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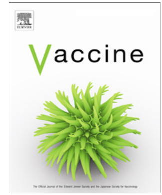

Vaccination of rhesus macaques with the live-attenuated HSV-1 vaccine VC2 stimulates the proliferation of mucosal T cells and germinal center responses resulting in sustained production of highly neutralizing antibodies

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ABSTRACT

We have shown that the live-attenuated HSV-1 VC2 vaccine strain with mutations in glycoprotein K (gK) and the membrane protein UL20 is unable to establish latency in vaccinated animals and produces a robust immune response capable of completely protecting mice against lethal vaginal HSV-1 or HSV-2 infections. To better understand the immune response generated by vaccination with VC2, we tested its ability to elicit immune responses in rhesus macaques. Vaccinated animals showed no signs of disease and developed increasing HSV-1 and HSV-2 reactive IgG_1 after two booster vaccinations, while IgG subtypes IgG_2 and IgG_3 remained at low to undetectable levels. All vaccinated animals produced high levels of cross protective neutralizing antibodies. Flow cytometry analysis of cells isolated from draining lymph nodes showed that VC2 vaccination stimulated significant increases in plasmablast ($CD27^{high}CD38^{high}$) and mature memory ($CD21^-IgM^-$) B cells. T cell analysis on cells isolated from draining lymph node biopsies demonstrated a statistically significant increase in proliferating ($Ki67^+$) follicular T helper cells and regulatory $CXCR5^+$ $CD8^+$ cytotoxic T cells. Analysis of plasma isolated two weeks post vaccination showed significant increases in circulating CXCL13 indicating increased germinal center activity. Cells isolated from vaginal biopsy samples collected over the course of the study exhibited vaccination-dependent increases in proliferating ($Ki67^+$) $CD4^+$ and $CD8^+$ T cell populations. These results suggest that intramuscular vaccination with the live-attenuated HSV-1 VC2 vaccine strain can stimulate robust IgG_1 antibody responses that persist for >250 days post vaccination. In addition, vaccination lead to the maturation of B cells into plasmablast and mature memory B cells, the expansion of follicular T helper cells, and affects in the mucosal immune responses. These data suggest that the HSV VC2 vaccine induces potent immune responses that could help define correlates of protection towards developing an efficacious HSV-1/HSV-2 vaccine in humans.

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1. Introduction

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are common human pathogens afflicting the oral and genital mucosa. Both viruses cause life-long infection with recurrent episodic reactivation that can manifest in ulcerations, neonatal infection, and in more severe instances meningitis, and encephalitis [1]. The acute infection of epithelial surfaces can manifest a range of pathogenesis more commonly resulting in asymptomatic infection, while blistering and ulceration are frequently observed. After replication in epithelial cells the virus enters via direct fusion with axonal membranes and is transported in a retrograde manner to the soma of the neuron where it establishes a latent infection. Reactivation from latency has been attributed to stress, UV irradiation, and immunosuppression. Asymptomatic reactivation and shedding results in the high global prevalence observed for these viruses. Recent data demonstrate that of the 3438 individuals in the control arm of the HERPEVAC Trial for Women in a 20 month period 183

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(3.7%) contracted herpes simplex virus infection [2]. The majority of infections occurred without clinical manifestation of disease however 83% of recognized cases were genital infections [2]. Global prevalence was estimated in 2012 to be 417 million people, 15–49 years old living with HSV-2 infection, of whom 267 million were women [3]. Importantly, human immunodeficiency virus (HIV) infection risk is three times higher in individuals infected with genital HSV-2 and antiviral therapy with acyclovir to control HSV-2 infection does not reduce this risk [4]. This significant risk and the general morbidity associated with symptomatic infection render the development of a successful prophylactic and therapeutic vaccine an important global health need.

Many HSV vaccine candidates have shown promise in preclinical and clinical trials, however none have resulted in an efficacious vaccine approach for the prevention of genital HSV-2 disease [Reviewed in: [5,6]]. Many of the issues observed in the clinical setting stem from the inability to reproduce preclinical results in humans warranting the development of models that better mimic the human response to vaccination. It is well known that there are significant differences between the immune systems of mice and that of humans resulting in conclusions that are not always applicable to understanding human immune responses [7,8]. As our closes evolutionary relatives NHPs represent the best animal model to study human biological processes. Old World monkeys, specifically rhesus macaques, are the most frequently used NHPs used in AIDS research and have many reagents and assays developed for studying immune responses.

The VC2 vaccine strain tested here expresses HSV-1 glycoprotein K (gK) carrying a deletion of amino acids 31–68 rendering this virus unable to infect neuronal axons and establish latency [9]. We have shown that the VC2 vaccine is safe and highly efficacious in providing protection against lethal intravaginal HSV-1 and HSV-2 challenge in mice [9]. In the current study we found that the VC2 vaccine elicited robust immune responses in rhesus macaques in agreement with our previous results.

2. Materials and methods

2.1. Animals

Four female Indian rhesus macaques (*Macaca mulatta*) were used for this study (age range: 5.8–13.9 years). Animals were housed under biosafety level two conditions at the Tulane National Primate Research Center (TNPRC) under the care of TNPRC veterinarians in accordance with the standards incorporated in the Guide for the Care and Use of Laboratory Animals and with the approval of the Tulane Institutional Animal Care and Use Committee. All procedures were performed only on sedated animals. Animals were healthy and negative for SIV, STLV, Simian Type D retroviruses, measles, and Herpes B virus at the beginning of the study.

2.2. VC2 vaccine preparation, immunization, tissue collection and tissue processing

Serum free VC2 virus stock was prepared as previously described [9], by infection of African green monkey kidney (VERO) cells at an MOI of 0.001 followed by replacement of DMEM growth medium without fetal bovine serum (FBS). Infection was allowed to progress for 48 h after which time virus was collected, titrated and aliquoted for vaccination purposes. All animals were vaccinated with the VC2 vaccine ($1x10^6$ PFU) at day 37, 65 and 93 through intramuscular route (Fig. 1). Whole blood and plasma samples were collected for all the time points shown in Fig. 1. Axillary/inguinal lymph node (LN) biopsies were collected at day 37, 51 (2 weeks following initial vaccination) and 79 (2 weeks following the second vaccination). Vaginal pinch biopsies were collected at day 37, 51, 79 and 107 from each animal. All tissues were processed for detecting cellular immune responses (Fig. 1).

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (LSM medium, Cellgro, Inc.) as described previously [10–13]. Axillary Lymph node (LN) lymphocytes were isolated by mincing into small pieces and gently pressing through 100 μm cell strainers and washed with PBS [10,12,14]. Vaginal lamina propria lymphocytes (LPL) were isolated using collagenase enzymatic treatment as reported earlier for jejunum LPL [10,12]. All freshly isolated lymphocytes were more than 90% viable and were used immediately for all cellular assays.

2.3. ELISA and plaque reduction neutralization test (PRNT)

ELISA plates (Pierce, Inc.) were coated with 1 μg/well of purified HSV-1 (McKrae) or HSV-2 (G) lysate in bicarbonate/carbonate buffer 100 mM (pH 9.6) and coating was allowed to bind with overnight rocking at 4 °C. Plates were then blocked for 1 h rocking at room temperature using 200 μL of 1% bovine serum albumin (BSA) in 1 x phosphate buffered saline (PBS). Serum samples were then diluted 1:1000 in 1% BSA PBS and 100 μL of each sample was applied to coated ELISA plate and allowed to bind rocking overnight at 4 °C. Plates were then washed and probed with anti-rhesus IgG_1, IgG_2, and IgG_3 (NIH Nonhuman Primate Reagent Resource), diluted to 1:1000 in 1% BSA in 1 x PBS. ELISAs were developed using TMB substrate and quenched with 1.6 M Sulfuric Acid. Colorimetric ELISAs were read at an absorbance of 450 nm on a VersaMax ELISA Microplate Reader (Molecular Devices) Relative concentration normalized to baseline controls as a fold change in absorbance.

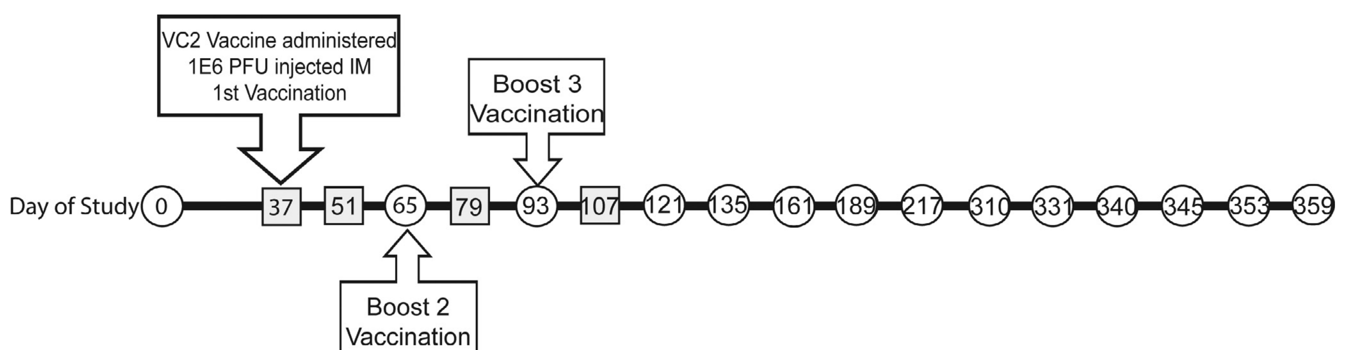

Fig. 1. Vaccine study outline. Four female rhesus macaques were vaccinated via intramuscular injections with the VC2 vaccine at 1×10^6 PFU/injection at 28-day intervals. PBMCs and plasma were collected at all time points (square/circle). Draining lymph nodes were collected on days 37 (1st Vaccination), 51 (2 weeks following initial inoculation) and 79 (2 weeks following the second inoculation) (square). Vaginal biopsies were collected on days 37, 51, 79, and 107 of the study (square).

Sera collected over the course of the study were tested by plaque reduction neutralization test (PRNT). Specifically, serum was diluted in complete DMEM containing 10% heat inactivated FBS to concentrations of 20%, 2%, and 0.2% plasma in a volume of 500 μL. 500 μL of media containing 100 plaque forming units (PFU) of either HSV-1 (McKrae) or HSV-2 (G) virus was then added to plasma dilutions of 10%, 1%, and 0.1%, respectively. Plasma/virus mixtures were then placed on a rocker at room temperature for 1 h and immediately plated on confluent monolayers of VERO cells. Virus was allowed to bind to the surface of the cells for 1 h with rocking at room temperature. Remaining supernatant was then aspirated and replaced with DMEM containing 1% methylcellulose, 1% FBS, and Primocin (InvivoGen, USA). Infection was allowed to progress for 2 days at which point cells were fixed with ice cold methanol. Immunohistochemistry was performed using polyclonal rabbit anit-HSV-1 primary antibody (Dako, Inc., Denmark), polyclonal goat anti-rabbit immunoglobulins horseradish peroxidase (HRP) conjugated secondary antibody (Dako, Inc., Denmark), and visualized using Vector NovaRED Substrate Kit (Vector, Inc., Burlingame, CA). Substrate was allowed to develop until sufficient staining of plaques was produced with minimal background. Anti-human CXCL13 ELISA kit (R&D systems, Inc., USA) was used to quantify plasma CXCL13 level at 2 weeks following the 0th, 1st and 2nd vaccination time points following the manufacturer's protocol.

2.4. Flow Cytometric staining and analysis

Fresh peripheral blood mononuclear cells (PBMCs), LN mononuclear cells and vaginal LPL were used for flow cytometry based assays. Typically, $0.5–1 \times 10^6$ cells were first stained with live/dead stain, followed by conjugated monoclonal/polyclonal antibodies to T cells and B cell markers. For the detection of T-cells phenotypes and proliferation, the antibody panel consisted of anti-CD3 (SP34-2, BD Biosciences, Inc.), anti-CD4 (L200, BD Biosciences, Inc.) and anti-CD8 (3B5, Invitrogen, Inc.), anti-CXCR5 (710D82.1, NHP reagent Resources) and anti-Ki67 (B56, BD Biosciences, Inc.) antibodies. For B cell subtypes detection, anti-CD20 (2H7, BD Biosciences, Inc.), anti-CD21 (Bly4, BD Biosciences, Inc.), anti-CD27 (O323, Biolegend, Inc.), anti-IgM (G20-127, BD Biosciences, Inc.) and anti-IgD (Southern Biotec, Inc.) were used. For surface staining, cells were incubated with surface antibody markers for 30 min at room temperature and washed with distilled PBS/BSA wash buffer.

For the detection of antigen-specific cytokine production, freshly isolated cells were stimulated for 6 h in complete RPMI-1640 medium containing 10% fetal calf serum (FCS) (R10) at 1×10^6 cells per ml with: gB (pp161–176 + pp566–580) and gD (pp70–78 + pp270–287 + pp278–286) peptide pools, R10 (used as a negative control) or PMA/ION (Sigma, Inc., used as a positive control) in the presence of 0.5 μg/ml of anti-CD28 (28.2, BD Biosciences, Inc.) and anti-CD49d (9F10, BD Biosciences, Inc.) monoclonal antibodies and Brefeldin A (10 μg/ml, Sigma, Inc.). After stimulation, cells were stained for live/dead stain, followed by cell surface markers with anti-CD3, anti-CD4 and anti-CD8 antibodies for 30 min at room temperature and washed with distilled PBS/BSA wash buffer. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained for intracellular monoclonal antibodies like anti-IFN-γ (4S.B3, BD Biosciences, Inc.), anti-TNF-α (MAb11, BD Biosciences, Inc.), anti-IL-2 (MQ1-17H12, Biolegend, Inc.) and anti-IL-17A (eBio64CAP17, Affymetrix eBioscience, Inc.) antibodies and incubated at room temperature for 30 min as described earlier (12, 15–17). For Ki67 staining, cells were fixed and permeabilized by using Cytofix/Cytoperm (BD Biosciences, Inc.), washed twice in Perm Buffer (BD Biosciences, Inc.) after the surface staining and stained with Ki67 and incubated at room temperature for 30 min as described earlier [12,15–17]. Cells were washed and fixed with 1X BD stabilizing fixative buffer (BD Biosciences, Inc.). Data were acquired within 24 h of staining using a BD Fortessa instrument (BD Immunocytometry System, Inc.) and FACSDiva software (BD Immunocytometry System, Inc.). For each sample, 50,000 events were collected by gating either on $CD3^+$ T cells or $CD20^+$ B cells. For cytokine analysis, 50,000 events were collected by gating on $CD3^+CD8^+$ T cells from each sample. Data analysis was performed using FlowJo software (version 9.9). For cytokine analysis, cells were gated first on singlets, followed by lymphocytes, live cells, $CD3^+$ T-cells and then on $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets. Gated $CD4^+$ and $CD8^+$ T-cells were further analyzed for cytokine production. Positive cytokine responses were determined based on the percentage of cytokine responses detected twofold higher than the medium control culture and greater than 0.05% responses for that specific cytokine and antigen.

2.5. Statistical analysis

Statistical analysis was conducted using the statistical software Prism Graph Pad (version 5) by Student's T test and Two-Way ANOVA with Bonferroni's posttest. P-values less than 0.05 are considered statistically significant. Correlation analysis was performed by plotting percent change in circulating IgG_1 reactive against HSV-1 or HSV-2 against the percentages of $CD3^+CD4^+CXCR5^+Ki67^+$ T cells. These scatterplots were then analyzed by linear trend correlation analysis.

3. Results

3.1. Safety and tolerability of vaccine

The VC2 vaccine was administered intramuscularly at the indicated time points (Fig. 1). All animals remained healthy for the duration of the study. There were no adverse effects or inflammation observed either systemically or visually at the injection site and none of the animals were euthanized preemptively before the end of the study. In addition, there were no significant changes in CBC (counts of neutrophil, lymphocytes, monocytes, eosinophils and basophils) or blood chemistry (sodium, potassium, chloride, total protein, albumin, globulin, blood urea nitrogen, glucose, creatinine, aspartate aminotransferase, alanine aminotransferase, calcium, total bilirubin, phosphorus, alkaline phosphatase, lactate dehydrogenase, cholesterol, triglycerides, gamma-glutamyl transferase, iron, C-reactive protein and microprotein) in any of the vaccinated animal (Supplemental Table 1).

3.2. Systemic antibody responses

Analysis of plasma isolated over the course of the study by ELISA indicated significant induction of HSV-1 and HSV-2 cross-reactive IgG antibodies. The relative increase in HSV-1- reactive antibodies over the course of the study was greater than that of HSV-2 antibodies. Antibody concentrations were maximal at 79–107 days post vaccination and were sustained throughout the post vaccination study period until day 359 when the study ended (Fig. 2A and B). The plasma was tested for ability to neutralize either HSV-1 (McKrae) or HSV-2 (G) by standardized plaque reduction neutralization test (PRNT). This analysis indicated that the antibodies produced were highly cross-reactive and cross-neutralizing, persisting for the duration of the study (Fig. 2C and D).

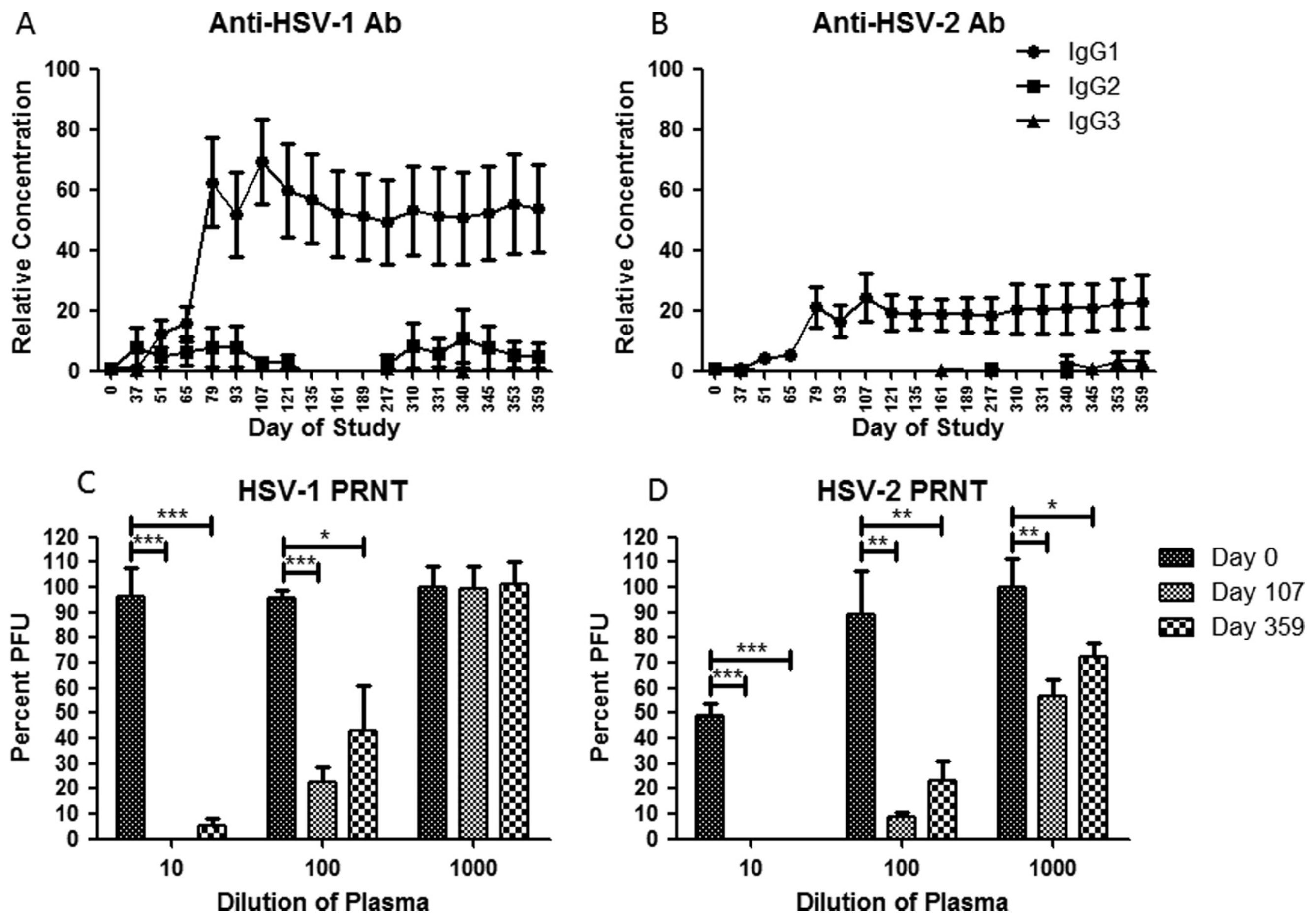

Fig. 2. Vaccine induced antibody responses. Plasma collected over the course of the study was used in HSV-1 (A) and HSV-2 (B) ELISAs. Antibodies bound to their respective assay plates were probed with secondary antibodies reactive to IgG_1, IgG_2, and IgG_3. Absorbance was normalized to day 0 absorbance and statistical analysis was conducted by two-way ANOVA and Bonferroni's test for multiple comparisons. Significance observed $p < 0.05$ at day 79 an onwards comparing IgG_1 vs IgG_2 and IgG_1 vs IgG_3. No significant differences were observed between IgG_2 vs IgG_3. Error bars represent standard error about the mean. Neutralizing affinity of plasma was measured by plaque reduction neutralization test (PRNT) for its ability to neutralize ~100 PFU of either HSV-1 McKrae (C) or HSV-2 G (D), with percentages of PFU normalized to no plasma controls. Statistical analysis conducted by Student's T-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Error bars represent standard error about the mean.

3.3. Vaccine induced changes in lymph nodes B cell subsets

To analyze the effect of VC2 vaccination on B cells, a modified version of the gating strategy for B cell typing was used as described previously [18] (Fig. S1). $CD20^+$ B cells from lymph nodes (LN) were characterized based on their surface expression of CD27 and IgD markers representing switched memory (SwMe, $CD27^+IgD^-$), Non Switched Memory (NSM, $CD27^+IgD^+$), Naïve (Naïve, $CD27^-IgD^+$), and Double Negative B cells (DN, $CD27^-IgD^-$). SwMe B cells can be further defined as plasmablasts ($CD27^{high}CD38^{high}$). The $CD38^+$ population of SwMe B cells can be further analyzed for their surface expression of CD21 and IgM, which are negatively correlated with antibody secreting B cells [18]. VC2 vaccination significantly induced the expansion of plasmablast and memory $CD38^+CD21^-IgM^-$ SwMe B cells in a vaccination-dependent manner (Fig. 3A and B). Significant decreases in the percentage of memory $CD38^+CD21^+IgM^+$ B cells were observed with multiple vaccinations (Fig. 3C).

3.4. T cell dynamics and antigen-specific T cell responses

To characterize the effect of VC2 vaccination on T-cell dynamics, the intercellular marker for cellular proliferation Ki67 was used to identify cells that are either currently undergoing proliferation or have recently proliferated. The representative gating strategy for T cell analysis is shown in the supplementary Fig. S2. Throughout the vaccination phase (up to day 107 of the study) animals displayed significant increases after each boost vaccination in proliferating T follicular helper cells (Tfh) defined as $CD3^+CD4^+CXCR5^+Ki67^+$ cells in the draining lymph node. Specifically, significant increases were observed at day 79 of the study in comparison to similar levels of cells observed at day 37 and day 51 (Fig. 4A). Similarly, significant increases in proliferating follicular cytotoxic T cells (Tfc, [19]) were also observed at day 79 of the study after 2 doses of the VC2 vaccine (Fig. 4B). Germinal center activity was monitored by measuring systemic changes in CXCL13 concentration [20]. Significant induction of CXCL13 was observed after the initial vaccination ($P < 0.05$) and approaching significance after the first booster vaccination ($p = 0.06$) (Fig. 4C). Correlation analysis between percent change in HSV-1 or HSV-2 reactive IgG_1 antibodies and percentages of $CD3^+CD4^+CXCR5^+Ki67^+$ cells indicated a strong correlation between the observed changes in T cell population and HSV-reactive antibodies (HSV-1: $p = 0.006$ 0.05 $r^2 = 0.54$, HSV-2: $p = 0.04$ $r^2 = 0.35$) (Fig. 4D). Vaccination also induced the proliferation of vaginal $CD4^+$ and $CD8^+$ T cells with multiple vaccinations, obtaining significance after 2 and 3 boost vaccinations (Fig. 5).

All vaccinated animals demonstrated substantial HSV-specific cytokine responses after stimulation of PBMCs and LNs with gB (pp161–176 + pp566–580) and gD (pp70–78 + pp270–287

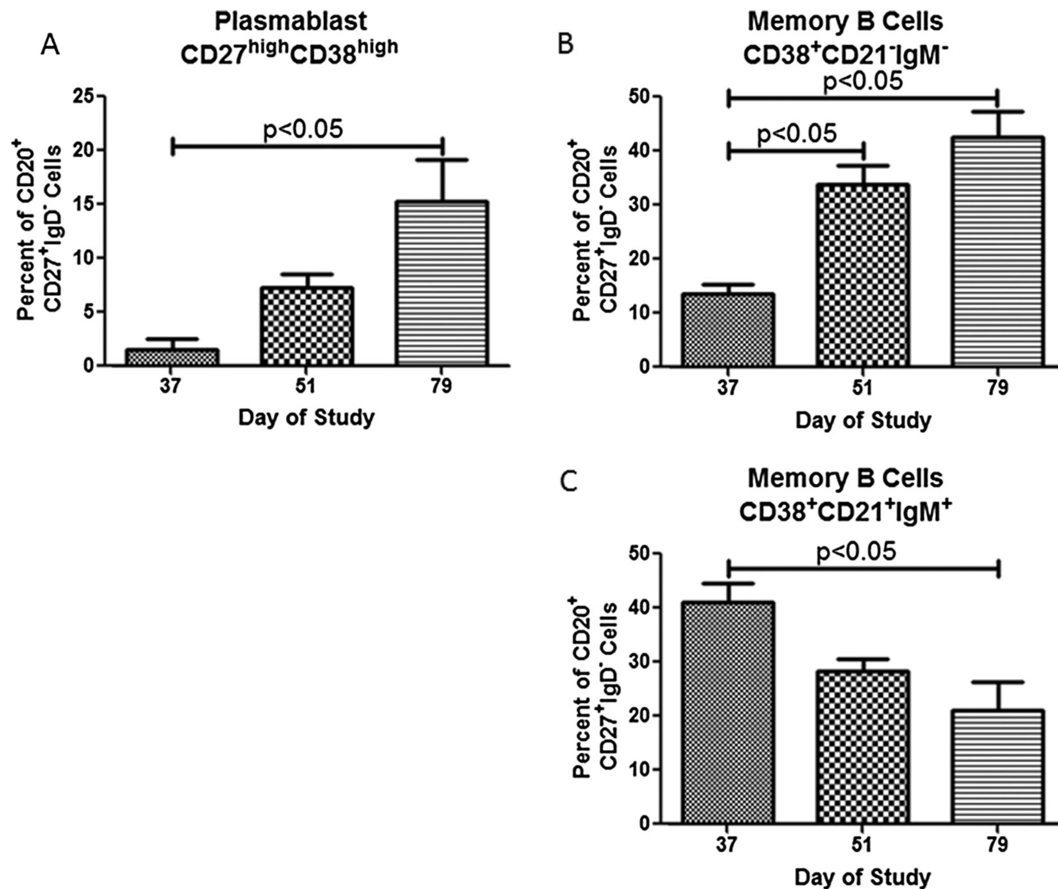

Fig. 3. Vaccine induced B-cell dynamics. Flow cytometry analysis on draining lymph nodes collected at the beginning of the study vaccination day 37 or 2 weeks following the 1st and second vaccination (days 51 and 79 respectively) of the VC2 vaccine analyzing change in the (A) Plasmablast ($CD27^{high}CD38^{high}$) population of switched memory B cells (B) $CD38^{+}$ $CD21^{-}IgM^{-}$ Memory B cells, and (C) $CD38^{+}$ $CD21^{+}IgM^{+}$ Memory B cells. Statistics calculated by Student's T-test. Error bars represent standard error about the mean.

+ pp278–286) peptide pools for both $CD4^{+}$ and $CD8^{+}$ T cells (Table 1). LN T-cells had more cytokine responses detected than the T cells from PBMC following vaccination. Overall, no HSV-specific IL-17a responses were detected. Mean responses to all cytokines except IL-2 were lower at day 79 time points compared to day 51 and day 37, possibly due to the lack of T-cell memory responses following boosting. Increased IL-2 expression was detected in $CD8^{+}$ cells in response to gD peptides in both PBMC and LN tissues following vaccination. Both gB and gD peptides were found immunogenic in inducing antigen specific cytokine responses. HSV-specific cytokine responses were also tested in vaginal LPL tissues, but the cytokine responses in both $CD4^{+}$ and $CD8^{+}$ T-cells were not detected (data not shown).

4. Discussion

We have previously shown that vaccination with the live-attenuated HSV-1 vaccine VC2 was non-toxic and protected mice from lethal vaginal infection with either virulent HSV-1 (McKrae) or HSV-2 (G). This protection was attributed to significant induction of cross neutralizing antibodies and cross reactive $CD4^{+}$ and $CD8^{+}$ T cells [9]. Mice represent an essential tool biomedical researchers have utilized for many life-changing discoveries however in the complex context of host pathogen interaction rodent models fall short. Rhesus macaque was shown to be a unique model to study HSV-2 pathogenesis and testing for vaccine efficacy [21]. In agreement with our previous results in mice, we show here that VC2 vaccination of rhesus macaques generated robust humoral responses characterized by the production of long-lasting neutralizing antibodies and proliferation of both $CD4^{+}$ and $CD8^{+}$ T cells. The VC2 virus is fully competent for virus replication in all cell types tested including African monkey kidney (Vero) cells [9] and primary Indian rhesus macaque vaginal epithelial cells (not shown). The VC2 virus was shown to be unable to infect rat neuronal axons in cell culture [22] and was defective in infecting ganglionic neurons of mice after ocular infection [23]. Therefore, it is expected that VC2 will have at least limited replication at the injection site, while unable to establish latency and prolonged virus shedding.

Vaccination of rhesus macaques with the live-attenuated HSV-1 VC2 vaccine significantly increased germinal center activity characterized by increased proliferation of follicular T helper cells (Tfh), follicular cytotoxic T cell, plasmablast, and $CD21^{-}IgM^{-}$ switched memory B cells resulting in robust humoral immune responses. The antibodies produced by the VC2 vaccine were highly neutralizing and persistent for >250 days following three vaccinations. Recently CXCL13 was shown to be a biomarker for germinal center activity. Specifically, increased CXCL13 production is correlated with lymph node germinal center formation and induction of broadly neutralizing antibody responses [20]. The CXCL13 cytokine selectively interacts with CXCR5 to promote chemotaxis of both B-1 and B-2 cells. The relative levels of CXCL13 were measured in plasma by ELISA to further validate the observations regarding T and B cell responses in the draining lymph node. CXCL13 production was increased and correlated with increased neutralizing antibody responses indicating germinal center devel-

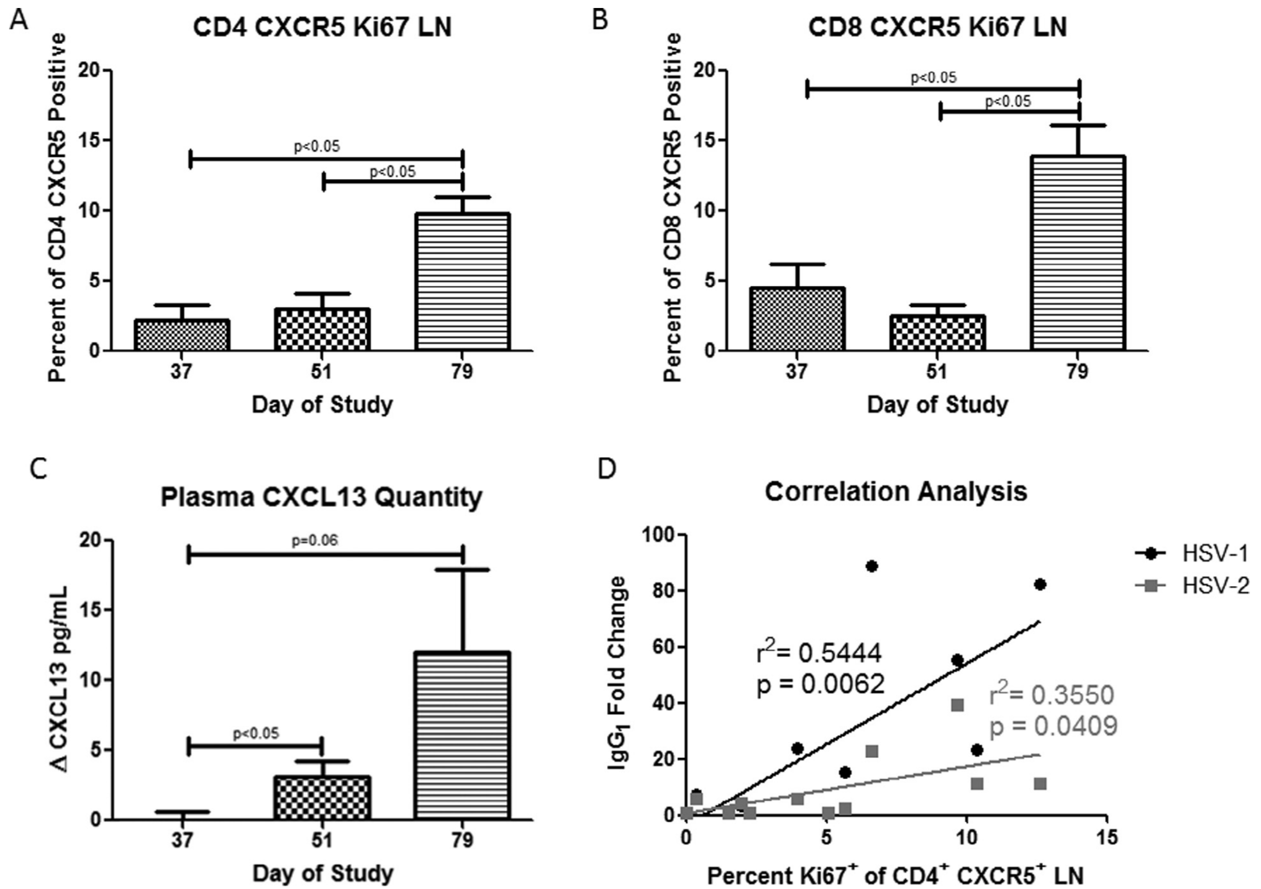

Fig. 4. Vaccine induced T-cell proliferation, plasma CXCL13 expression and correlation with proliferating Tfh cells. Flow cytometry analysis on draining lymph nodes collected at the beginning of the study vaccination day 37 or 2 weeks following the 1st and second vaccination (days 51 and 79 respectively) of the VC2 vaccine analyzing change in the (A) Ki67+ population of CD4+ CXCR5+ T cells, (B) the Ki67+ population of CD8+ CXCR5+ T cells, (C) the change in systemic CXCL13, and (D) correlation analysis between percentage of Ki67+ population of CD4+ CXCR5+ T cells and the change in HSV-1 and HSV-2 reactive IgG_1. Statistics calculated by Student's T-test and linear regression analysis. Error bars represent standard error about the mean.

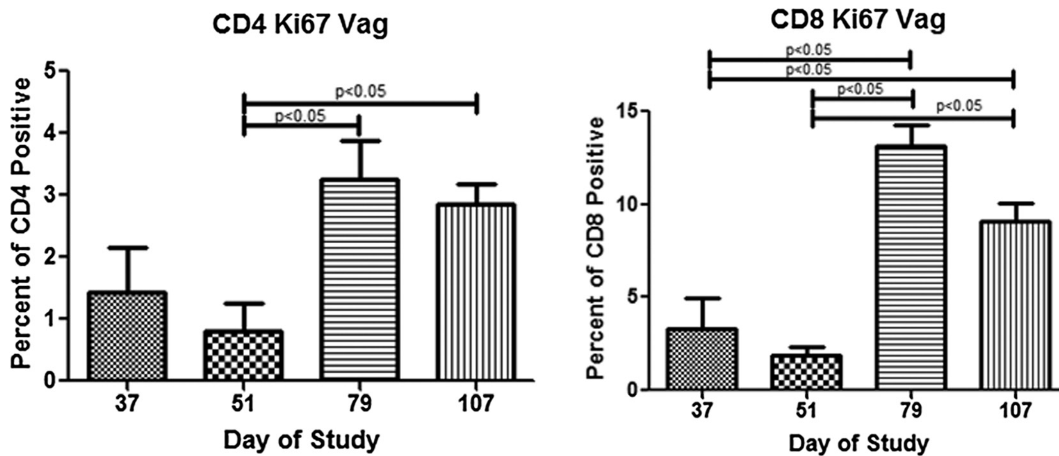

Fig. 5. Proliferation of vaginal LPL CD4+ and CD8+ T cells. Flow cytometry analysis on vaginal LPL isolated from vaginal biopsies collected at the beginning of the study vaccination 0 or 2 weeks following the 1st, 2nd, and 3rd vaccination of the VC2 vaccine analyzing change in the Ki67+ population of CD4+ T cells (A), and the Ki67+ population of CD8+ T cells (B). Statistics were calculated by Student's T test. Error bars represent standard error about the mean.

opment and induction of memory B cells, as observed in LN memory B cells.

Long lived B cells such as plasmablast and SwMe B cells are responsible for the persistent secretion of antibodies more typically associated with defense against microbial pathogens. Specifically, CD27 (TNF receptor family) is a cell surface marker indicating cells that have undergone somatic hypermutation. Interactions of these cells with Tfh cells stimulate the secretion of high affinity antibodies through the interaction of several costimulatory molecules between the Tfh cell and the B cell and by secretion of IL-4 and IL-21 [24]. Vaccination with VC2 may induce inflammatory responses that facilitate the maturation of naïve T cells into

Table 1
Frequency of HSV antigen-specific cytokine producing cells in vaccinated animals.[a]

Tissue	HSV antigen	Days post vaccination	CD4				CD8		
			IFNγ (%)	IL-2 (%)	IL-17A (%)	TNFα (%)	IFNγ (%)	IL-2 (%)	TNFα (%)
PBMC	gB	d37	0.00	0.00	0.03	0.00	0.00	0.06	0.00
		d51	0.43	0.00	0.00	0.00	0.00	0.06	0.00
		d79	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	gD	d37	0.00	0.00	0.11	0.00	0.00	0.12	0.00
		d51	0.53	0.07	0.00	0.00	0.22	0.09	0.00
		d79	0.11	0.00	0.00	0.00	0.00	0.58	0.00
LN	gB	d37	0.27	0.00	0.00	0.23	0.81	0.00	0.36
		d51	0.00	0.68%	0.00	0.09	0.19	0.03	0.28
		d79[b]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	gD	d37	0.38	0.00	0.00	0.20	0.74	0.06	0.07
		d51	0.10	0.06	0.00	0.14	0.15	0.56	0.04
		d79[b]	0.00	0.19	0.00	0.00	0.00	2.06	0.00

All values were subtracted from medium control before the analysis.
[a] Percentages are calculated means for each animal group.
[b] Mean percentages from 2 animals for the d79 timepoint.

Tfh cells which help persistence and maintenance of B cells that secrete highly neutralizing HSV-1/HSV-2 cross reactive antibodies. The VC2 vaccination demonstrated a propensity to stimulate HSV-1/HSV-2 cross reactive IgG_1 in vaccinated rhesus macaques. This particular IgG subtype is the primary IgG subtype found in the blood but is also associated with the highest affinity to initiate antibody dependent cellular cytotoxicity (ADCC) [25]. Several groups have published on the importance of ADCC in anti-HSV immunity [26–29].

No significant changes were detected in the circulating PBMC Tfh cell population over the course of the study. This indicates that the proliferative Tfh phenotype is restricted to the lymph nodes. Tfh cells were shown to be the key subset of $CD4^+$ T cells, which are responsible for generating neutralizing antibodies by providing B cell help [30]. Increased production of IgG_1 antibodies and virus neutralizing antibodies also correlated with increased proliferation of Tfh cells in LN suggesting that vaccine-induced Tfh cells proliferation correlates with B cell maturation and potent neutralizing antibody production, in agreement with previous observations [31].

The VC2 vaccine was able to induce both T and B cell responses. However, B cell responses were surprisingly long-lasting. The vaccinated animals were able to generate strong neutralizing antibodies and induce Tfh cell proliferation. Overall, this data suggest that the vaccine has the potential to induce strong and long-lasting neutralizing antibody based against HSV-1 and HSV-2 infection. Unfortunately, we were unable to follow up the T-cell responses beyond day 79 due to limitations of LN sampling and the limited number of animals of the study, and therefore cannot comment on the status of the long term T cell memory in these animals. A substantial limitation of our T cell analysis is the use of a limited number of gB and gD peptides, which may not adequately represent T cell responses induced by the VC2 vaccination. Surprisingly, intramuscular vaccination with VC2 stimulated the expansion of vaginal $CD4^+$ and $CD8^+$ T cells. It is well known that tissue specific dendritic cells are capable of stimulating the expression of homing molecules to traffic T cells to target tissues [32,33] however vaginal homing is as of yet not fully explored. This phenotype of recruitment of proliferating T cell after intramuscular vaccination to the vaginal mucosa warrants further investigation.

Preclinical animal models are innately flawed predictors of how humans will respond to vaccination. Currently, it is estimated that bringing a vaccine from research and development to market can cost between 200 and 500 million US dollars making proof of concept in animal models essential to the further development of the vaccine [34]. We have shown that the HSV live-attenuated VC2 vaccine generates robust immune responses in mice, guinea pigs (Stanfield et al., manuscript in preparation) and rhesus macaques increasing confidence that it will be efficacious in humans.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.12.018.

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