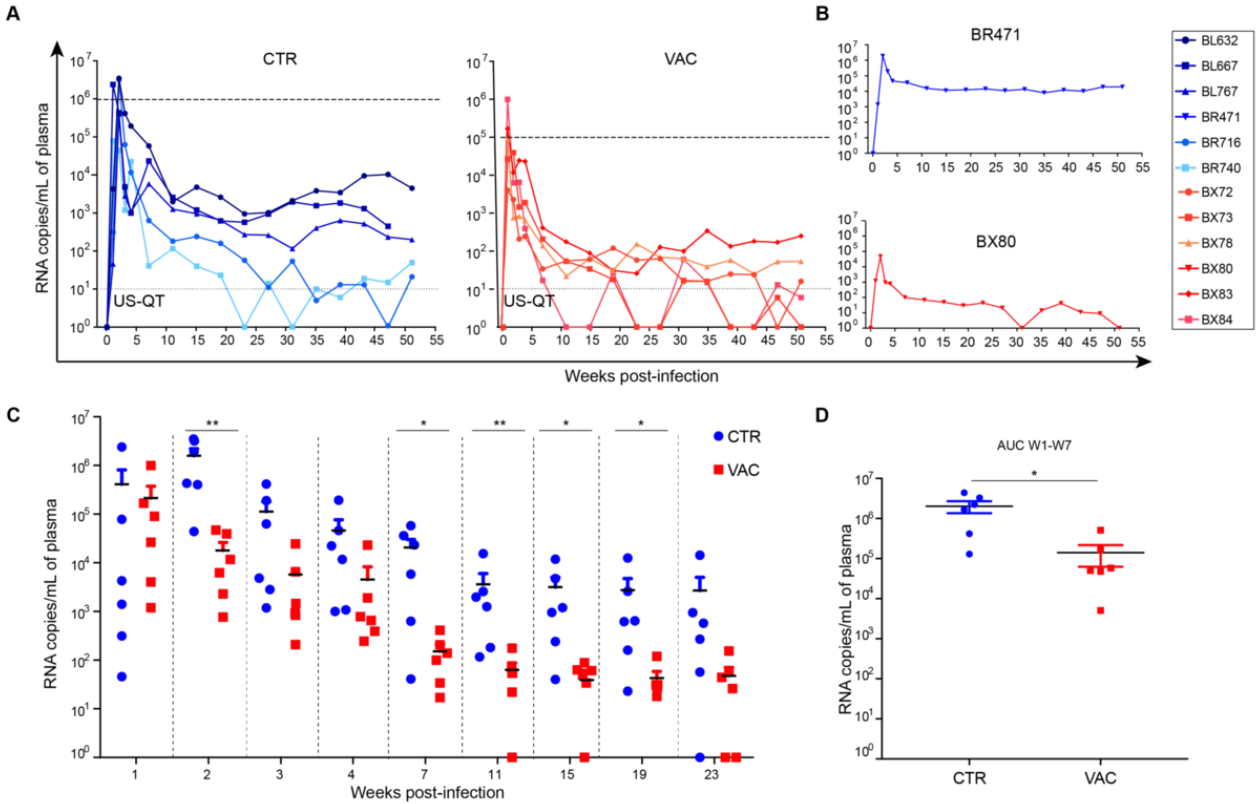
Sections of inguinal lymph nodes taken after necropsy from both control (CTR) and vaccinated (VAC) animals were double-stained with anti-CD4 or CD8 and anti-PD-1 mAbs via immunohistochemistry (IHC). Representative PD-1 (black) and CD4 or CD8 (Fuchsin-red) stainings (20 × and 200×) in one representative vaccinated (A, B top panel) and one control (C, D bottom panel) cynomolgus macaques are shown.

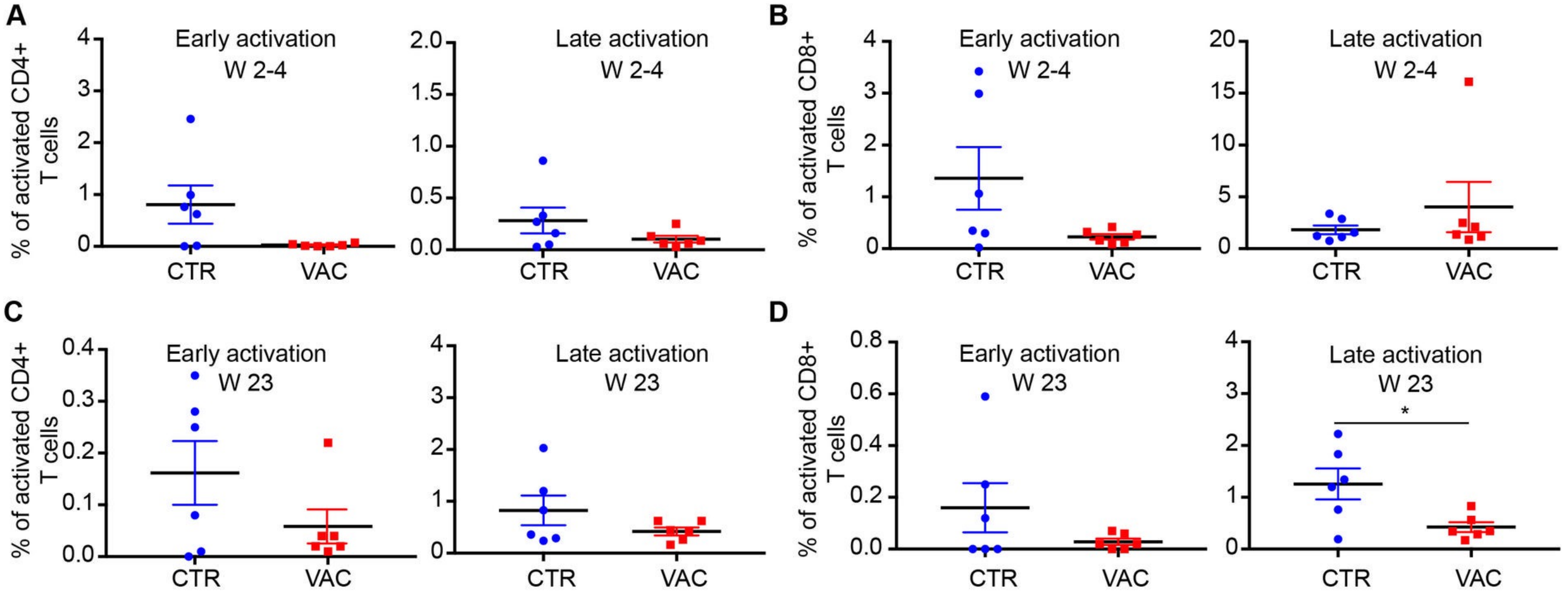
A


Immunization
SIVmac251 challenge
(IR) ..... (IV)
CAL-SHIV-IN-
IM / ID + EP
Vaccinated
macaques
(n = 6)
0.5
1
2
50 AID50
Control
macaques
(n = 6)
0
Weeks post-immunization
82
92
96
100

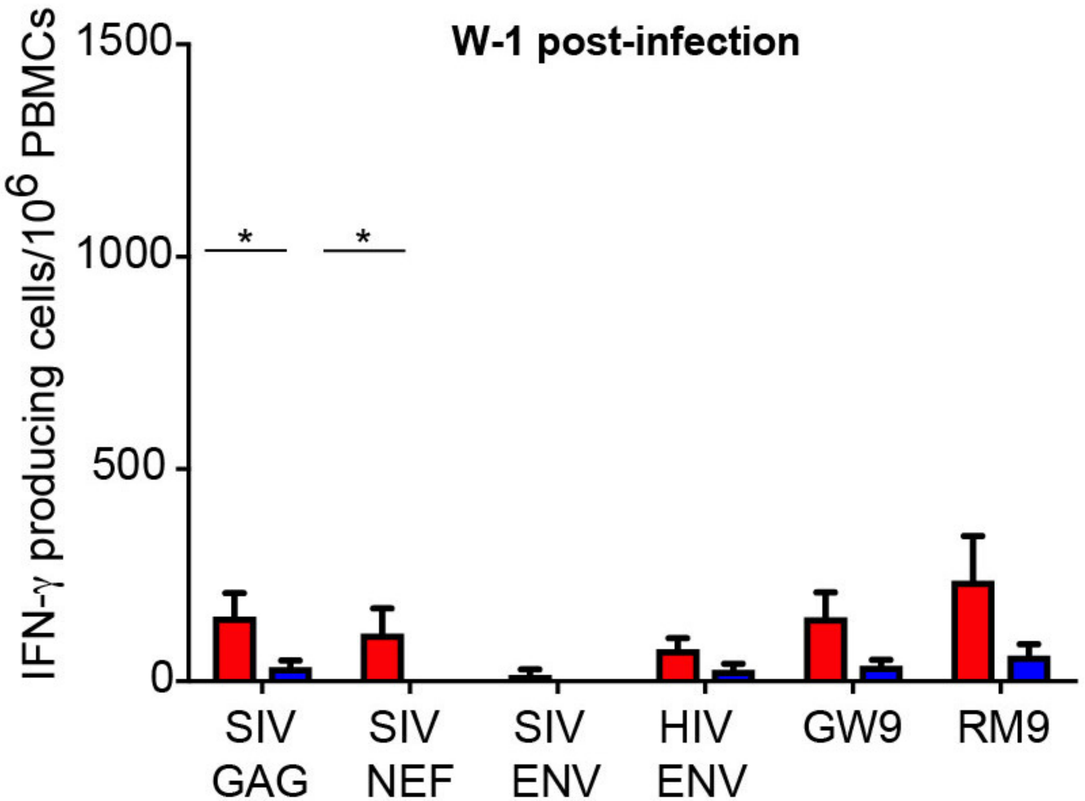
B


Percentage of infection (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Weeks post-1st challenge
CTR
VAC
0.5 AID50 IR
1 AID50 IR
2 AID50 IR
50 AID50 IV

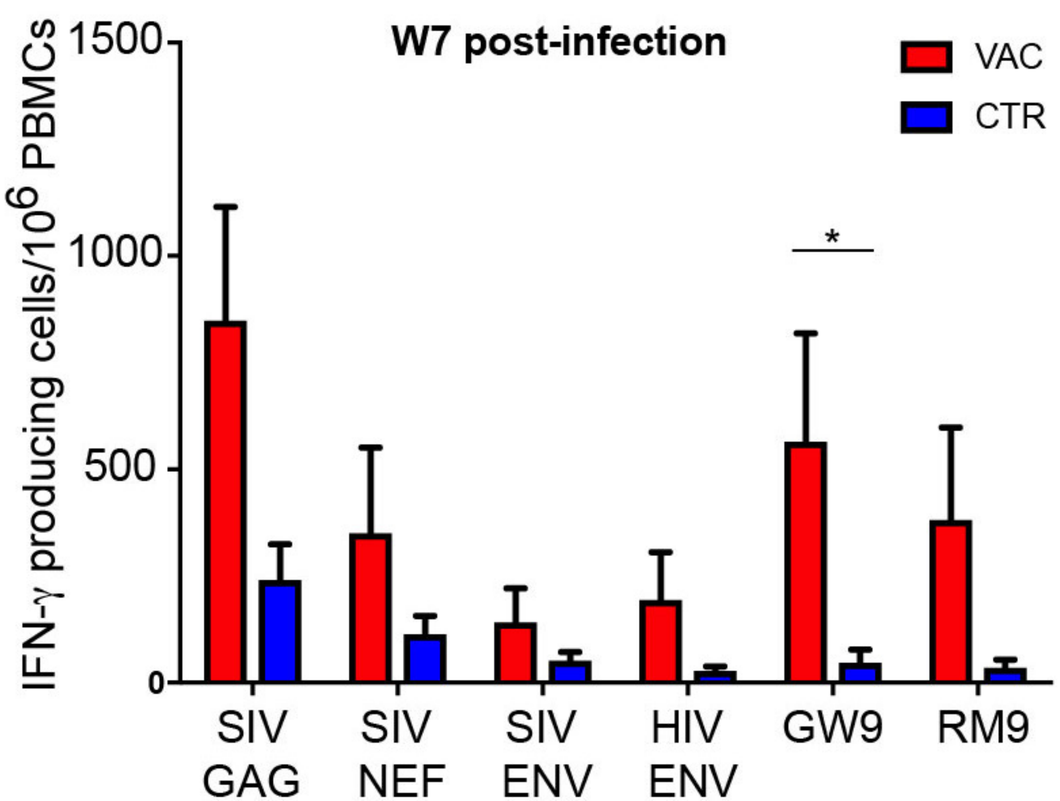

A
CTR
VAC
US-QT
US-QT
RNA copies/mL of plasma
Weeks post-infection
B
BR471
BX80
BL632
BL667
BL767
BR471
BR716
BR740
BX72
BX73
BX78
BX80
BX83
BX84
C
CTR
VAC
RNA copies/mL of plasma
Weeks post-infection
D
AUC W1-W7
RNA copies/mL of plasma
CTR
VAC


A
% of activated CD4+ T cells
Early activation
W 2-4
CTR
VAC
Late activation
W 2-4
CTR
VAC
B
% of activated CD8+ T cells
Early activation
W 2-4
CTR
VAC
Late activation
W 2-4
CTR
VAC
C
% of activated CD4+ T cells
Early activation
W 23
CTR
VAC
Late activation
W 23
CTR
VAC
D
% of activated CD8+ T cells
Early activation
W 23
CTR
VAC
Late activation
W 23
*
CTR
VAC

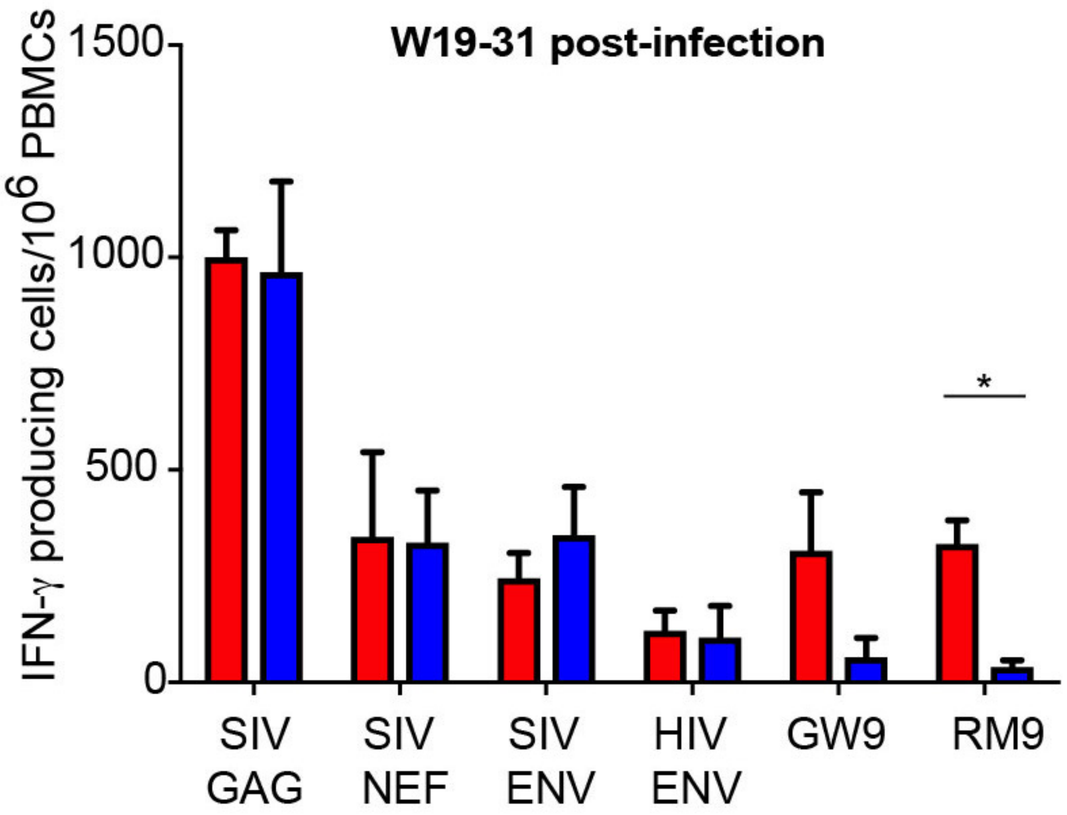
A


W-1 post-infection
IFN-γ producing cells/10^6 PBMCs
1500
1000
500
0
*
*
SIV GAG
SIV NEF
SIV ENV
HIV ENV
GW9
RM9

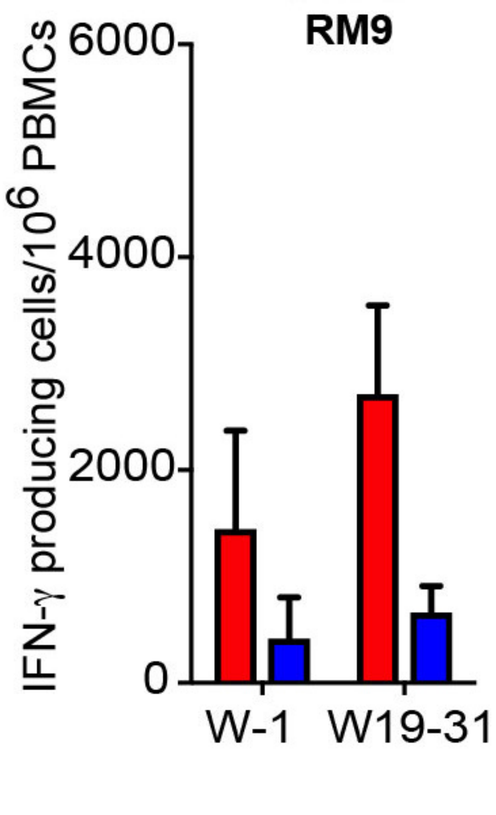
B


W7 post-infection
VAC
CTR
IFN-γ producing cells/10^6 PBMCs
1500
1000
500
0
*
SIV GAG
SIV NEF
SIV ENV
HIV ENV
GW9
RM9

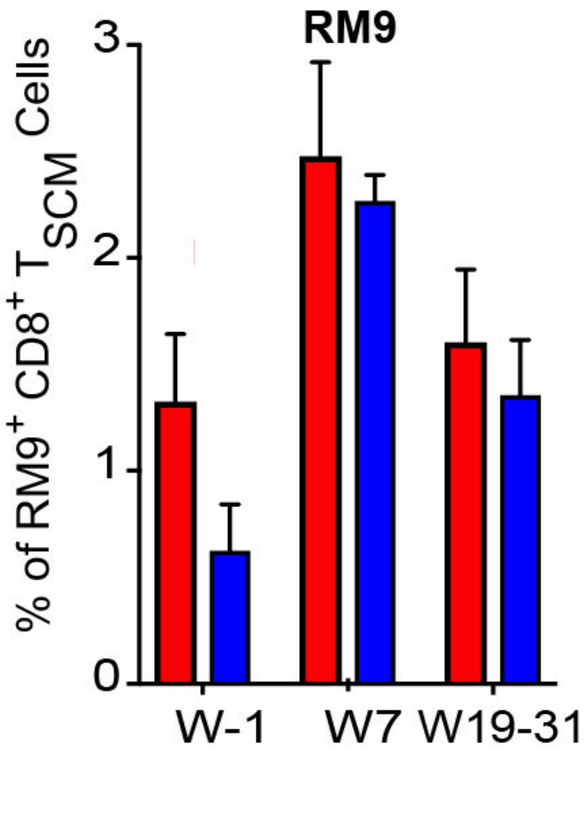
C

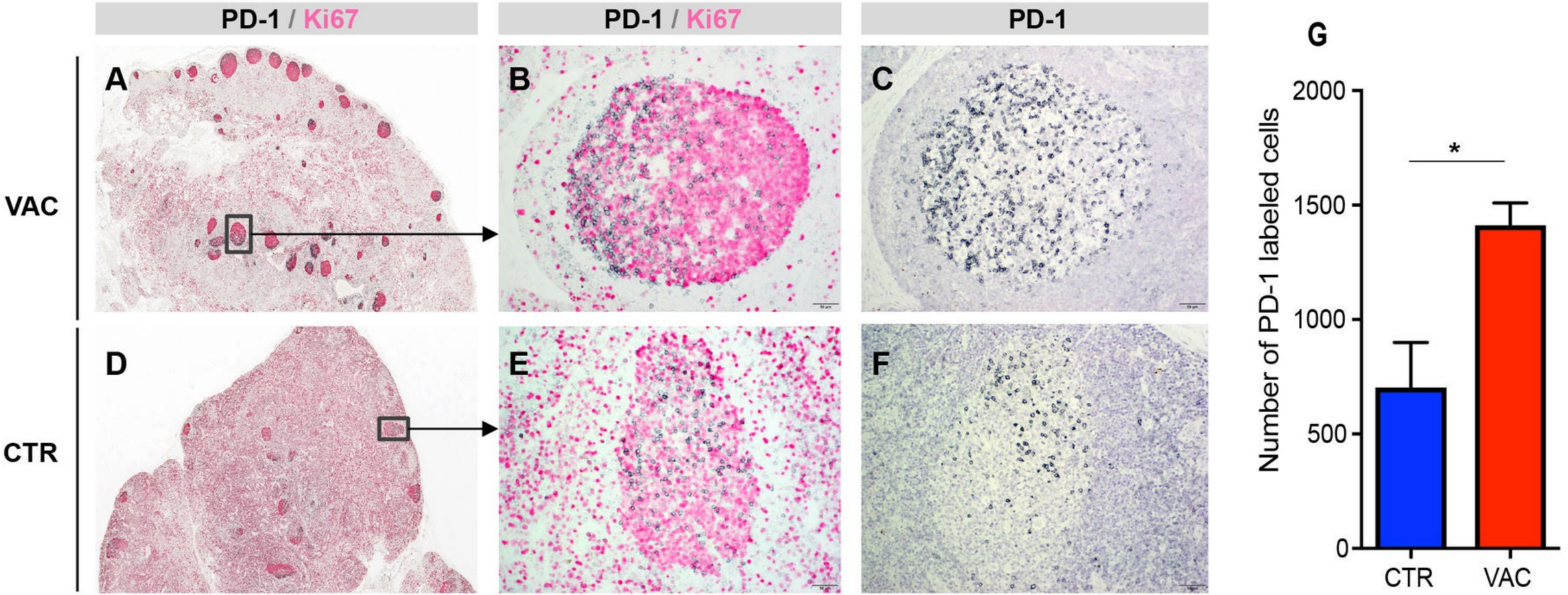

W19-31 post-infection
IFN-γ producing cells/10^6 PBMCs
1500
1000
500
0
*
SIV GAG
SIV NEF
SIV ENV
HIV ENV
GW9
RM9

D


PHPC
RM9
IFN-γ producing cells/10^6 PBMCs
6000
4000
2000
0
W-1
W19-31

E


TSCM
RM9
% of RM9+ CD8+ TSCM Cells
3
2
1
0
W-1
W7
W19-31


PD-1 / Ki67
PD-1 / Ki67
PD-1
VAC
CTR
A
B
C
D
E
F
G
Number of PD-1 labeled cells
2000
1500
1000
500
0
*
CTR
VAC