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Authors

Quadiri, Afshana
Prakash, Swayam
Dhanushkodi, Nisha
et al.

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Therapeutic prime/pull vaccination of HSV-2-infected guinea pigs with the ribonucleotide reductase 2 (RR2) protein and CXCL11 chemokine boosts antiviral local tissue-resident and effector memory CD4+ and CD8+ T cells and protects against recurrent genital herpes

Afshana Quadiri,[1] Swayam Prakash,[1] Nisha Rajeswari Dhanushkodi,[1] Mahmoud Singer,[1] Latifa Zayou,[1] Amin Mohammed Shaik,[1] Miyo Sun,[1] Berfin Suzer,[1] Lauren Su Lin Lau,[1] Amruth Chilukurri,[1] Hawa Vahed,[1,2] Hubert Schaefer,[3] Lbachir BenMohamed[1,2,4]

AUTHOR AFFILIATIONS See affiliation list on p. 18.

ABSTRACT Following acute herpes simplex virus type 2 (HSV-2) infection, the virus undergoes an asymptomatic latent infection of sensory neurons of dorsal root ganglia (DRG). Chemical and physical stress cause intermittent virus reactivation from latently infected DRG and recurrent virus shedding in the genital mucosal epithelium causing genital herpes in symptomatic patients. While T cells appear to play a role in controlling virus reactivation from DRG and reducing the severity of recurrent genital herpes, the mechanisms for recruiting these T cells into DRG and the vaginal mucosa (VM) remain to be fully elucidated. The present study investigates the effect of CXCL9, CXCL10, and CXCL11 T-cell-attracting chemokines on the frequency and function of DRG- and VM-resident CD4+ and CD8+ T cells and its effect on the frequency and severity of recurrent genital herpes in the recurrent herpes guinea pig model. HSV-2 latent-infected guinea pigs were immunized intramuscularly with the HSV-2 ribonucleotide reductase 2 (RR2) protein (*Prime*) and subsequently treated intravaginally with the neurotropic adeno-associated virus type 8 expressing CXCL9, CXCL10, or CXCL11 chemokines to recruit CD4+ and CD8+ T cells into the infected DRG and VM (*Pull*). Compared to the RR2 therapeutic vaccine alone, the RR2/CXCL11 prime/pull therapeutic vaccine significantly increased the frequencies of functional tissue-resident and effector memory CD4+ and CD8+ T cells in both DRG and VM tissues. This was associated with less virus in the healed genital mucosal epithelium and reduced frequency and severity of recurrent genital herpes. These findings confirm the role of local DRG- and VM-resident CD4+ and CD8+ T cells in reducing virus shedding at the vaginal site of infection and the severity of recurrent genital herpes and propose the novel prime-pull vaccine strategy to protect against recurrent genital herpes.

IMPORTANCE The present study investigates the novel prime/pull therapeutic vaccine strategy to protect against recurrent genital herpes using the latently infected guinea pig model. In this study, we used the strategy that involves immunization of herpes simplex virus type 2-infected guinea pigs using a recombinantly expressed herpes tegument protein-ribonucleotide reductase 2 (RR2; prime), followed by intravaginal treatment with the neurotropic adeno-associated virus type 8 expressing CXCL9, CXCL10, or CXCL11 T-cell-attracting chemokines to recruit T cells into the infected dorsal root ganglia (DRG) and vaginal mucosa (VM) (pull). We show that the RR2/CXCL11 prime-pull therapeutic vaccine strategy elicited a significant reduction in virus shedding in the vaginal mucosa and decreased the severity and frequency of recurrent genital herpes. This protection

Editor Anna Ruth Cliffe, University of Virginia, Charlottesville, Virginia, USA

Address correspondence to Lbachir BenMohamed, Lbenmoha@uci.edu.

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This work is dedicated to the memory of the late Professor Steven L. Wechsler "Steve" (1948-2016), whose numerous pioneering works on herpes infection and immunity laid the foundation for this line of research.

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was associated with increased frequencies of functional tissue-resident (T_{RM} cells) and effector (T_{EM} cells) memory $CD4^+$ and $CD8^+$ T cells infiltrating latently infected DRG tissues and the healed regions of the vaginal mucosa. These findings shed light on the role of tissue-resident and effector memory $CD4^+$ and $CD8^+$ T cells in DRG tissues and the VM in protection against recurrent genital herpes and propose the prime-pull therapeutic vaccine strategy in combating genital herpes.

KEYWORDS genital herpes, chemokines, vaginal mucosa, T cells, therapeutic, prime/pull vaccine

Herpes simplex virus type 2 (HSV-2) affects both women and men; however, women are more susceptible to the infection (1). Approximately 315 million women aged 5–49 years old are currently infected globally (2). After exposure of vaginal mucosa (VM) to HSV-2, the virus replicates in the mucosal epithelial cells leading to the development of acute genital herpetic lesions (2–7). Once the acute primary infection is cleared, the virus establishes a lifelong latent infection. The virus enters the nerve termini innervating peripheral vaginal tissues and is subsequently transported by retrograde to the nucleus of the sensory neurons of dorsal root ganglia (DRG), where it establishes a dormant state within the neuronal cells (8).

More than 80% of HSV-2-seropositive women are unaware of their infection as they never develop any apparent recurrent symptoms. In contrast, the symptomatic women often display sporadic reactivation, leading to recurrent genital lesions and painful blisters that can burst and form ulcers. The virus occasionally reactivates and sheds asymptomatically, even without visible lesions. Such women, being unaware, can contribute significantly to the transmission of the virus, emphasizing the need for an antiviral therapeutic vaccine to prevent or reduce virus reactivation and/or its shedding in the genital tract. In addition, neonatal infections can be severe and result in serious diseases with high rates of morbidity and mortality. Despite the availability of many intervention strategies, such as sexual behavior education, barrier methods, and antiviral drug therapies (e.g., acyclovir and derivatives), eliminating or at least reducing recurrent genital herpes remains a challenge (9–14). Besides, antiviral drugs, such as acyclovir, can neither prevent *de novo* infection (initial infection) nor clear the virus completely. They work primarily in reducing the severity and duration of symptoms by inhibiting viral replication during active outbreaks. An effective antiviral therapeutic vaccine may serve as the best approach to protect from recurrent genital herpetic disease (15). An antiviral therapeutic vaccine stimulates the immune system to target and control an existing infection. Ideally, a therapeutic vaccine for genital herpes would help reduce virus reactivation, limit shedding, and potentially decrease the frequency and severity of recurrent outbreaks.

The acquired immune responses that develop following exposure to the virus are not sufficient for protection against recurrent genital herpes (16–18). More recently, studies are investigating a successful therapeutic vaccine that can boost immune responses stronger and/or different than the acquired immunity induced by the virus (19). In 1988, a study by Myers et al. provided early evidence that a vaccine could reduce the number of recurrent genital herpes outbreaks using the guinea pig model of genital herpes (20). This study demonstrated the efficacy of immunization in reducing the frequency of recurrences (20). In recent years, several sub-clinical experiments have supported these preclinical findings using protein-based subunit vaccines, often administered with a potent adjuvant (a substance that enhances the immune response) (21). Vaccination with these proteins has been found to reduce the rates of recurrent lesions (visible outbreaks) or recurrent shedding by approximately 50% (22).

Over the last two decades, only a single subunit protein vaccine strategy, based on the HSV-2 glycoprotein D (gD), delivered with or without gB, has been tested and retested in clinical trials. Despite inducing strong neutralizing antibodies, this subunit gD ± gB vaccines proved unsuccessful in clinical trials (23). Subsequent studies have

identified other HSV-2 tegument proteins by screening the over 80+ open-reading frames (ORFs) of the HSV-2 152 kb genome with antibodies and T cells from HSV-2 seropositive individuals (10). Aside from three reports, first by our group in 2012 and later by Genocea Biosciences, Inc. in 2014 (24, 25), the comparison of the repertoire of HSV-2 proteins, encoded by the 84 ORFs, recognized by antibodies and T cells from HSV-2-seropositive symptomatic versus asymptomatic individuals is largely unknown. Previously, we found that the ribonucleotide reductase 2 (RR2) protein was frequently and highly recognized by antibodies and T cells in naturally "protected" asymptomatic individuals (24, 26). We later demonstrated that the HSV-2-specific RR2 protein-based subunit therapeutic vaccine elicited a significant reduction in virus shedding and decreased the severity and frequency of recurrent genital herpes lesions. Moreover, the protein boosted the number and function of antiviral tissue-resident memory $CD4^+$ and $CD8^+$ T_{RM} cells, locally within the DRG and vaginal mucocutaneous tissues, leading to better protection against recurrent herpes.

In the present study, we sought to improve protein-based vaccination by combining it with a prime and pull strategy. The strategy involves conventional parenteral vaccination using HSV-2-specific RR2 protein to elicit systemic T cell responses (prime), followed by recruitment of activated T cells via administration of an adenovirus-expressing chemokine or T cell attractant (pull), for those T cells to establish long-term protective immunity. The adeno-associated virus type 8 (AAV8) was used to express the chemokines as it efficiently targets the enteric nervous system in guinea pigs. The immunization results showed that the primary $CD8^+$ T cell responses were of similar magnitudes in the spleen, whereas the frequency and number of $CD8^+$ T cells in the vaginal mucocutaneous tissue were significantly higher in vaccinated mice treated with adenovirus-expressing chemokine as compared with the control immunized mice without the "pull." Furthermore, the action of the chemokine pull was not restricted to the genital mucosa, as $CD8^+$ T cell recruitment was also observed in the DRG. Importantly, the prime and pull strategy conferred near complete protection against the primary challenge of genital HSV-2 infection compared with the prime alone. In this study, we extend this application to therapeutic vaccines and demonstrate that the frequency of recurrent disease and recurrent vaginal shedding is reduced most effectively by the combination of prime (protein vaccine) and pull (adenovirus-expressing chemokine).

MATERIALS AND METHODS

Animals

Female guinea pigs (*Hartley strain*, Charles River Laboratories, San Diego, CA) weighing 275–350 g (5–6 weeks old) were housed at the University of California, Irvine, vivarium.

Vaccine candidate

We used recombinantly expressed 'RR2' protein antigens from HSV2 as RR2 is highly recognized by T cells from naturally protected asymptomatic individuals.

Vector preparation strategy

A schematic representation of the adeno-associated virus type 8 vectors expressing CXCL9, CXCL10, and CXCL11, synthesized by Vector Builder Inc (Chicago, IL, USA), is provided in Fig. S2. The neurotropic AAV-8 vector was selected because (i) it is safe. Clinical trials using AAV8 vectors in gene therapies have shown only mild and transient inflammation while demonstrating clinical benefits: (ii) it is efficient and specific in delivering chemokines to sensory neurons of the DRG, and (iii) it can accommodate up to 4.7 kb of DNA expressing multiple chemokines. The AAV-8 were packaged using the classic triple transfection method and HEK293T cells. Viral particles harvested from cell lysis were further purified and concentrated by cesium chloride (CsCl) gradient

ultracentrifugation. CsCl was removed by dialysis, and the buffer was eventually changed to PBS buffer (pH 7.4) supplemented with 200 mM NaCl and 0.001% pluronic F-68 (10, 15, 27, 28).

Infection and immunization of guinea pigs

Guinea pigs (n = 30) were infected intravaginally with 5×10^5 pfu of HSV-2 (strain MS). The virus was diluted to a final volume of 100 µL using sterile PBS and administered using a butterfly infusion tubing (BD Vacutainer-368656) attached to a 25G pipette tip. Subsequently, the 30 guinea pigs were distributed into five groups, i.e., RR2 + CXCL9 (n = 6), RR2 + CXCL10 (n = 6), RR2 + CXCL11 (n = 6), RR2 alone (n = 6), and non-immunized (n = 6). We performed the power analysis and showed six guinea pigs per group are enough to produce significant results with a power >80%. Additionally, the experiments were replicated twice. Once the acute infection was resolved, latently infected animals were vaccinated intramuscularly twice in the right hind calf muscle on day 15 and day 25 post-infection. We used a BD 1 mL tuberculin syringe 25G for intramuscular injection of the RR2 vaccine. Animals were immunized on day 15 post-infection with RR2 protein (20 µg/guinea pig) mixed with 100 µg/guinea pig of the oligonucleotide (5′-TCGTCG TTGTCGTTTTGTCGTT-3′) (Trilink Inc., Santa Fe Springs, CA) and 150 µg/guinea pig of alum (Alhydrogel, Accurate Chemical and Scientific Corp., Westbury, NY) adjuvants. The components were diluted to a final 100 µL/guinea pig volume using dPBS. The vaccine components were mixed and kept overnight on a rocker at 4°C. Mock-immunized guinea pigs were received 100 µg CpG/guinea pig and 150 µg alone.

One week later of immunization with HSV-2 RR2 protein, on day 32 of experimental timeline (Fig. 3A), three different groups of guinea pigs were treated intravaginally with AAV8 containing (i) CXCL9 (1×10^{10} GC), (ii) CXCL10 (1×10^{10} GC), and (iii) CXCL11 (1×10^{10} GC). While the other HSV-2 RR2 protein-alone immunized group was treated with AAV8-GFP vector. Mock-vaccinated guinea pigs were used as negative control and received Alum + CpG adjuvants alone followed by AAV8-GFP vector (Mock).

Monitoring of recurrent HSV-2 disease in guinea pigs

Following intravaginal HSV-2 infection, the female guinea pigs display primary acute lesions within 4–5 days of viral inoculation. The acute genital lesions usually resolve within 2 weeks post-infection, and the virus goes into latency in the neurons of DRG. Starting day 28–30 post-infection, the virus spontaneously reactivates from latently infected neurons of DRG, and sporadic reoccurrences start and go over the ensuing months (recurrent phase). Some herpes latently infected symptomatic female guinea pigs exhibit recurrent genital herpetic disease with symptoms similar to recurrent genital herpes in women infected with HSV-2 (symptomatic shedding). Other latently infected asymptomatic female guinea pigs intermittently shed virus without developing visible genital herpetic lesions (asymptomatic shedding). The clinical and subclinical shedding of HSV-2 DNA can be determined by quantitative PCR from vaginal swabs collected during the recurrent phase. Following RR2 immunization, with and without chemokine treatment, the female guinea pigs were observed for 80 days post-infection, for recurrent herpetic disease by examining the severity and size of vaginal herpetic lesions. The lesions were recorded daily from each animal on an arbitrary scale of 0–4, where 0 reflects no disease, 1 reflects redness, 2 reflects a single lesion, 3 reflects coalesced lesions, and 4 reflects ulcerated lesions.

Bulk RNA sequencing on sorted $CD8^+$ T cells

RNA was isolated from 100,000 sorted $CD8^+$ T cells using the Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA) according to the manufacturer's instructions. RNA concentration and integrity were determined using the Agilent 2100 Bioanalyzer. Sequencing libraries were constructed using TruSeq Stranded Total RNA Sample Preparation Kit (Illumina, San Diego, CA). Briefly, rRNA was first depleted using the RiboGone rRNA

Removal Kit (Clonetech Laboratories, Mountain View, CA) before the RNA was fragmented, converted to double-stranded cDNA and ligated to adapters, amplified by PCR, and selected by size exclusion. Following quality control for size, quality, and concentrations, libraries were multiplexed and sequenced to paired-end 100 bp sequencing using the Illumina HiSeq 4000 platform.

Differential gene expression analysis

Differentially expressed genes (DEGs) were analyzed using integrated differential expression and pathway analysis tools (29). These system tools seamlessly connect 63 R/Bioconductor packages, 2 web services, and comprehensive annotation and pathway databases for guinea pigs. The expression matrix of DEGs was filtered and converted to Ensembl gene identifiers, and the preprocessed data were used for exploratory data analysis, including k-means clustering and hierarchical clustering. The pairwise comparison of immunized and non-immunized guinea pigs was performed using the DESeq2 package with a single-Andover rate threshold (<0.5 and fold change and >1.5).

Moreover, a hierarchical clustering tree and network of enriched GO/KEGG terms were constructed to visualize the potential relationship. Gene set enrichment analysis (GSEA) method was performed to investigate the related signal pathways activated among protective and non-protective groups. The parametric gene set enrichment analysis method was applied based on data curated in gene ontology and KEGG. Pathway significance cutoff with a false discovery rate ≥0.2 was used (29, 30).

Real-time qPCR for HSV-2 quantification from vaginal swabs and dorsal root ganglia

Vaginal swabs were collected daily using a Dacron swab (type 1; Spectrum Laboratories, Los Angeles, CA) starting from day 35 until day 65 post-challenge. Individual swabs were transferred to a 2-mL sterile cryogenic vial containing 1-mL culture medium and stored at −80°C until use. On day 80 post-challenge, 12 lower lumbar and sacral dorsal root ganglia per guinea pig were collected by cutting through the lumbar end of the spine. DNA was isolated from the collected vaginal swab and DRG of guinea pigs by using DNeasy Blood and Tissue Kits (Qiagen). The presence of HSV-2 DNA was quantified by real-time PCR (StepOnePlus Real-Time PCR System) with 50–100 ng vaginal swab DNA or 250 ng of DRG DNA. HSV-2 DNA copy number was determined using purified HSV-2 DNA (Advanced Biotechnologies, Columbia, MD) and based on a standard curve of the C_T values that were generated with 50,000, 5,000, 500, 50, and 5 copies of DNA and run in triplicates. Samples with <150 copies/mL by 40 cycles or only positive in one of two wells were reported as negative. Primer and probe sequences for HSV-2 Us9 were primer forward, 5′-GGCAGAAGCCTACTACTCGGAAA-3′, and reverse 5′-CCATGCGCACGAGGAAGT -3′, and probe with reporter dye 5′-FAM-CGAGGCCGCCAAC-MGBNFQ-3′ (FAM, 6-carboxyfluorescein). All reactions were performed using TaqMan gene expression master mix (Applied Biosystems), and data were collected and analyzed on StepOnePlus real-time PCR system.

Splenocyte isolation

Spleens were harvested from guinea pigs at day 80 post-infection. Spleens were placed in 10 mL of cold PBS with 10% fetal bovine serum (FBS) and 2× antibiotic-antimycotic (Life Technologies, Carlsbad, CA). Spleens were minced finely and sequentially passed through a 100-µm mesh and a 70-µm mesh (BD Biosciences, San Jose, CA). Cells were then pelleted via centrifugation at 400 × *g* for 10 minutes at 4°C. Red blood cells were lysed using a lysis buffer and washed again. Isolated splenocytes were diluted to 1×10^6 viable cells per milliliter in RPMI media with 10% (vol/vol) FBS and 2× antibiotic-antimycotic. Viability was determined by Trypan blue staining.

Isolation of lymphocytes from the guinea pig's vaginal mucosa and dorsal root ganglia

Vaginal mucosa and DRG were minced into fine pieces on a Petri Dish using a fine scalpel. The tissue was then subjected to collagenase treatment (7 mg/mL) and allowed to digest at 37°C for 1 hour on a rocker. After 1 hour, suspensions of the digested tissue were passed through a 100 µm cell strainer, followed by centrifugation and washing with RPMI media containing 10% FBS. Lymphocytes in the cell pellets were then suspended in 40% Percoll, layered on top of 70% Percoll, and centrifuged at 900 × *g* at room temperature for 30 minutes with the brake-off. The lymphocytes at the interface layer between 40% and 70% Percoll layers were harvested, washed with three volumes of RPMI, and spun down at 740 × *g*.

Flow cytometry analysis

Mononuclear cells from vaginal mucosa and splenocytes were analyzed by flow cytometry using the following antibodies: mouse anti-guinea pig CD8 (Cat # MCA752F, Bio-Rad Laboratories), mouse anti-guinea pig CD4 (Cat # MCA749PE, Bio-Rad Laboratories), anti-mouse CRTAM (clone 11–5, Cat # 142008, Biolegend), anti-mouse/human CD44 (clone IM7, Cat # 103028, Biolegend), anti-mouse CD69 (clone H1.2F3, Cat # 552879, BD Biosciences), anti-mouse CXCR3 (clone CXCR3-173, Cat # 126531, Biolegend), and anti-mouse CD103 (clone 2E7, Cat # 121442, Biolegend). For surface staining, mAbs against various cell markers were added to a total of 1×10^6 cells in phosphate-buffered saline containing 1% FBS and 0.1% sodium azide [fluorescence-activated cell sorter (FACS) buffer] and left for 45 minutes at 4°C. At the end of the incubation period, the cells were washed twice with FACS buffer. A total of 100,000 events were acquired by the LSRII (Becton Dickinson, Mountain View, CA), followed by analysis using FlowJo version 10 software (TreeStar, Ashland, OR). The overall gating strategy used to select the stained population is provided in Fig. S2B.

Statistical analyses

The groups of guinea pigs were compared using the analysis of variance (ANOVA) applied using multiple comparison-based Tukey test and Student's *t*-test with GraphPad Prism version 10.1.0 (La Jolla, CA). The differences between the groups of guinea pigs were then identified and expressed as the mean ± SD. Results were considered statistically significant at $P < 0.05$.

RESULTS

CXCL11/CXCR3 axis for T cell activation: a target for novel "prime-pull" therapeutic herpes vaccine

Guinea pigs (n = 12) were infected intravaginally with 5×10^5 pfu of HSV-2 (strain MS). Once the acute infection was resolved, latently infected animals were vaccinated intramuscularly twice, on days 15 and 25 post-infection, with individual HSV-2 antigen RR2 emulsified in Alum + CpG adjuvants (n = 6). Mock-vaccinated guinea pigs, which received Alum + CpG adjuvants alone, were used as negative control (Mock) (n = 6). Using bulk RNA sequencing, we detected differential expression of 752 (upregulated = 427, downregulated = 325) genes in tissue-resident $CD8^+$ T cells FACS-sorted from vaginal mucosa of HSV-2-immunized and non-immunized guinea pigs (Fig. 1A through C). With gene set enrichment analysis on these differentially expressed genes, we found T cell activation (P = 0.02, logFC = 4.12), T cell proliferation (P = 0.02, logFC = 3.68), TCR signaling (P = 0.01, logFC = 2.15), and cytokine secretion (P = 0.01, logFC = 2.01) pathways to be upregulated in immunized group of guinea pigs (Fig. 1D). We detected a significant up-regulation of the gene for T cells attracting chemokine receptors in HSV-specific $CD8^+$ T_{RM} cells from an immunized group of guinea pigs suggestive of a heightened activation of T cell-attracting chemokine receptors may lead to increased infiltration/retention of protective antiviral T_{RM} cells observed in the VM of

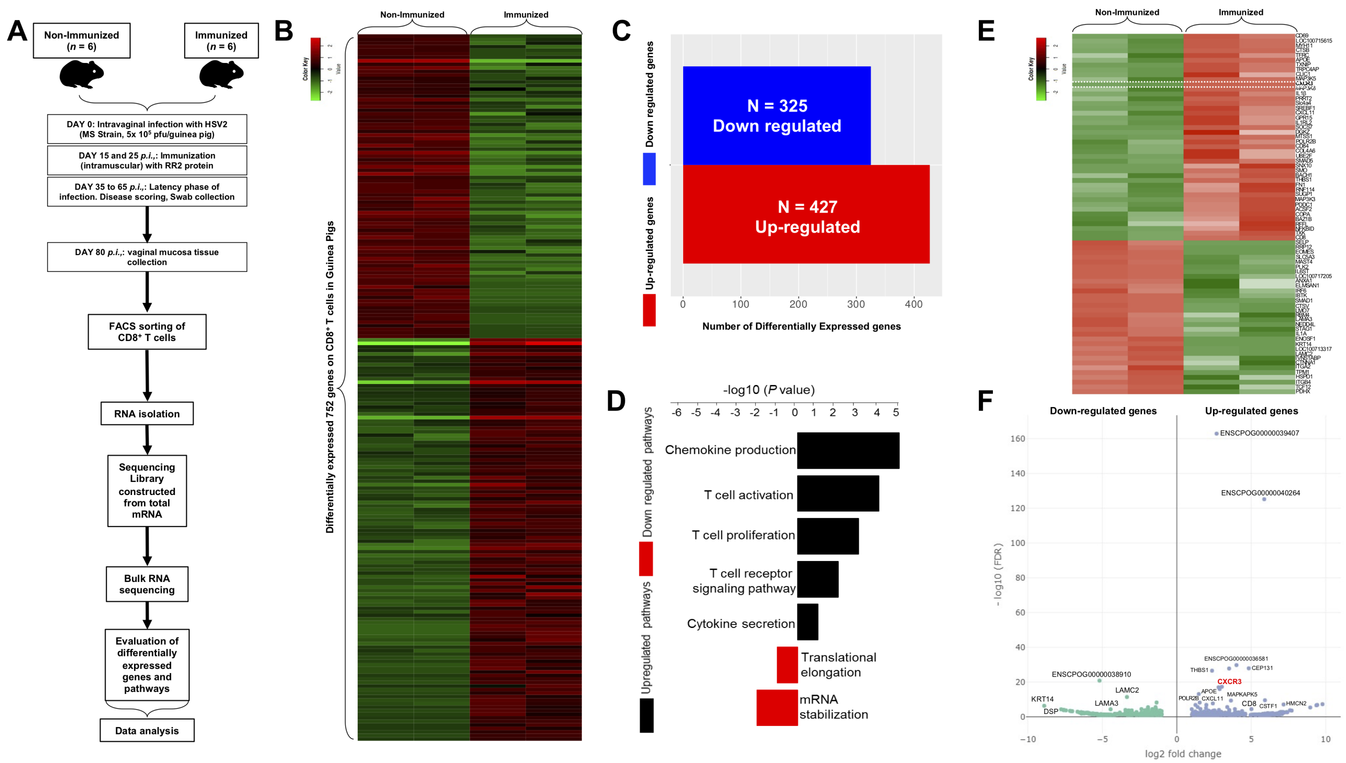

FIG 1 Higher frequency of tissue-resident CD8⁺ T cells in healed vaginal mucosal cells of protected guinea pigs is associated with stimulation of the CXCL11/CXCR3 axis. (A) Guinea pigs (*n* = 12) were infected intravaginally with 5 × 10^5 pfu of HSV-2 (strain MS). Once the acute infection was resolved, latently infected animals were divided into two groups. The first group of guinea pigs was vaccinated intramuscularly twice, on days 15 and 25 post-infection with HSV-2 RR2 antigen emulsified in Alum + CpG adjuvants (*n* = 6). The second group of guinea pigs received Alum + CpG adjuvants alone and used as negative control (Mock vaccinated) (*n* = 6). (B) Heatmap showing differential gene expression analysis in guinea pig $CD8^+$ T cells. (C) Histogram showing the distribution of significantly differentially expressed genes among immunized and non-immunized guinea pigs. (D) GSEA analysis showing differentially expressed immunological pathways in $CD8^+$ T cells associated with HSV-2 infection in guinea pigs. (E) GSEA analysis showing differentially upregulated genes in chemokine production pathway in immunized guinea pigs. Upregulation of CXCR3 observed among guinea pigs infected with HSV-2 in the data obtained from bulk RNA sequencing. (F) Volcano plot showing upregulated m-RNA fold change expression for CXCR3 and other immune cell markers in immunized guinea pigs challenged with HSV-2.

the immunized group of guinea pigs. The gene found to be significantly upregulated among immunized guinea pigs was CXCR3 (*P* = 4.79E−18, logFC = 3.02) (Table S1) (Fig. 1E and F), which is a receptor for CXCL9, CXCL10, and CXCL11. These results suggest that CXCL9/10/11-CXCR3 activates tissue-resident $CD8^+$ T cell responses locally in the genital tissues.

DRG samples were collected from both immunized and non-immunized infected guinea pigs and subjected to mRNA expression, immunostaining (IHC), and western blotting for CXCR3 and corresponding chemokines CXCL9, CXCL10, and CXCL11. Results indicated a significantly higher mRNA expression of CXCR3 in the infected-immunized group compared to the infected non-immunized group (Fig. 2A). Further higher frequency of $CXCR3^+CD8^+$ T cells was detected by FACS from DRG of immunized and non-immunized guinea pigs (Fig. 2B). The IHC and western blot data also suggested a higher magnitude of CXCR3, CXCL9, CXCL10, and CXCL11 in the DRG tissue of immunized animals subjected to HSV-2 challenge (Fig. 2C and D).

Therapeutic immunization of HSV-2-infected guinea pigs with vaccine candidate RR2 and treatment with adenovirus containing CXCL11 protect better against recurrent genital disease

AAV vectors are useful vehicles for delivering transgenes to infected tissues and organs *in vivo*. Recently, there has been much progress in delivering AAV vectors

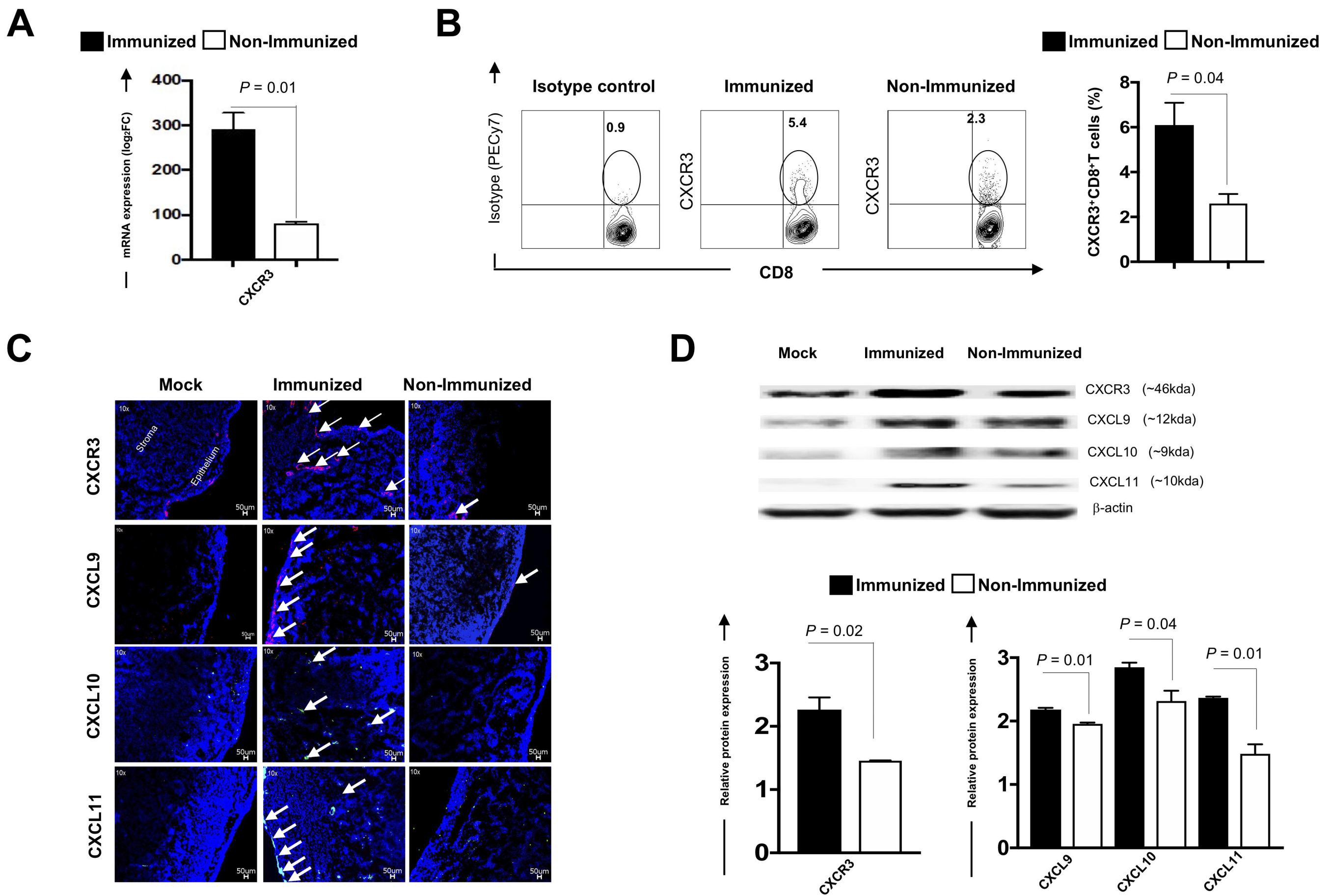

FIG 2 Frequent HSV-specific tissue-resident CXCR3$^+$CD8$^+$ T cells in DRG tissue of immunized guinea pigs are associated with stimulation of the CXCL9, CXCL10, CXCL11/CXCR3 axis. (A) Expression profile of CXCR3 gene detected in DRG of immunized and non-immunized guinea pigs, (B) frequency of CXCR3$^+$CD8$^+$ T cells detected by FACS from DRG of immunized and non-immunized guinea pigs, and (C) expression levels of CXCR3, CXCL9, CXCL10, and CXCL11 proteins detected by IHC (indicated by white arrows) and (D) by western blot in DRG-resident CD8$^+$ T cells from immunized versus non-immunized guinea pigs.

to sensory neurons following application to the peripheral epithelium. We compared AAV1-GFP, AAV8-GFP, and AAV9-GFP vectors in HSV-2-infected guinea pig and discovered the AAV8-GFP vector's superiority at 4 weeks after intra-vaginal inoculation (Fig. S1A). Moreover, the neurotropic AAV8 expressing GFP under the neurotropic human synapsin 1 gene promoter (hSYN) (AAV8-hSYN-eGFP-hSYN- vector) resulted in higher GFP expression in DRG and VMC tissue of guinea pigs (Fig. S1B and C). We hypothesized that the AAV8 expressing guinea pig T cell-attracting chemokines such as CXCL9, CXCL10, and CXCl11 under the neurotropic human synapsin 1 gene promoter (AAV8-hSYN-eGFP-hSYN- gpCXCL9/10/11 vector) could result in higher expression of these genes in the HSV-2-infected DRG tissues (pull). The presence of chemoattractant at and near DRG and VM tissue could increase the number of DRG- and VM-resident CD4$^+$ and CD8$^+$ T cells and thus reduce the severity and rate of recurrent genital herpes. Therefore, AAV8 expressing CXCL9, CXCL10, or CXCL11 was used to treat guinea pigs post-immunization. Detailed structures of the AAV8-expressing chemokine vectors are provided in Fig. S2A.

Guinea pigs (n = 30) were infected intravaginally with 5 × 10^5 pfu of HSV-2 (strain MS) (Fig. 3A). Once acute infection was resolved, latently infected animals were randomly divided into five groups (n = 6) and then vaccinated twice intramuscularly on days 15 and 25 post-infection with HSV-2 protein RR2 emulsified in Alum + CpG adjuvants. One week later, three groups were treated with an adenovirus containing CXCL9, CXCL10, and CXCL11 separately, while the other group remained untreated. Mock-vaccinated

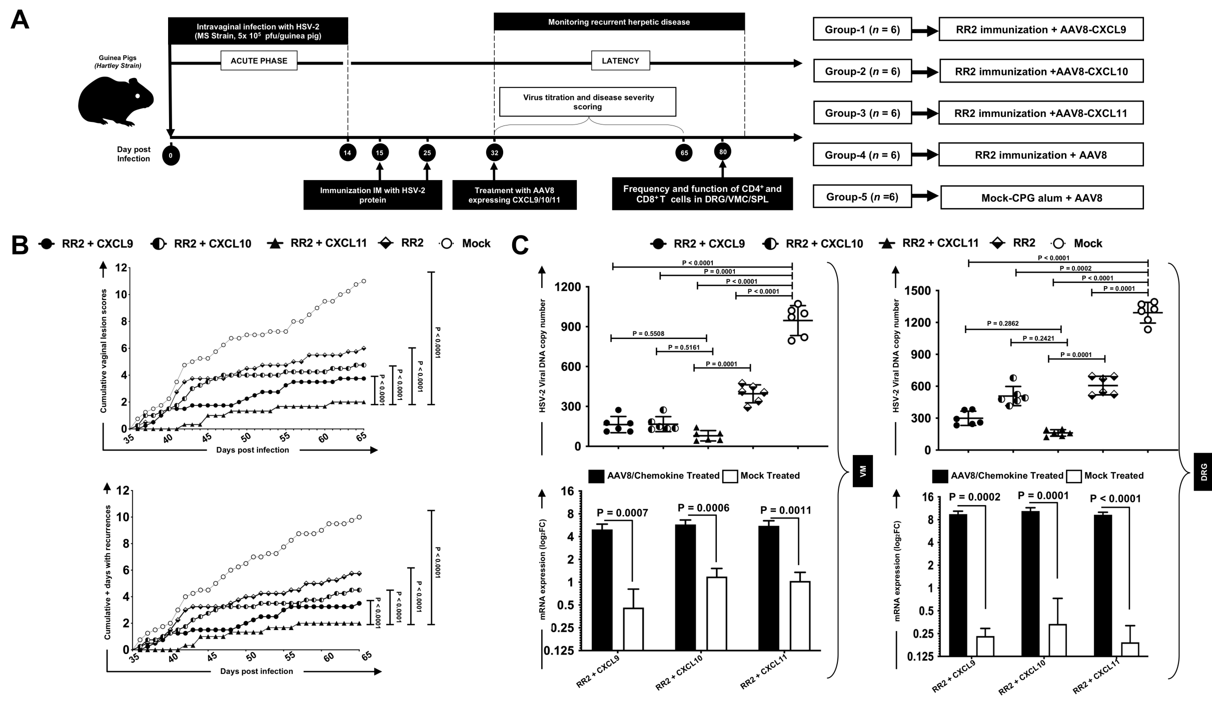

FIG 3 Protection against recurrent genital herpes infection and disease in HSV-2-infected guinea pigs following therapeutic prime/pull vaccination with the RR2 protein and adenovirus-expressing chemokine CXCL9/10/11. (A) Timeline of HSV-2 infection, immunization, immunological, and virological analyses. Guinea pigs (n = 30) were infected intravaginally on day 0 with 5 × 10^5 pfu of HSV-2 (strain MS). Once acute infection was resolved, the remaining latently infected animals were randomly divided into five groups (n = 6) and then vaccinated intramuscularly twice, on day 15 and day 25 post-infection, with 10 µg of the HSV-2 protein-based subunit vaccine-RR2 emulsified in Alum + CpG adjuvants. One Mock-vaccinated guinea pig that received Alum + CpG adjuvants alone was used as negative control (*Mock*). One week later, on day 32, one group was treated with an adenovirus-expressing CXCL9, second group was treated with an adenovirus-expressing CXCL10, third group was treated with an adenovirus-expressing CXCL11, while the other groups remained untreated (RR2 alone). From day 35 to day 80 post-infection (i.e., from day 10 to day 55 after final immunization), vaccinated and non-vaccinated animals were observed daily for (i) the severity of genital herpetic lesions scored on a scale of 0–4, and pictures of genital areas taken; and (ii) vaginal swabs were collected daily from day 35 to day 65 post-infection (i.e., from day 10 to day 40 after final immunization) to detect virus shedding and to quantify HSV-2 DNA copy numbers. (B) Top panel: cumulative scoring of vaginal lesions observed during recurrent infection; bottom panel: cumulative positive days with recurrent genital lesions. The severity of genital herpetic lesions was scored on a scale of 0–4, where 0 reflects no disease, 1 reflects redness, 2 reflects a single lesion, 3 reflects coalesced lesions, and 4 reflects ulcerated lesions. (C) Top left panel: HSV-2 DNA copy numbers detected in the VM of each vaccinated and mock-vaccinated guinea pig group; top right panel: HSV-2 DNA copy numbers detected in the DRG of each vaccinated and mock-vaccinated guinea pig group; bottom left panel: expression profile of CXCL9, CXCL10, CXCL11 genes detected in VM of AAV8-expressing chemokines in comparison to mock-treated guinea pigs; bottom right panel: expression profile of CXCL9, CXCL10, CXCL11 genes detected in DRG of AAV8-expressing chemokines in comparison to mock-treated guinea pigs.

guinea pigs (n = 6), who received alum plus CpG adjuvants alone, were used as negative controls. Starting on day 35 until day 65, the guinea pigs were observed and scored regularly for genital lesions. RR2-vaccinated animals treated with chemokine and RR2-alone-vaccinated animals exhibited significantly lower cumulative vaginal lesions (Fig. 3B, left panel) and an overall significant reduction in cumulative positive days of recurrence compared to the mock-vaccinated controls (Fig. 3B, right panel). RR2-vaccinated animals treated with CXCL11 displayed lowest cumulative vaginal lesions and an overall reduced cumulative positive days of recurrence compared to the other vaccinated and chemokine-treated animals (Fig. 3B).

On day 80 post-infection, the VM and DRG were harvested from the guinea pigs, and the HSV-2 DNA copy number was determined by qPCR. RR2/chemokine-treated and RR2-alone-vaccinated guinea pigs exhibited lower HSV-2 DNA copy numbers in the VM (Fig. 3C, top left panel) and DRG (Fig. 3C, top right panel) in comparison to mock-vaccinated controls, which were associated with a significant reduction in cumulative virus vaginal shedding in the vaccinated group as compared to the mock-vaccinated

control group. Furthermore, more than twofold gene expression was observed for CXCL9 (P = 0.0007), CXCL10 (P = 0.0006), CXCL11 (P = 0.0011) genes in guinea pigs treated with AAV8-expressing chemokines in comparison to mock-treated guinea pigs (Fig. 3C, bottom left panel). Similarly, significantly high log2fold change expression was observed in DRG tissue for CXCL9 (P = 0.0002), CXCL10 (P = 0.0001), CXCL11 ($P < 0.0001$) genes of guinea pigs treated with AAV8-expressing chemokines in comparison to mock-treated guinea pigs (Fig. 3C, bottom right panel). The groups of vaccinated and mock-vaccinated guinea pigs were compared by the ANOVA using multiple comparison-based Tukey test and Student's *t*-test. A significant reduction in both cumulative vaginal lesions and cumulative positive days of recurrences was observed in the VM of RR2/CXCL11-prime/pull-vaccinated guinea pigs compared to RR2/CXCL9-prime/pull-vaccinated guinea pigs, to RR2/CXCL10-prime/pull-vaccinated guinea pigs, and RR2 alone ($P < 0.0001$) (Fig. 3B). In addition, relatively lower viral copy numbers were observed in the DRG and VM of RR2/CXCL11-prime/pull-vaccinated guinea pigs compared to RR2/CXCL9-prime/pull-vaccinated guinea pigs, to RR2/CXCL10-prime/pull-vaccinated guinea pigs, and RR2 alone (Fig. 3C). Altogether, these results indicate that therapeutic immunization with RR2 and treatment with AAV8 containing CXCL11 protected HSV-2-seropositive guinea pigs against recurrent genital herpes infection and disease.

Therapeutic prime/pull vaccination of HSV-2-infected guinea pigs with RR2 protein/CXCL11 increased the frequencies of tissue-resident CD4$^+$ and CD8$^+$ T cells

Subsequently, we determined the frequencies of CD4$^+$ and CD8$^+$ T cells in the spleen (SPL), vaginal mucosa, and dorsal root ganglia. Guinea pigs were infected and immunized, as detailed above. On day 80, after the second and final immunization treatment with AAV-8 containing CXCL9/10/11, vaccinated alone and control animals were euthanized, and the frequencies of SPL, DRG, and VM tissue-resident CD4$^+$ and CD8$^+$ T cells were detected by fluorescence-activated cell sorting. There were no significant differences observed in the frequencies of CD4$^+$ and CD8$^+$ T cells in the SPL of vaccinated guinea pigs compared to the mock-vaccinated group. However, significantly higher frequencies of CD4$^+$ and CD8$^+$ T cells were induced in the RR2-vaccinated group following treatment with the chemokine, especially CXCL11, compared to RR2-vaccinated group alone and compared to those with the mock-vaccinated group (i.e., adjuvant alone) in the VM and DRG tissues (Fig. S3A and B).

Therapeutic prime/pull vaccination of HSV-2-infected guinea pigs with RR2 protein followed by CXCL11 treatment protected by bolstering effector and CXCR3 responses

We next determined the expression of CXCR3 on the CD8$^+$ T cells. On day 80, after the second and final therapeutic prime/pull vaccination and treatment with chemokines, guinea pigs were euthanized, and single-cell suspensions from the VM (Fig. 4A), DRG (Fig. 4B), and spleen (Fig. 4C) tissue were obtained, and the effector function of SPL, VM-resident, and DRG-resident CD8$^+$ T cells was analyzed by observing the expression of CXCR3 on CD8$^+$ T cells by FACS analysis. A non-significant difference was observed in the frequencies of CXCR3$^+$ CD8$^+$ T cells in the SPL of guinea pigs vaccinated with RR2 and treated with chemokines and RR2 protein alone compared to the mock-vaccinated group (Fig. 4C). A higher frequency of CXCR3$^+$CD8$^+$ T cells was induced by the RR2-vaccinated group treated with chemokines, followed by vaccination with the RR2 alone compared to those with the mock-vaccinated group (i.e., adjuvant alone) in the VM and DRG tissues (Fig. 4A and B). Relatively higher frequency of CXCR3$^+$CD8$^+$ T cells was found in CXCL11, in comparison to CXCL9- and CXCL10-treated groups.

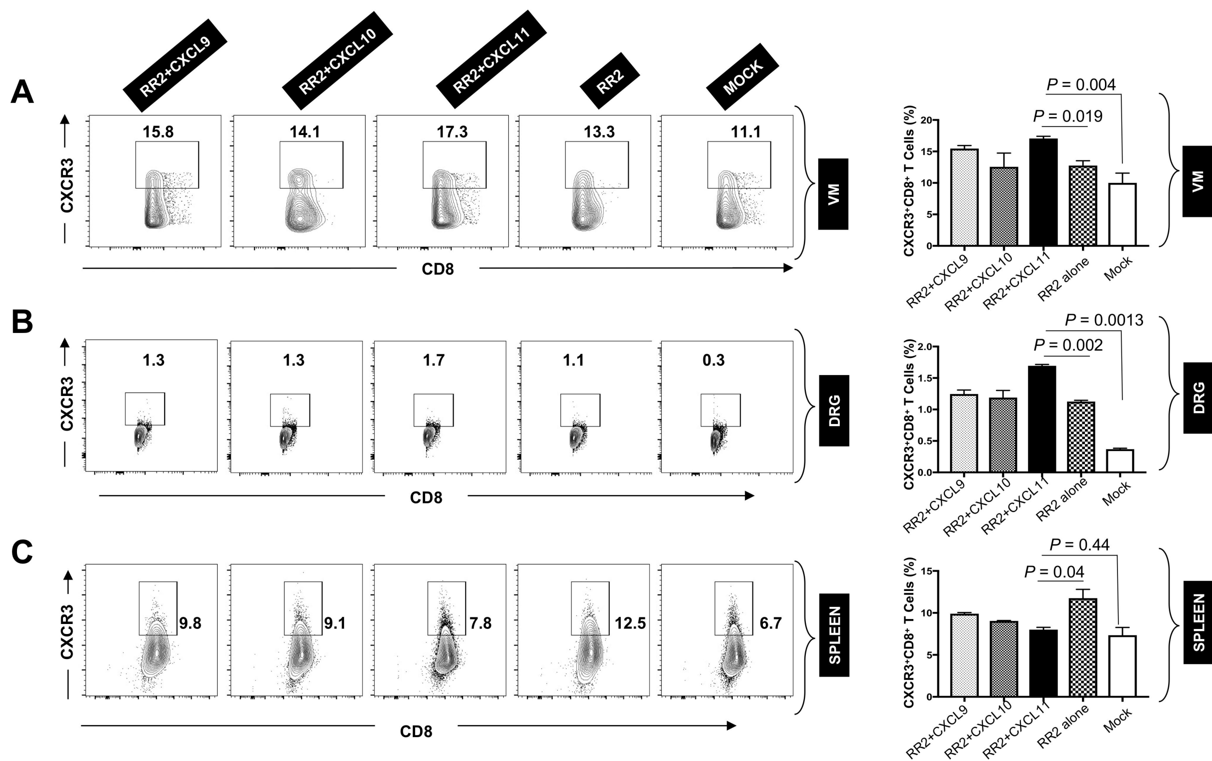

FIG 4 Increased frequencies of CD8$^+$CXCR3$^+$ T cells in the vaginal mucosa and DRG of HSV-2 infected in guinea pigs following therapeutic "prime-pull" vaccination with RR2/CXCL11. (A) Eighty days post-infection, guinea pigs were euthanized, and single-cell suspensions from the spleen, vaginal mucosa, and DRG were obtained after collagenase treatment. The SPL, VM, and DRG cells were stained for CD8$^+$ and CXCR3$^+$ expressing T cells and then analyzed by FACS. Representative FACS data (left panel) and average frequencies (right panel) of CD8$^+$ CXCR3$^+$ T cells detected in the (A) VM, (B) DRG, and (C) spleen of RR2/CXCL9/10/11- and RR2-alone-vaccinated and mock-vaccinated animals. Cells were analyzed using a BD LSR Fortessa Flow Cytometry system with 4 $\times$ 10^5 events. The indicated *P* values performed by *t*-test for significance show statistical significance between prime/pull-vaccinated versus RR2-alone and prime/pull-vaccinated versus mock-vaccinated control groups.

Therapeutic prime/pull vaccination of HSV-2-infected guinea pigs with RR2 protein followed by CXCL11 treatment efficiently generates CXCR3-dependent total memory T cells in the VM and DRG

We next sought to determine the association of various protection parameters with effector memory in the vaginal mucocutaneous and DRG tissue of HSV-2-infected RR2 + chemokine and RR2-alone-vaccinated guinea pigs. On day 80, after the second and final therapeutic immunization and treatment with chemokines, guinea pigs were euthanized, and single-cell suspensions from the SPL, VM, and DRG tissues were obtained. The effector function of SPL-, VM-, and DRG-specific CD8$^+$ T cells was analyzed by observing the expression of CD44 on CD8$^+$ T cells by FACS. Significantly higher frequencies of CD44$^+$ T cells were induced by RR2-vaccinated group treated with chemokines, followed by vaccination by RR2 alone compared to those with the mock-vaccinated group (i.e., adjuvant alone) in the VM tissues and DRG (Fig. 5A through C). Relatively higher frequency of CD8$^+$CD44$^+$ T cells was found in CXCL11, in comparison to CXCL9- and CXCL10-treated groups.

Therapeutic prime/pull vaccination of HSV-2-infected guinea pigs with RR2 protein followed by CXCL11 treatment efficiently generates CXCR3-dependent tissue-resident memory in the VM and DRG

We next determined the association of various protection parameters (i.e., virus shedding and severity and frequency of recurrent genital herpes lesions) with tissue-resident

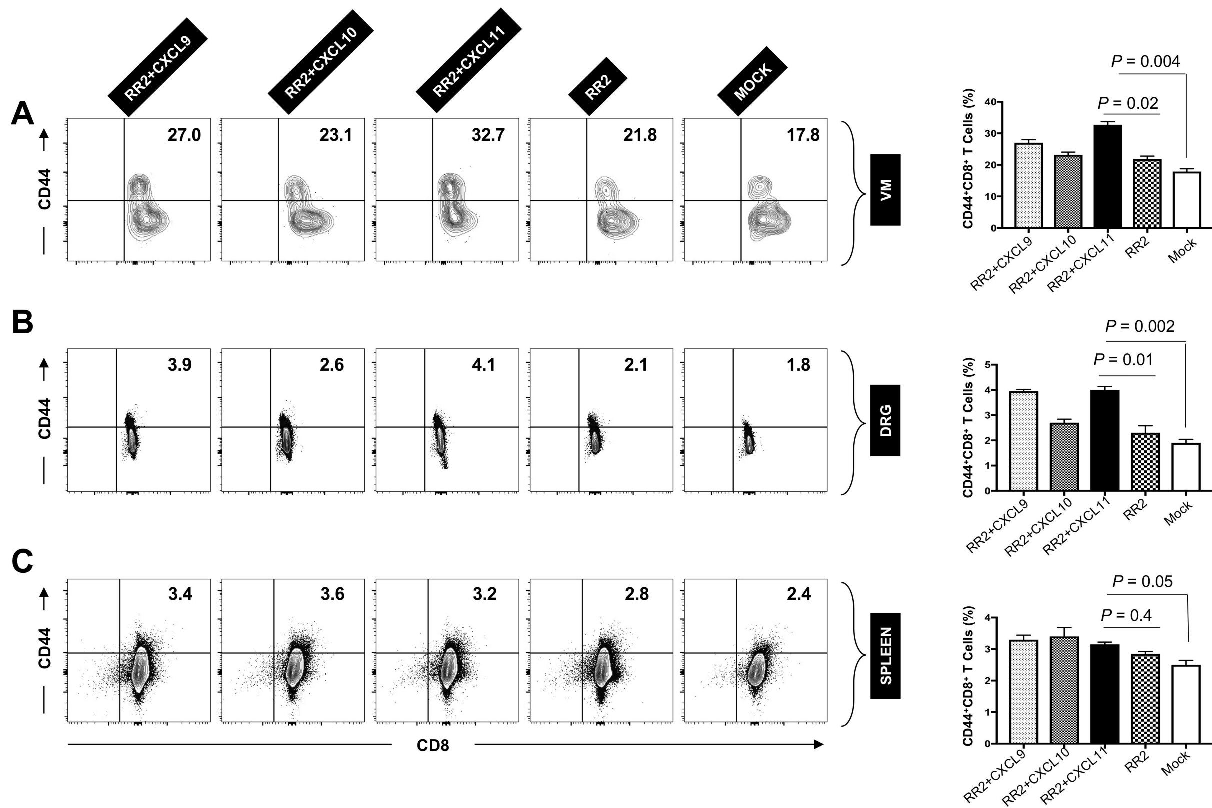

FIG 5 Therapeutic prime/pull vaccination of HSV-2-infected guinea pigs with RR2 protein followed by AAV-8 expressing chemokine, especially CXCL11 treatment efficiently bolsters $CD44^{+}$ T cells. Eighty days post-infection, guinea pigs were euthanized, and single-cell suspensions from the spleen, vaginal mucosa, and DRG were obtained after collagenase treatment. The SPL, VM, and DRG cells were stained for $CD44^{+}$ expressing T cells and then analyzed by FACS. Representative FACS data (left panel) and average frequencies (right panel) of $CD44^{high}$ $CD8^{+}$ T cells were detected in the (A) VM, (B) DRG, and (C) spleen of RR2/CXCL9/10/11- and RR2-alone-vaccinated and mock-vaccinated animals. Higher frequencies of $CD44^{high}$ $CD8^{+}$ T cells were detected in the (A) VM and (B) DRG of prime/pull-vaccinated guinea pigs, especially the RR2 + CXCL11 chemokine-treated group. The indicated *P* values performed by *t*-test for significance show statistical significance between "prime-pull"-vaccinated versus RR2-alone and prime/pull-vaccinated versus mock-vaccinated control group.

memory that resides at the vaginal mucocutaneous and DRG tissue of HSV-2-infected RR2 + chemokine and RR2-vaccinated guinea pigs. On day 80, after the second and final therapeutic immunization and treatment with chemokine, guinea pigs were euthanized, and single-cell suspensions from the SPL, VM, and DRG tissues were obtained. The effector function of SPL-resident, VM-resident, and DRG-resident $CD8^{+}$ T cells was analyzed using both production of CD69 and CD103 expression by FACS. No significant difference was observed in the frequencies of $CD69^{+}CD103^{+}CD8^{+}$ T cells in the SPL of guinea pigs vaccinated with RR2 and treated with CXCL11 and RR2 protein alone compared to the mock-vaccinated group. However, significantly higher frequencies of $CD69^{+}CD103^{+}CD8^{+}$ T cells were induced by RR2-vaccinated group treated with CXCL11, followed by CXCL9, CXCL10, and RR2 alone compared to those with the mock-vaccinated group (i.e., adjuvant alone) in the VM and DRG tissues (Fig. 6A and B).

Induced protection from HSV-2 infection following therapeutic prime/pull vaccination with RR2 and treatment with adenovirus containing CXCL11 is associated with more functional tissue-resident IFN-γ^{+}CRTAM^{+} $CD8^{+}$ T cells

We next compared the function of $CD8^{+}$ T cells in the SPL, VM, and DRG of HSV-2-infected guinea pigs following therapeutic prime/pull vaccination with RR2 and treatment with chemokine. On day 80, after the second and final therapeutic immunization and treatment with chemokines, guinea pigs were euthanized, single-cell suspensions from

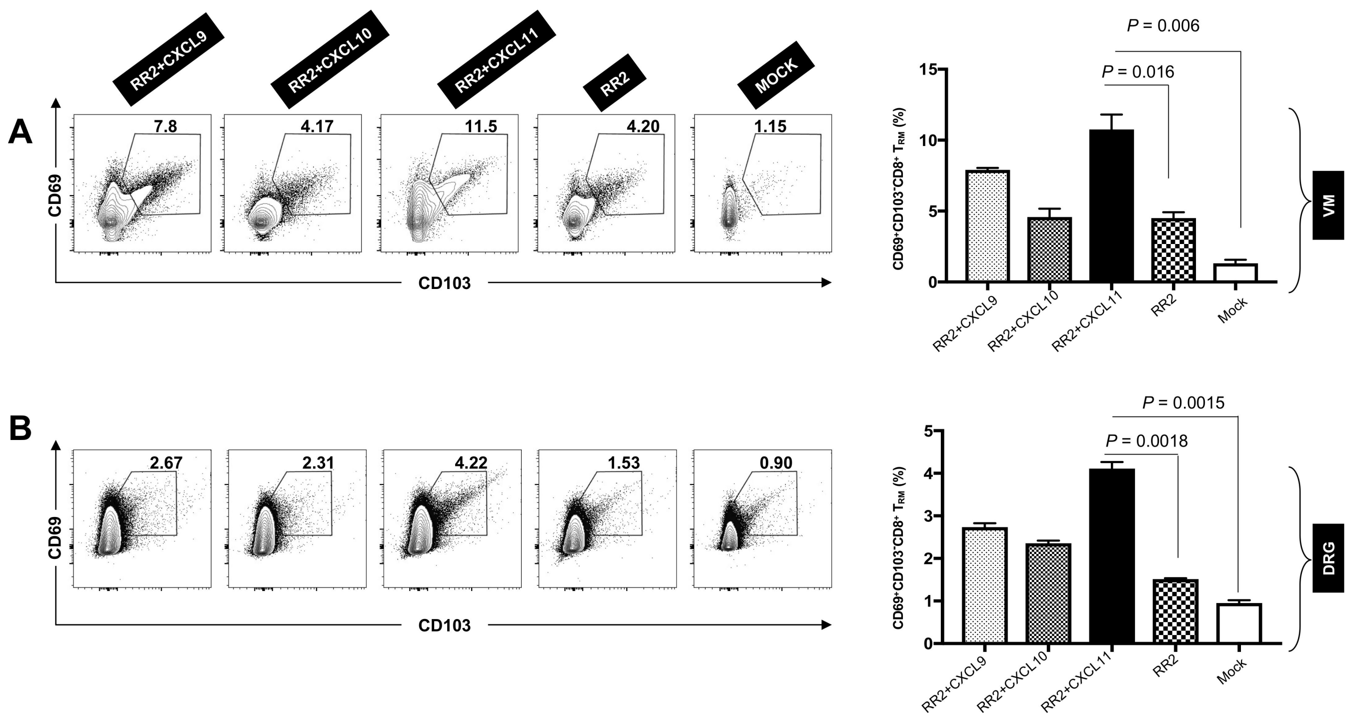

FIG 6 Therapeutic prime/pull vaccination of HSV-2-infected guinea pigs with RR2 protein followed by adenovirus-expressing chemokine, especially CXCL11, efficiently bolsters tissue-resident memory CD69$^+$CD103$^+$CD8$^+$ T_{RM} cells in the VM and DRG of guinea pigs. (A) Single-cell suspensions obtained post-infection and immunization from VM and DRG were stained for memory markers CD69, CD103, and CD8 and analyzed by FACS. Representative FACS data (left panel) and average frequencies (right panel) of CD69$^+$CD103$^+$CD8$^+$ T cells were detected in the (A) VM and (B) DRG of RR2/CXCL9/10/11- and RR2-alone-vaccinated and mock-vaccinated animals. Higher frequencies of CD44^{high}CD62L^{low} CD8$^+$ T_{EM} were detected in the VM (A) and DRG (B) of prime/pull-vaccinated guinea pigs, especially the RR2$^+$CXCL11 chemokine-treated group.

the SPL, VM, and DRG tissues were obtained, and the function of SPL, VM-resident, and DRG-resident CD8$^+$T cells was analyzed using both production of IFN-γ and CRTAM expression by FACS. We detected a non-significant difference in the frequencies of IFN-γ-producing CD8$^+$ T cells in the SPL of guinea pigs that were vaccinated with "RR2 + chemokine" and RR2 protein compared to the mock-vaccinated group (Fig. 7A and C). However, significantly high frequencies of IFN-γ-producing CD8$^+$ T cells were observed to be induced by the RR2-vaccinated group treated with chemokines, followed by the RR2-alone-vaccinated group compared to those with the mock-vaccinated group in the VM and DRG tissues (Fig. 7A and C). Relatively higher frequency of CD8$^+$IFN-γ$^+$ T cells was found in CXCL11, in comparison to CXCL9- and CXCL10-treated groups (Fig. 7A and C). Similarly, no significant difference was observed in the frequencies of CRTAM expression gated on CD8$^+$ T cells in the SPL of guinea pigs vaccinated with RR2 and treated with chemokines and RR2 protein compared to the mock-vaccinated group (Fig. 7B and D). However, significantly high frequencies of CRTAM on CD8$^+$ T cells were induced by the RR2-vaccinated group treated with CXCL11, followed by CXCL9, CXCL10, and RR2 alone compared to those with the mock-vaccinated group in the VM and DRG tissues (Fig. 7B and C) were detected. In conclusion, these results indicate that therapeutic "prime-pull" vaccination of HSV-2-infected guinea pigs with RR2 protein and treated with CXCL11 induced more IFN-γ$^+$CRTAM$^+$CD8$^+$ T cells within the vaginal mucocutaneous tissue and DRG tissue associated with significant protection against recurrent genital herpes.

DISCUSSION

The morbidity and the socioeconomic burden related to recurrent genital herpes highlight the need for a therapeutic herpes vaccine that can mitigate the impact of

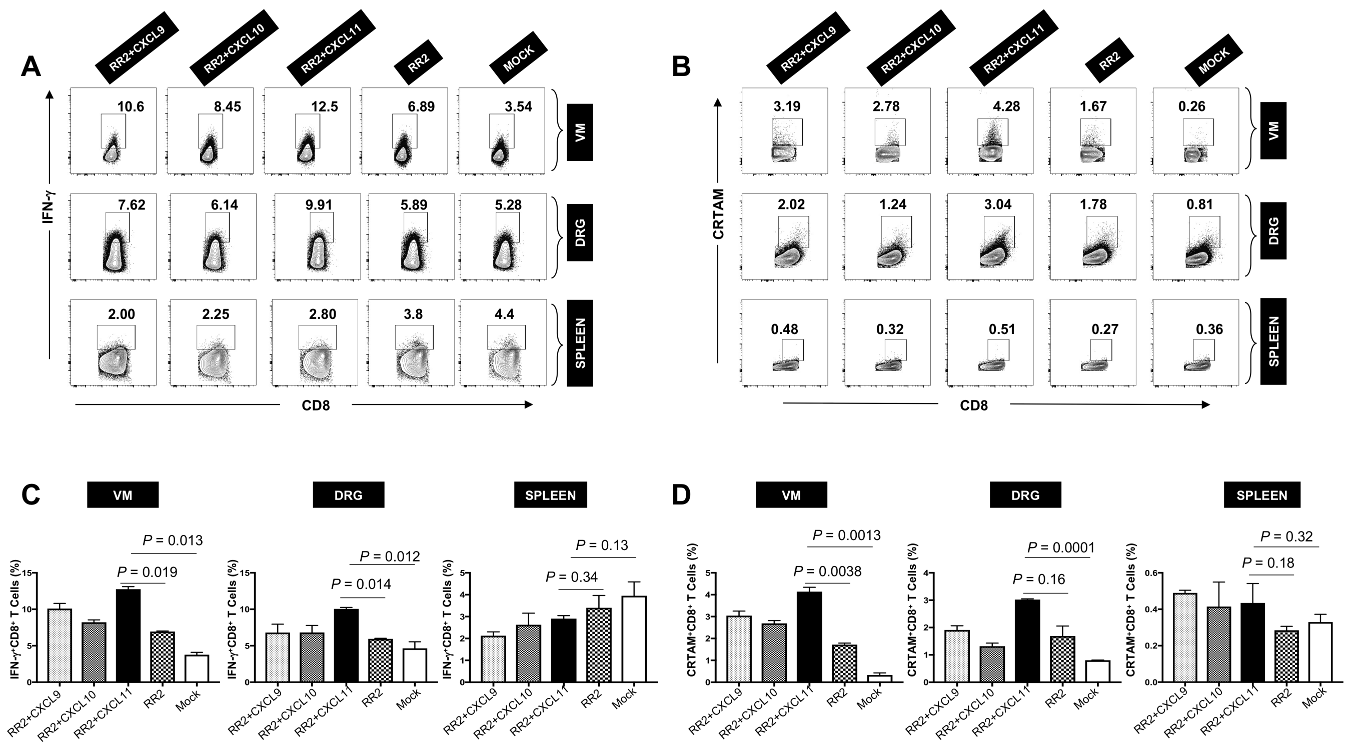

FIG 7 Increased frequencies of functional tissue-resident CD8$^+$ T cells observed in HSV-2-infected guinea pigs following therapeutic prime/pull vaccination with the RR2 protein/ chemokine, especially CXCL11. Functional analysis of CD8$^+$ T cells from HSV-2-infected guinea pigs following therapeutic prime/pull vaccination with RR2 protein/chemokine CXCL9/10/11, RR2 alone, and mock-vaccinated animals. Single-cell suspensions from SPL, VM, and DRG were obtained post-infection and immunization. Single cells from SPL, VM, and DRG were stained for functional marker IFN-γ, activation marker CRTAM, and CD8 and analyzed by FACS. Representative FACS data (upper panel) and average frequencies (lower panel) of IFN-γ$^+$CD8$^+$ T cells were detected in the spleen, VM, and DRG of RR2$^+$ chemokine, RR2-alone-vaccinated groups, and the mock-vaccinated group. (A and C) Frequency of IFN-γ$^+$CD8$^+$ T cells per tissue-specific total cells. Representative FACS data (A) and average frequencies (B) of IFN-γ$^+$CD8$^+$ T cells were observed in the RR2$^+$ chemokine group compared to RR2-alone-vaccinated groups and the mock-vaccinated group. (B and D) Similarly, frequency of CRTAM$^+$CD8$^+$ T cells per tissue-specific total cells. Representative FACS data (B) and average frequencies (D) of CRTAM$^+$CD8$^+$ T cells were observed in the RR2$^+$ chemokine group comparison compared to RR2-alone-vaccinated groups and the mock-vaccinated group.

the disease. Despite its toll, herpes has generally been seen as a “marginal” disease. At present, only a handful of pharmaceutical companies and academic institutions have invested in herpes vaccine research over the past few decades (12). The past clinical trials that used glycoproteins gB and gD as the primary antigen in herpes vaccines met failure. Although these glycoproteins are commonly targeted by the immune system, they did not provide sufficient protection against recurrent herpetic disease when included in the vaccine (12). In the present study, lessons learned from the failed gB/gD-based vaccines led us to hypothesize therapeutic subunit vaccine containing non-gB/gD “asymptomatic” HSV-2 proteins that can selectively induce CD4$^+$ and CD8$^+$ T_{RM} cells from naturally “protected” asymptomatic women would decrease the frequency and severity of the recurrent herpetic disease.

Increased number and function of DRG- and VMC-resident CD4$^+$ and CD8$^+$ T cells were associated with reduced severity and rate of recurrent genital herpes. The reduction in the HSV-2 DNA copy numbers in the DRG of the infected vaccinated animals has been shown earlier. However, to date, no study has reported the effect of T cell-attracting chemokines on the frequency of DRG-resident T cell subsets and how that affects recurrent genital herpes. To address this, we have chosen the novel prime/pull vaccine strategy, in which we (i) “primed” peripheral anti-viral CD4$^+$ and CD8$^+$ T cells with HSV-1 RR2 antigen and (ii) subsequently “pulled” the primed CD4$^+$ and CD8$^+$ T cells into DRG tissue by AAV8 vectors expressing CXCL9, CXCL10, or CXCL11 T cell-attracting

chemokines (CXCR3 ligands). AAV vectors are useful vehicles for delivering transgenes to infected tissues and organs *in vivo*. Recently, there has been much progress in delivering AAV vectors to sensory neurons following application to the peripheral epithelium, such as skin or eye. Results from our earlier experiments showed that the neurotrophic AAV8 serotype is an optimal candidate for expressing the GFP transgene in DRG and VMC tissues in comparison to AAV1 and AAV9 serotypes (Fig. S1). To date, no study has reported the effect in attracting more functional DRG-resident $CD4^+$ and $CD8^+$ T cells using CXCL11 chemokine affects recurrent genital herpes infection and disease. In this study, we found that AAV8 neurotropic serotype efficiently delivered the guinea pigs' T cell-attracting chemokines (CXCL9, CXCL10, CXCL11) into DRG and VM tissues (Fig. 3C). Moreover, the AAV8 vector expressing the guinea pigs' CXCL9, CXCL10, and CXCL11 chemokines specifically in infected DRG tissue reduced recurrent genital herpes lesions associated with an increased number of antiviral tissue-resident T_{RM} cells. The expression of these T cell-attracting chemokines is likely to be associated with the reduction of HSV-2 DNA in the DRG. While the present study demonstrated that increased frequencies of antiviral functional $CD4^+$ and $CD8^+$ T cell in the DRG are associated with a reduction in virus shedding and recurrent genital herpes in latently infected guinea pigs. However, evidence that the presence of DRG-resident $CD4^+$ and $CD8^+$ T cells directly reduces spontaneous active replication would require extensive *in vivo* and *in vitro* studies. Thus, the mechanism behind this is yet to be explored and will be the subject of future report.

Recurrent genital herpetic disease is one of the most common sexually transmitted diseases, with a worldwide prevalence of infection predicted to be over 3.5 billion individuals for HSV-1 and over 536 million for HSV-2 (12). However, given the staggering number of individuals already infected with HSV-1 and HSV-2, there is a significant unmet medical need for a therapeutic herpes vaccine to reduce viral shedding and alleviate herpetic disease in symptomatic patients. Historically, it has been harder to develop therapeutic vaccines because the viruses HSV-1 and HSV-2 employ many strategies to evade the host immune system, which are in place in already infected individuals. Thus, scientists relied on developing prophylactic vaccines to prevent new infections in seronegative individuals. Moreover, the potential for recombination between HSV-1 and HSV-2 has given rise to HSV recombinant strains that are widely circulating (12). The majority of HSV-1- and HSV-2-seropositive individuals are asymptomatic, implying that such individuals can readily and quietly transmit the virus to their partners. Global herpes prevalence stresses the urgency of developing a therapeutic vaccine. In our previous research, the protective efficacy of eight HSV-2 proteins that were highly recognized by the immune system from naturally protected asymptomatic individuals as therapeutic vaccine candidates was compared. Among others, we found that immunization using HSV-2 RR2 protein demonstrated significant protection against recurrent genital herpes disease. Moreover, RR2 protein-induced HSV-2-specific antibody and T cell responses correlated with reduced viral shedding and disease severity. In addition, previous research has identified a $CD8^+$ T cell population located near nerve endings that control reactivated HSV (12). This suggests that the presence and activity of $CD8^+$ T cells in the mucosa are critical for controlling HSV reactivation and subsequent disease outcomes.

The HSV-2 ribonucleotide reductase is a major target of HSV-2-specific $CD8^+$ T cells in humans. It consists of two heterologous protein subunits. The small subunit (RR2) is a 38-kDa protein encoded by the UL40 gene, and the large subunit (RR1), designated ICP10, is a 140-kDa protein encoded by the UL39 gene (31). RR2 protein can boost neutralizing antibodies and increase the numbers of functional IFN-γ^+CRTAM$^+$ CD8 T cells within the VM tissues. While systemic memory T cells can migrate freely through organs such as the spleen and liver, others such as the intestines, lung airways, central nervous system, skin, and vagina are restrictive for memory T cell entry. In the latter tissues, inflammation or infection is often required to permit entry of circulating activated T cells to establish a tissue-resident memory T cell pool that composes a separate compartment from the circulating pool. Given that the occurrence of inflammation in the

reproductive tissue may preclude the infection or reactivation of the virus, we investigated an alternative approach to recruit virus-specific T cells into the vaginal mucosa without inducing local inflammation or infection, i.e., using chemokines. This strategy referred to as the "prime-pull" strategy involves priming the immune system with an initial vaccination and then "pulling" the immune response to the site of infection using specific chemokines (12). The goal is to increase the number of HSV-specific $CD8^+$ T cells that can effectively control reactivated HSV and reduce recurrent disease and viral shedding. Chemokines are naturally produced by our immune system and could serve as safer and more reliable molecules to attract immune cells. The CXC chemokine ligand 10 (CXCL10)/CXC chemokine receptor 3 (CXCR3) pathways are critical in promoting T cell immunity against many viral infections. A "prime-pull" therapeutic vaccine can boost neutralizing IgG/IgA antibodies and boost the number and function of antiviral $CD4^+$ and $CD8^+$ T_{RM} cells within the cervical genital mucocutaneous (CGMC) and DRG tissues and thus expected to help stop the virus reactivation from latently infected DRG, virus shedding, and virus replication in CGMC, thus curing or reducing recurrent genital herpes disease. In one of the "prime-pull" strategies, a topical chemokine was applied to the genital mucosa after subcutaneous vaccination to pull HSV-specific $CD8^+$ T cells that were found to be associated with decreased disease upon challenge with HSV-2 (32). The chemokine/CXCR3 pathway also affects TG- and cornea-resident $CD8^+$ T cell responses to recurrent ocular herpes virus infection and disease. Chemokines can also be co-delivered in a DNA vaccine for immunomodulation. Pre-clinical studies in HSV have shown immuno-potentiation of DNA vaccines by co-delivery of chemokines such as CCR7 ligands and IL-8, RANTES delivered to the mucosa (33). In this study, we establish the therapeutic efficacy of the "prime-pull" strategy using HSV-2 protein for immunization (prime) and a chemokine (pull) to increase the number of HSV-2-specific T cells in the vaginal mucosa of guinea pigs. The localized increase in T cells' frequency was attained by using adenovirus-expressing guinea pig-specific mucosal chemokine CXCL9/10/11. Guinea pigs were immunized (primed) using previously studied RR2 protein, and RR2-specific T cells were pulled in VM using CXCL9/10/11. We hypothesized that the chemokine-based pulling would increase the functional, tissue-resident, IFN-γ-producing T cells in the VM. Our studies demonstrate that the chemokine CXCL9/10/11 treatment could pull the RR2-specific $CD4^+$ and $CD8^+$ T cells in the VM. However, CXCL11 was relatively better in comparison to CXCL9 and CXCL10. The CXCL11, also known as IFN-inducible T cell α chemoattractant, mediates the recruitment of T cells, natural killer (NK) cells, and monocytes/macrophages at sites of infection, predominantly through the cognate G-protein-coupled receptor CXCR3 (34, 35). This signaling axis has been implicated in several physiological activities, including immune cell migration, differentiation, and activation. In addition, the affinity of CXCL11 for CXCR3 is the highest among the three selective ligands, i.e., CXCL9, CXCL10, and CXCL11. We show an increase in the number and function of tissue-resident memory $CD8^+$ T cells in the vaginal mucosa of vaccinated guinea pigs treated with CXCL11 than that observed with other chemokines and with RR2 alone (12). The pulling of T cells manifested in a significant reduction of virus shedding and decreased recurrent genital herpes lesions. The viral reduction was significantly higher in vaccinated guinea pigs treated with CXCL11 than that observed with RR2 alone. Upon evaluation of the expression of CXCR3 on $CD8^+$ T cells in the VM, DRG, and spleen of guinea pigs, we observed an increased number and percentage of $CXCR3^+CD8^+$ T cells in the VM and DRG of guinea pigs treated with chemokine/RR2 compared to RR2 alone. The reduced frequency of $CXCR3^+CD8^+$ T cells in mock and RR2 alone highlights the role of chemokine/RR2 in the migration of $CD8^+$ T cells in the VM and DRG of chemokine-treated guinea pigs. Moreover, the expression of CXCR3 on $CD8^+$ T cells was predominant in the DRG of vaccinated guinea pigs treated with CXCL11. We believe that CXCL11-dependent therapy may be a potential approach for viral infections.

Over the last three decades, it has not been technically feasible to perform phenotypic, functional, and transcriptional profiling of memory $CD4^+$ and $CD8^+$ T cell subsets in the guinea pig model. One major limitation has been the unavailability of monoclonal

antibodies (mAbs) specific to guinea pigs' T cell subsets, surface CD, cytokines, and chemokines. Our laboratory has recently advanced T cell immunology frontiers in guinea pigs. Over the last 6 years, we have identified, tested, and validated the specificity of many commercially available mAbs and immunological reagents to study the phenotype and function of T cell subsets in the guinea pig model. In addition, we have teamed up with Dr. Hubert Schaefer, at the Robert Koch-Institute, Berlin, Germany, who provided many "in-house" developed mAbs specific to guinea pigs' immune cells and T cell subsets used in our guinea pigs' T cell studies (12). In the present study, like our previous report (12), we have tested and validated many of these mAbs including markers of T cell subsets from the guinea pigs. In the absence of anti-CD3 mAb, one cannot exclude the heterogeneous $CD4^+$ and $CD8^+$ T cells used in our gated cell population since the CD4 and CD8 markers used to identify the T cells are also expressed by other immune cell populations (i.e., NK cells, NKT cells, ILC cells, macrophages, and DCs). However, (i) the gated cell population presented the shape and granularity (FSC/SSC) characteristics of T cells and (ii) the isolated cells expression of CXCR3 chemokine receptor, of CD103/CD69 markers, of CD44 receptor, of CRTAM marker, and the production of IFN-γ (Fig. 4 to 7; Fig. S3), all support a T cell phenotype and function. These T cell studies in the guinea pigs' model are supported by previous human studies showing that the CXCR3 chemokine receptor is highly expressed on effector T cells and plays an important role in T cell trafficking and function (36–38). CXCR3 is rapidly induced on naïve T cells following activation and preferentially remains highly expressed on Th1-type $CD4^+$ T cells and effector $CD8^+$ T cells (39, 40). The present T cell studies in guinea pigs agree with human studies showing that CRTAM is one of the most highly expressed surface markers of activated human $CD4^+$ and $CD8^+$ T cells and upregulation of the CRTAM on both $CD4^+$ T cells (41) and $CD8^+$ T cells correlated with efficient lysis of infected cells (42–45). Besides, present study confirms our previously reported phenotype and function of $CD4^+$ and $CD8^+$ T cells in the guinea pig model using the same validated mAbs. Therefore, based on our expertise in guinea pig immunology, the present function and exhaustion of T cell assays, including IFN-γ-ELISpot, CFSE-based proliferation, surface markers of $CD4^+$ and $CD8^+$ T cell subsets, activation markers CD25, CD44, CD69, and CRTAM, and T cell exhaustion markers PD-1, LAG-3, and TIM-3, can be readily used to study T cells in the guinea pig model (12).

In conclusion, our study demonstrates that immunizing guinea pigs with an immunogenic HSV-2 protein RR2 and treatment with adenovirus-expressing chemokine provide better protection against recurrent genital herpes. This strategy seems to prevent the migration of HSV-2 from mucosa to neurons leading to decreased reactivation and viral shedding. We hypothesize that $CD8^+$ T cells reduce neuronal infection or viral replication within neurons. To date, no study has reported the effect in attracting more functional DRG-resident $CD4^+$ and $CD8^+$ T cells using CXCL11 chemokine affects recurrent genital herpes infection and disease. In the present study, we showed that increased number and function of DRG-resident $CD4^+$ and $CD8^+$ T cells were associated with a reduction in the HSV-2 DNA copy numbers in the DRG, reduced severity, and rate of recurrent genital herpes in latently infected vaccinated guinea pigs. The presence of T cells near infected neurons may likely affect the viral replication and/or reduced recurrences of HSV-2 from latency, which in turn reduces the severity of recurrent genital herpes. Moreover, the presence and increased number of functional $CD4^+$ and $CD8^+$ T cells in vaginal mucosa may also affect viral replication at the entry site. However, providing mechanistic evidence of the direct implication of DRG- and VM-resident $CD4^+$ and $CD8^+$ T cells in reduction of spontaneous active replication would require extensive *in vivo* and *in vitro* studies, and this will be the subject of future reports. This type of immunotherapy can help recruit and establish resident T cells that can provide protection not only against genital herpes but also other types of sexually transmitted diseases.

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AUTHOR AFFILIATIONS

[1]Laboratory of Cellular and Molecular Immunology, Gavin Herbert Eye Institute, University of California Irvine, School of Medicine, Irvine, California, USA
[2]Department of Vaccines and Immunotherapies, TechImmune, LLC, University Lab Partners, Irvine, California, USA
[3]Intracellular Pathogens, Robert Koch-Institute, Berlin, Germany
[4]Institute for Immunology, University of California Irvine, School of Medicine, Irvine, California, USA

AUTHOR ORCIDs

Afshana Quadiri http://orcid.org/0000-0003-0212-5854

Lbachir BenMohamed http://orcid.org/0000-0001-9468-2521

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AUTHOR CONTRIBUTIONS

Afshana Quadiri, Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft | Swayam Prakash, Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing – original draft | Nisha Rajeswari Dhanushkodi, Data curation, Methodology, Writing – original draft | Mahmoud Singer, Formal analysis, Methodology, Software | Latifa Zayou, Software, Visualization, Writing – original draft | Amin Mohammed Shaik, Data curation, Formal analysis | Miyo Sun, Methodology | Berfin Suzer, Methodology | Lauren Su Lin Lau, Methodology | Amruth Chilukurri, Methodology | Hawa Vahed, Methodology | Lbachir BenMohamed, Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review and editing.

DATA AVAILABILITY

The RNA-Seq data obtained in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) under the accession number GSE261599.

ETHICS APPROVAL

The Institutional Animal Care and Use Committee of the University of California, Irvine, reviewed and approved the protocol for these studies (IACUC # AUP-22-086).

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental figures (JVI01596-23-s0001.pdf). Fig. S1 to S3.
Table S1 (JVI01596-23-s0002.xlsx). Differentially expressed chemokine signaling pathway-specific genes in CD8+ T cells.

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