HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2024 July 01.

Published in final edited form as:

J Immunol. 2023 July 01; 211(1): 118–129. doi:10.4049/jimmunol.2300093.

Mucosal CCL28 Chemokine Improves Protection Against Genital Herpes Through Mobilization of Antiviral Effector Memory CCR10^{+}CD44^{+} CD62L^{-}CD8^{+} T_{EM} cells and Memory CCR10^{+}B220^{+}CD27^{+} B Cells Into the Infected Vaginal Mucosa

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Abstract

Four major mucosal-associated chemokines, CCL25, CCL28, CXCL14, and CXCL17 play an important role in protecting mucosal surfaces from infectious pathogens. However, their role in protection against genital herpes remains to be fully explored. The CCL28 is a chemoattractant for the CCR10 receptor-expressing immune cells and is produced homeostatically in the human vaginal mucosa (VM). In the present study, we investigated the CCL28/CCR10 chemokine axis's role in mobilizing protective antiviral B- and T-cell subsets into the VM site of herpes infection. We report a significant increase in the frequencies of HSV-specific memory CCR10^{+}CD44^{+}CD8^{+} T cells, expressing high levels of CCR10, in herpes-infected asymptomatic (ASYMP) women compared to symptomatic (SYMP) women. Similarly, a significant increase in the CCL28 chemokine (a ligand of CCR10), was detected in the VM of herpes-infected ASYMP B6 mice, associated with the mobilization of high frequencies of HSV-specific effector memory CCR10^{+}CD44^{+} CD62L^{-}CD8^{+} T_{EM} cells and memory CCR10^{+}B220^{+}CD27^{+} B cells in the VM of HSV-infected asymptomatic mice. Inversely, compared to wild-type (WT) B6 mice, the CCL28 knockout (CCL28$^{(-/-)}$) mice: (*i*) Appeared more susceptible to intravaginal infection and re-infection with HSV-2; (*ii*) Exhibited a significant decrease in the frequencies of HSV-specific effector memory CCR10^{+}CD44^{+} CD62L^{-}CD8^{+} T_{EM} cells and of memory CD27^{+}B220^{+} B cells

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Conflict of interest: The authors have declared that no conflicts of interest exist.

in the infected VM. These findings suggest a critical role of the CCL28/CCR10 chemokine axis in the mobilization of anti-viral memory B and T cells within the VM to protect against genital herpes infection and disease.

Keywords

HSV-2; genital herpes; CCL28; mucosa; memory $CD8^+$ T cells; memory B cells

INTRODUCTION

Genital herpes caused by herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) affects over 490 million (13%) people 15–49 years of age worldwide (1). Over the past several decades, considerable efforts have been made to develop a herpes simplex vaccine, but such a vaccine remains an unmet medical need (2). This results in a significant global health and financial burden. Approximately forty to sixty million individuals are infected with HSV-2 in the United States alone, with nearly six to eight hundred thousand reported annual clinical cases (3–8). HSV-2 and HSV-1 replicate predominantly in the mucosal epithelial cells and establish latency in the sensory neurons of the dorsal root ganglia (DRG) where, in symptomatic individuals, they reactivate sporadically causing recurrent genital herpetic disease (4, 9, 10). Both HSV-1 and HSV-2 cause genital herpes disease through infection of the mucosa of the genital tract. Genital herpes can produce genital ulcers increasing the risk of acquiring and transmitting HIV infection (11–13).

In response to HSV-1 and HSV-2 infections, the vaginal epithelial cells secrete soluble factors including chemokines that mobilize and guide leukocytes of the innate and adaptive immune system, such as the NK cells, neutrophils, monocytes, B and T cells to the site of infection, vaginal mucosa (VM), or DRG the site of reactivation. Apart from their role in the mobilization of immune cells, chemokines can signal through specific membrane-bound receptors that lead to the activation of cellular pathways that can eliminate the virus. Out of all 48 known human chemokines, CCL25, CCL28, CXCL14, and CXCL17 mucosal chemokines are especially important in mucosal immunity because they are homeostatically expressed in mucosal tissues (14–17). The chemokine expression in the vagina mucosa influences the mobilization and activation of innate immune cells that facilitate adaptive immune responses (4, 9).

Local B and T cell responses within the VM play an important role in the defense against herpes infection and disease (18–22). *However, the VM tissue appears to be immunologically restricted and mostly resistant to accepting homing B and T cells that could be traveling from the draining lymph nodes and circulation.* (23–26). Major gaps within the current literature include the identity of involved chemokines and the underlying mechanisms through which these chemokines and their receptors mobilize the protective memory B and T cell subsets into the infected and inflamed vaginal mucosal tissues. Several chemokines are produced in the vaginal mucosa following genital HSV-2 infection (23–26), but whether and how these chemokines affect mucosal B and T cell responses in the vaginal mucosa remains to be fully elucidated.

In this study, we first performed bulk RNA sequencing of HSV-specific $CD8^+$ T cells to determine any differential regulation of the chemokine pathways in HSV-infected symptomatic (SYMP) asymptomatic vs. (ASYMP) women. Subsequently, we identified the CCL28, also known as mucosae-associated epithelial chemokine (MEC), (a chemoattractant for CCR10 expressing B and T cells), as being highly expressed in HSV-infected ASYMP women. Moreover, using the CCL28 knockout mouse model, we confirmed the role of the CCL28/CCR10 chemokine axis in protective B and T cell immunity against genital herpes. In this report, we demonstrate the role of the CCL28/CCR10 chemokine axis in the mobilization of circulating B and T memory cells into the VM site of infection and the underlying CCL28/CCR10 chemokine axis-mediated mechanism of action. In this study, we discussed the potential use of the mucosal chemokine CCL28 to improve genital herpes immunity and protect against infection and disease caused by HSV, and potentially other sexually transmitted viruses.

MATERIALS AND METHODS

Virus propagation and titration:

Rabbit skin (RS) cells (from ATCC, VA, USA) grown in Minimum Essential Medium Eagle with Earl's salts and L-Glutamine (Corning, Manassas, VA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin was used for virus propagation. HSV-2 strain186 was propagated in RS cells as described previously (20–22). The virus was quantified by plaque assay in RS cells. The HSV-2 strain 186 was originally isolated from a genital lesion from an individual attending a sexually transmitted disease clinic in Houston, Texas, in the 1960s. Strain 186 is used in this study as it is a highly pathogenic herpes virus (87).

Mice:

Female C57BL/6 (B6) wild-type mice (6-8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME) and $CCL28^{(-/-)}$ KO mice breeders were a kind donation by Dr. Takashi Nakayama, Kindai University, Japan). $CCL28^{(-/-)}$ KO mice breeding was conducted in the animal facility at UCI where female mice at 6-8 weeks were used. Animal studies conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. Animal studies were conducted with the approval of the Institutional Care and Use Committee of the University of California-Irvine (Irvine, CA) and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (IACUC protocol #19-111).

Genital infection of mice with HSV-2:

All animals were injected subcutaneously with 2mg progesterone (Depo-Provera®), to synchronize the ovarian cycle and increase susceptibility to herpes infection, and then received an IVAG HSV-1 challenge. Previous studies have shown that estrogen might have a crucial role in the protection against genital infection by regulating MEC/CCL28 expression in the uterus (58). Since immune responses in the VM compartment appear to be under the influence of sex hormones, future studies will compare the phase of the menstrual cycle/estrous cycle in mice as well as in symptomatic and asymptomatic women. Mice

were intravaginally infected with 5 x 10^3 pfu of HSV-2 strain 186 in 20μL sterile PBS. Following genital infection, mice were monitored daily for genital herpes infection and disease progression. For genital inflammation and ulceration examination, pictures were taken at the time points listed in the figure legends using a Nikon D7200 camera with an AF-S Micro NIKKOR 105mm f/2.8 lens and a Wireless Remote Speedlight SB-R200 installed. CCL28 KO and WT that survived the primary infection were re-infected with 5 x 10^3 pfu HSV-2 strain186 at day 30 p.i. At day 10 post-re-infection, mice were euthanized and immune cells from VM and spleen were used for flow cytometry. Single-cell suspensions from the mouse vaginal mucosa (VM) after collagenase treatment (15mg/ml) for 1 hour were used for FACS staining.

Monitoring of genital herpes infection and disease scoring in mice:

Virus shedding was quantified in vaginal swabs collected on days 3, 5, 7, and 10 p.i. Infected mice were swabbed using moist type 1 calcium alginate swabs and frozen at −80 °C until titrated on RS cell monolayers, as described previously (30–34). Mice were scored every day from day 1 to day 9 p.i for pathological symptoms. Stromal keratitis was scored as 0- no disease; 1- cloudiness, some iris detail visible; 2- iris detail obscured; 3- cornea opaque; and 4- cornea perforation. Mice were evaluated daily and scored for epithelial disease (erythema, edema, genital ulcers, and hair loss around the perineum) and neurological disease (urinary and fecal retention and hind-limb paresis/paralysis) on a scale that ranged from 0 (no disease) to 4 (severe ulceration, hair loss, or hind-limb paralysis) (88, 89). Mice that reached a clinical score of 4 were euthanized.

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

RNA was isolated from the 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 single-end 100-bp sequencing using the Illumina HiSeq 4000 platform.

Differential gene expression analysis:

Differentially expressed genes (DEGs) were analyzed by using integrated Differential Expression and Pathway analysis tools. Integrated Differential Expression and Pathway analysis seamlessly connect 63 R/Bioconductor packages, two web services, and comprehensive annotation and pathway databases for homo sapiens and other species. The expression matrix of DEGs was filtered and converted to Ensemble gene identifiers, and the preprocessed data were used for exploratory data analysis, including *k*-means clustering and hierarchical clustering. The pairwise comparison of symptomatic and asymptomatic groups was performed using the DESeq2 package with a threshold of false discovery rate < 0.5. and fold change >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 symptomatic and asymptomatic groups. The Parametric Gene Set Enrichment Analysis (PSGEA) method was applied based on data curated in Gene Ontology and KEGG. The pathway significance cutoff with a false discovery date (FDR) ≥ 0.2 was applied.

Flow cytometry:

Single-cell suspensions from the mouse VM after Collagenase D (Millipore Sigma, St. Louis, MO) treatment (15mg/ml) for 1h at 37C were used for FACS staining. The following antibodies were used: anti-mouse CD3 (clone 17A-2, BD Biosciences), CD45 (clone 30-F11, BD Biosciences), CD4, CD8, CD44, CD62L, B220and CD27 (BD Biosciences). For surface staining, mAbs were added against various cell markers to a total of 1 x 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. Cells were washed again with FACS buffer and fixed in PBS containing 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO).

HSV-2-specific ASC ELISPOT assay:

Immune cells isolated from VM of HSV-2 infected mice (2 million cells/ml) were stimulated in B-cell media containing mouse polyclonal B cell activator (Immunospot) for 5 days. CTL Mouse B-Poly-S are stock solutions containing Resiquimod and either recombinant Human IL-2 or recombinant Mouse IL-2 respectively, used for the polyclonal expansion of memory B cells Subsequently, cells were washed in RPMI medium and plated in specified cell numbers in ELISPOT membrane plates coated with heat-inactivated HSV-2. The ASC-secreting cells were detected after 48 hours of the addition of cells to ELISPOT plates. The ELISpot plates were detected by imaging using an ELISPOT reader (ImmunoSpot). The spots were detected and quantified manually.

Immunohistochemistry for human VM tissue:

For immunohistochemistry, human vaginal mucosa sections were used for CCL28 staining. Sections were deparaffinized and rehydrated before the addition of primary antibody anti-human CCL28 for overnight incubation. HRP-labeled secondary antibodies (Jackson Immunoresearch, PA) were used before the addition of substrate DAB. Hematoxylin was used for counterstaining these slides. Subsequently, after thoroughly washing in PBS 3 times slides were mounted with a few drops of mounting solution. Images were captured on the BZ-X710 All-in-One fluorescence microscope (Keyence).

Virus titration in vaginal swabs:

Vaginal swabs (tears) were analyzed for viral titers by plaque assay. RS cells were grown to 70% confluence for plaque assays in 24-well plates. Transfer medium in which vaginal swabs were stored was added after appropriate dilution at 250 ul per well in 24-well plates. Infected monolayers were incubated at 37°C for 1 hour, rocked every 15 minutes for viral adsorption, and then overlaid with a medium containing carboxymethyl cellulose. After 48 hours of incubation at 37°C, cells were fixed and stained with crystal violet, and viral

plaques were counted under a light microscope. Positive controls were run with every assay using our previously tittered laboratory stocks of McCrae.

Statistical analysis:

Data for each assay were compared by ANOVA and Student's *t*-test using GraphPad Prism version 5 (La Jolla, CA). As we previously described, differences between the groups were identified by ANOVA and multiple comparison procedures (33, 34). Data are expressed as the mean ± SD. Results were considered statistically significant at a P value of ≤ 0.05.

RESULTS

1. Increased expression of CCR10, the receptor of CCL28 chemokine, on HSV-specific $CD8^+$ T cells from herpes-infected asymptomatic women compared to symptomatic women:

We first determined whether there are differential expressions of chemokine and chemokine receptor pathways in HSV-specific $CD8^+$ T cells from herpes-infected symptomatic women compared to symptomatic women. $CD8^+$ T cells specific to HSV-2 $gB_{561\text{-}569}$ and VP11-$12_{220\text{-}228}$ epitopes were sorted from PBMC of HSV-infected SYMP and ASYMP women and subjected to bulk-mRNA sequencing. As shown in Fig. 1A major chemokine and chemokine receptor-specific pathways were significantly upregulated among HSV-infected ASYMP women compared to HSV-infected SYMP women ($P < 0.05$) (Supplementary Table 1). In Figs. 1B and 1C, particularly, both the heatmaps (*top panels*) and the volcano plots (bottom *panels*) showed a significant upregulation of CCR10, the receptor of CCL28 chemokine, in $CD8^+$ T cell-specific to HSV-2 $gB_{561\text{-}569}$ epitope (Fig. 1B) and HSV-2 VP11-$12_{220\text{-}228}$ epitope (Fig. 1C) isolated from ASYMP women, compared to SYMP women. Using flow cytometry, we confirmed high frequencies of CCR10 expressing immune cells in HSV-infected ASYMP women ($n = 9$) compared to HSV-1 infected SYMP women ($n = 9$) (Fig. 1D). There was a significant increase in frequencies of CCR10 positive lymphocytes detected in HSV-infected ASYMP women compared to low frequencies of CCR10 positive lymphocytes in SYMP women (i.e., 4.9% vs. 2.6%, $P = 0.04$, Fig. 1D *top panels*). Moreover, higher frequencies of $CCR10^+CD8^+$ T cells, but not of $CCR10^+CD4^+$ T cells, were detected in HSV-2 infected ASYMP women as compared to HSV-2 infected SYMP women (0.61% vs. 0.27%, $P = 0.03$, Fig. 1D *bottom panels*). High levels of CCL28 chemokine expression were found in the epithelial cells of the VM in HSV-2-infected women. As detected by immunohistochemistry, the CCL28 is specifically expressed within the Stratum Corneum (SC) and Sub layer of the epithelium in the human VM (Fig. S1).

Altogether, these results indicate a significant upregulation of CCR10, the receptor of CCL28 chemokine, on HSV-specific $CD8^+$ T cells is associated with asymptomatic genital herpes. Additionally, Supplementary Table 1 shows the differential gene expression (DGE) in HSV-specific $CD8^+$ T cells from herpes-infected symptomatic women compared to symptomatic women.

2. The CCL28 chemokine is highly produced in the vaginal mucosa of HSV-2-infected B6 mice and is associated with asymptomatic genital herpes:

We next determined whether the CCL28 chemokine would be associated with the protection against genital herpes seen in HSV-infected asymptomatic (ASYMP) mice following genital infection with HSV-2. B6 mice ($n = 20$) were infected intra-vaginally (IVAG) with 2 x 10^5 pfu of HSV-2 (strain MS) (Fig. 2A). The vaginal mucosa (VM) was harvested at day 14 post-infection (dpi) and cell suspensions were assayed by flow cytometry for the frequencies of $CD8^+$ T cells expressing CCR10, the receptor of CCL28 among total cells (Fig. 2B). The level of CCL28 was compared in the VM cell extracts from (*i*) HSV-infected symptomatic (SYMP) mice; (*ii*) HSV-infected asymptomatic (ASYMP) mice; and (*iii*) non-infected control (naive) mice, using ELISA, Immunohistochemical (IHC), and western blot (Fig. 2C to 2E). As shown in Fig. 2B, there was a significant increase in the frequency of $CCR10^+CD8^+$ T cells expressing CCR10, the receptor of CCL28 in the VM of ASYMP HSV-infected B6 mice (*HSV-2*) compared to the SYMP HSV-infected B6 mice ($P = 0.002$). Moreover, increased levels of CCL28 chemokine were detected by ELISA quantification in the VM extracts of HSV-2-infected ASYMP mice as compared to HSV-2-infected SYMP mice (Fig. 2C). We confirmed an increased expression of CCL28 in HSV-2-infected ASYMP mice compared to HSV-2-infected SYMP mice by the IHC staining of VM sections (Fig. 2D) and by Western blot analysis of VM lysates (Fig. 2E).

Altogether, these results indicate that: (*i*) The intravaginal infection with HSV-2 mobilized higher frequencies of $CCR10^+CD8^+$ T cells expressing CCR10, the receptor of CCL28 in the VM of infected B6 mice; and (*ii*) A significant production of the CCL28 chemokine in the VM of HSV-2 infected B6 mice is associated with asymptomatic genital herpes. These results suggest a role for the CCL28/CCR10 chemokine axis in the protection against symptomatic genital herpes.

3. CCL28 deficiency is associated with severe genital herpes and increased virus replication following intravaginal HSV-2 re-infection:

To further substantiate the role of the CCL28 chemokine in genital herpes immunity, we studied the functional consequences of CCL28 deficiency in protection against genital herpes infection and disease in mice. CCL28 knockout mice ($CCL28^{(-/-)}$ mice) and WT mice ($n = 12$) were IVAG infected on day 0 with 5 x 10^3 pfu of HSV-2 (strain 186) (Fig. 3A). Mice were scored every day for 14 days p. I for signs of genital herpes and the severity of genital herpes scored, as described in *Material and Methods* (Fig. 3A). The disease was scored as 0- no disease, 2- swelling and redness of external vagina, 3- severe swelling and redness of vagina and surrounding tissue and hair loss in the genital area, 4- ulceration and hair loss in the genital and surrounding tissue. Vaginal swabs were collected on days 3, 5, and 7 p.i. to determine virus titers (Fig. 3A). As shown in Fig. 3B, following primary HSV-2 infection, there was no significant difference detected in the severity of genital herpes between $CCL28^{(-/-)}$ and WT mice at day 8 p.i. and no significant difference observed in the survival of $CCL28^{(-/-)}$ and WT mice following IVAG infection with HSV-2 (Fig. 3C). In addition, we did not detect any significant difference in virus replication detected in the vaginal swabs collected at day 2, 5, and 7 post-infection from $CCL28^{-/-}$ and WT mice following IVAG infection with HSV-2 (Fig. 3D and E).

We further determined a potential role of CCL28 chemokine in genital herpes immunity following recall of memory immune responses. The CCL28 knockout mice ($CCL28^{(-/-)}$ mice) and WT mice (n =3) were subject to a second IVAG infection with 5 x 10^3 pfu of HSV-2 (strain 186 delivered on day 28 post-primary infection) (Fig. 3A). On day 28 post-primary infection, some animals (n = 3) were re-infected once. Mice were scored every day for 14 days p.i. for the severity of genital herpes, survival, and virus replication. Following the reinfection with HSV-2, we observed a significant increase in disease severity in $CCL28^{(-/-)}$ mice compared to WT mice detected on day 8 post-re-infection (P = 0.05, Fig. 3F and H). Moreover, compared to WT mice, there was a significant increase in virus replication measured by plaque assay in vaginal swabs collected in the $CCL28^{(-/-)}$mice at days 3, 5, 7, and 10 post-re-infection (P < 0.05, Fig. 3G).

These results: (*i*) Demonstrate a functional consequence of CCL28 deficiency that led to severe genital herpes disease caused by HSV-2 re-infection; (*ii*) Confirm that CCL28 mucosal chemokine plays an important role in protective immunity against genital herpes infection and disease.

4. CCL28 deficiency is associated with decreased frequencies of both $CCR10^+CD4^+$ and $CCR10^+CD8^+$ T cells within the vaginal mucosa following HSV-2 infection and re-infection:

We next examined whether CCL28 deficiency, which was associated with severe genital herpes and increased virus replication following HSV-2 re-infection (Fig. 3 above), would be the consequence of lower frequencies of $CD4^+$ and $CD8^+$ T cells within the VM. CCL28 knockout mice ($CCL28^{(-/-)}$mice) and WT mice (n =12) were IVAG infected on day 0 with 5 x 10^3 pfu of HSV-2 (strain 186) and then re-infected on day 28 with 5 x 10^3 pfu HSV-2 strain186. On day 10 post-re-infection, mice were euthanized and cell suspensions from VM and spleen were analyzed by flow cytometry for the frequency of $CCR10^+CD4^+$ and $CCR10^+CD8^+$ T cells. As shown in Fig. 4A, we detected significantly lower frequencies of $CD8^+$ T cells (P = 0.01, *left panels*) and $CD4^+$ T cells (P < 0.01, *right panels*) in the VM of CCL28 knockout mice ($CCL28^{(-/-)}$mice) compared to WT mice following re-infection with HSV-2. Moreover, we detected significantly lower frequencies of $CCR10^+$ T cells (P = 0.007, *top panels*), $CCR10^+CD8^+$ T cells (P = 0.02, *middle panels*), and $CCR10^+CD4^+$ T cells (P = 0.02, *bottom panels*) in the VM of CCL28 knockout mice ($CCL28^{(-/-)}$mice) compared to WT mice following re-infection with HSV-2 (Fig. 4B). The CCL28 deficiency specifically affected the frequencies of $CCR10^+$ T cells, $CCR10^+CD8^+$ T cells and $CCR10^+CD4^+$ T cells within the VM (*left panels*) but not within the spleen (*right panels*).

These results: (*i*) demonstrate that CCL28 deficiency is associated with decreased frequencies of $CCR10^+CD4^+$ and $CCR10^+CD8^+$ T cells specifically in the vaginal mucosa (not in the spleen) following HSV-2 infection and re-infection; and (*ii*) suggest that CCL28 mucosal chemokine plays a critical role in the mobilization of protective memory $CCR10^+CD4^+$ and $CCR10^+CD8^+$ T cells, which express the CCR10 receptor of CCL28 chemokine, into the infected VM which likely protects locally against genital herpes infection and disease.

5. CCL28 deficiency is associated with decreased frequencies of effector memory CCR10$^+$CD8$^+$ T_{EM} cell subset, but not of central memory CCR10$^+$CD8$^+$ T_{CM} cell subset, within the vaginal mucosa following HSV-2 re-infection:

We next examined whether CCL28 deficiency would affect the frequencies of specific subsets of memory CD4$^+$ and CD8$^+$ T cells within the VM, namely the effector memory T_{EM} and central memory T_{CM} cell subsets. CCL28 knockout mice (CCL28$^{(-/-)}$ mice) and WT mice (n =12) were IVAG infected on day 0 with 5 x 10^3 pfu of HSV-2 (strain 186) and then re-infected on day 28 with 5 x 10^3 pfu HSV-2 strain186. On day 10 post-re-infection, mice were euthanized and cell suspensions from the VM and spleen were analyzed by flow cytometry for the frequency of effector memory T_{EM} and central memory T_{CM} cell subsets of both CD4$^+$ T cells and CD8$^+$ T cells. As shown in Fig. 5A, significantly lower frequencies of total memory CD8$^+$ T cells (P = 0.01, *left panels*) were detected in the VM of CCL28 knockout mice (CCL28$^{(-/-)}$ mice) compared to WT mice following re-infection with HSV-2. Moreover, the CCL28 deficiency was associated with decreased frequencies of effector memory CCR10$^+$CD8$^+$ T_{EM} cell subset, but not of central memory CCR10$^+$CD8$^+$ T_{CM} cell subset, within the vaginal mucosa following HSV-2 re-infection (Fig. 5A). However, deficiency in CCL28 neither affected the frequencies of effector memory CCR10$^+$CD4$^+$ T_{EM} cell subset nor of central memory CCR10$^+$CD4$^+$ T_{CM} cell subset within the vaginal mucosa following re-infection with HSV-2 (Fig. 5B).

These results suggest that CCL28/CCR10 chemokine axis plays a major role in the mobilization of effector memory CCR10$^+$CD44$^+$ CD8$^+$ T_{EM} cells within the VM site of herpes infection.

6. Decreased frequency of memory CD27$^+$B220$^+$ B cells in the vaginal mucosa of CCL28$^{(-/-)}$ knockout mice compared to wild type B6 mice following HSV-2 infection and re-infection:

Since antibodies and B cells also play a role in protection against genital herpes infection and disease, we finally examined whether CCL28 deficiency would affect the frequencies of total B cells and memory B cell subsets. CCL28 knockout mice (CCL28$^{(-/-)}$ mice) and WT mice (n =12) were IVAG infected on day 0 with 5 x 10^3 pfu of HSV-2 (strain 186) and then re-infected on day 28 with 5 x 10^3 pfu HSV-2 strain186. On day 10 post-re-infection, mice were euthanized and cell suspensions from VM and spleen were analyzed by flow cytometry for the frequency of effector memory T_{EM} and central memory T_{CM} cell subsets of both CD4$^+$ T cells and CD8$^+$ T cells. There were significantly lower frequencies of CCR10$^+$B220$^+$ B cells (P = 0.01), CCR10$^+$B220$^+$CD27$^+$ memory B cells (P = 0.05) were detected in the VM of CCL28$^{(-/-)}$ mice compared to WT mice following re-infection with HSV-2 (Fig. 6A). As expected, the decrease in the frequencies of CCR10$^+$B220$^+$ B cells and CCR10$^+$B220$^+$CD27$^+$ memory B cells specifically affected the CCR10 expressing B cells (P = 0.04, Fig. 6B). As shown in ELISPOT, the HSV-2-specific memory B cell response further confirmed a significant decrease in the function of HSV-specific memory B cells in CCL28$^{(-/-)}$ mice compared to WT mice following re-infection with HSV-2 (P = 0.04, Fig. 6C). Our findings suggest that the CCL28/CCR10 chemokine axis functions through the infiltration of memory B cells to the site of re-activation, the VM.

These results suggest that: (*i*) CCL28/CCR10 chemokine axis affects the mobilization and function of memory CCR10^{+}CD27^{+}B220^{+} B cells, in addition to memory CCR10^{+}CD44^{+} CD8^{+} T_{EM} cells, within the VM site of herpes infection; and (*ii*) CCL28 mucosal chemokine plays an important role in the mobilization of protective memory CCR10^{+}CD8^{+} T_{EM} cells and CCR10^{+}CD27^{+}B220^{+} B cells, both expressing the CCR10 receptor of CCL28 chemokine, into the infected VM, which likely protect locally against genital herpes infection and disease.

DISCUSSION

The four major mucosal-associated epithelial chemokines, CCL25, CCL28, CXCL14, and CXCL17, are expressed homeostatically in many mucosal tissues and play an important role in protecting mucosal surfaces from incoming infectious pathogens. Since the CCL28 mucosal chemokine is a chemoattractant for CCR10 expressing B and T cells and is highly expressed in the vaginal mucosa (VM), we investigated the role of the CCL28/CCR10 chemokine axis in the mobilization of HSV-specific memory B and T cells into VM site of herpes infection and its association with protection against genital herpes. We compared the differential expression of the CCR10, the receptor of CCL28, on herpes-specific CD8^{+} T cells from SYMP and ASYMP HSV-1 infected individuals using bulk RNA sequencing and flow cytometry. Genital herpes infection and disease were compared in CCL28 knockout (CCL28$^{(-/-)}$) mice and wild-type B6 mice (WT) following genital herpes infection and re-infection with HSV-2 (strain186) genital infection. Frequencies of CCR10 expressing memory B and T cells within the VM were studied by flow cytometry and ELISPOT in SYMP and ASYMP HSV-1 infected mice. We found a significant increase in the frequencies of HSV-specific memory CD44^{+}CD8^{+} T cells, expressing high levels of the CCR10 receptor, in herpes-infected ASYMP compared to SYMP individuals. Similarly, we detected significantly increased expression levels of the CCL28 chemokine in the VM of herpes-infected ASYMP mice compared to SYMP mice. Moreover, compared to WT mice, the CCL28 knockout (CCL28$^{(-/-)}$) mice: (*i*) Appeared more susceptible to intravaginal infection and re-infection with HSV-2; (*ii*) Exhibited a decrease in frequencies of HSV-specific effector memory CCR10^{+}CD44^{+} CD62L^{-}CD8^{+} T_{EM} cells, infiltrating the infected VM; and (*iii*) presented a decrease in the frequency of memory CD27^{+} B220^{+} B cells. Increased levels of CCL28 chemokine in asymptomatic herpes suggests a role of the CCL28/CCR10 mucosal chemokine axis in protection against genital herpes infection and disease through mobilization of high frequencies of both CCR10^{+}B220^{+}CD27^{+} memory B cells and HSV-specific memory CCR10^{+}CD44^{+} memory CD8^{+} T cells within the infected vaginal mucosa.

Herpes simplex virus is one of the most common sexually transmitted viral infections worldwide (27). Globally, more women than men are infected by HSV-2 (28, 29), including ~ 31 million in the U.S., and >300 million worldwide (30–32). Except for antiviral prophylaxis only available in developed countries, genital herpes simplex lacks effective treatment and there is no effective vaccination.

Studies that explore the correlates of protective immune response in HSV-infected but asymptomatic individuals would significantly aid in developing immune interventions

to protect from herpes infection and disease in symptomatic patients. After the initial vaginal exposure, the virus replicates in vaginal epithelial cells (VEC), causing painful mucocutaneous blisters (33–39). Newly infected seronegative pregnant women can vertically transmit the virus to their newborns, causing encephalitis and death (40–42). Genital HSV-2 infection has also played a major role in driving the HIV prevalence (43–47), and there is no herpes vaccine or immunotherapy (27, 32, 48–50). Therefore, infected individuals rely on sustained or intermittent antiviral drugs (Acyclovir and derivatives), restrained sexual activity, and barrier methods to limit the spread of HSV-2 (51, 52).

In this study, we performed bulk RNA sequencing of herpes-specific $CD8^+$ T cells isolated from PBMC of HSV-infected SYMP and ASYMP women. Our analysis revealed a unique differential regulation of the chemokine pathway and a significantly increased expression of CCR10 in ASYMP as compared to SYMP herpes-infected women. We further confirmed this result downstream by flow cytometry analysis of immune cells from PBMCs of SYMP and ASYMP HSV-infected women. Our results demonstrated an increased expression level of CCR10 on HSV-specific $CD8^+$ T cells from ASYMP compared to SYMP women both transcriptionally and translationally. Based on these mRNA sequencing and flow cytometry results from SYMP and ASYMP HSV-infected women, we also explored the role of the mucosal chemokine CCL28/CCR10 chemokine axis in protection against genital herpes infection and disease using the mouse model. We used SYMP and ASYMP mice infected intravaginally with HSV and found an increased expression of CCL28 chemokine in the VM was associated with protection in ASYMP mice, but not in SYMP mice. We further confirmed this increased expression of chemokine CCL28 in VM of HSV-2 infected ASYMP mice by western blot and immunohistochemistry. The corresponding increase in the CCR10-expressing memory B and T cells was shown by flow cytometry, further suggesting a critical role of the mucosal chemokine CCL28/CCR10 chemokine axis in the protective immunity against genital herpes.

Chemokines are small, secreted polypeptides with chemotactic properties that regulate the trafficking of immune cells in homeostasis and inflammation (53). Inflammatory chemokines regulate inflammatory responses (54). Homeostatic chemokines are involved in T-cell immunity and immunopathology. They guide, attract, and relocate specific subsets of CD8+ T cells within and between lymphoid organs and non-lymphoid infected tissues (53, 55). Chemokines and their functions can be redundant and may not contribute to disease protection *in-vivo*. To further understand if CCL28 played a profound role associated with protection from disease severity in genital herpes, we used the $CCL28^{(-/-)}$mice to understand if the absence of CCL28 can increase the severity of HSV-2 genital herpes. In addition, we studied whether the $CCL28^{(-/-)}$ mice were more susceptible to genital re-infection with HSV-2 compared to WT mice, as reactivation of HSV-2 infection is the cause of recurrent genital herpes. *Since CCL28 chemokine appears to play a key role in the infiltration of memory immune cells into the VM compartment, we hypothesized that the recall of memory immune cells into the VM in re-infected CCL28 knock-out mice would be compromised.* It is also noteworthy to mention that the $CCL28^{(-/-)}$ mice did not show any differences in disease or pathology during primary infection, but only showed increased susceptibility during re-infection. This could be due to CCL28 eliciting a better memory response by attracting more memory T cells to the site of infection during

re-infection. This confirms previous reports showing that CCL28 regulates the migration of T cells that express the CCR10 receptor (56). CCL28 binds to both CCR10 and CXCR3, which are highly expressed on mucosal epithelia cells (56–65). The underlying mechanism of how the CCL28 improved the frequencies of antiviral $CD8^+$ T cells in the VM is currently unknown. Nevertheless, our finding implies that delivering mucosal chemokines, such as CCL28, intravaginally using "mucosal tropic" adenovirus vectors in symptomatic mice could: (a) "re-open" this otherwise "immunologically closed compartment," allowing infiltration by circulating $CD8^+$ T cells; and/or (b) promote the formation, retention, and expansion of protective vaginal mucosa-resident $CD8^+$ T_{RM} cells, which will suppress local HSV-2 replication, and hence prevent or reduce genital herpes disease. In future experiments, we will use AAV8 vectors expressing CCL28 mucosal chemokine that will be delivered intravaginally in HSV-2 infected mice, and examine recruitment, formation, retention, and expansion of HSV-specific $CD8^+$ T_{RM} cells to the vaginal mucosa. We anticipate that sustained expression of CCL28 mucosal chemokine locally will be critical in mobilizing vaginal mucosal tissues-resident protective HSV-specific $CD8^+$ T_{RM} cells that should control genital HSV-2 infection and disease. Those results will be the subject of a future report. Also, previous studies have shown that estrogen might have a crucial role in the protection against genital infection by regulating MEC/CCL28 expression in the uterus (58). The effect of sex hormones like estrogen on the functions of CCL28 will be an interesting area of research.

The immune profile of cells in the VM of infected mice showed that the $CCL28^{(-/-)}$ mice had a decrease in CCR10 expressing $CD8^+$ and $CD4^+$ T cells and a decreased frequency of $CCR10^+CD44^+$ memory $CD8^+$ T cells compared to WT mice. The role of the CCL28/CCR10 chemokine axis in the mobilization of IgA-secreting cells in mucosa has been well-established in the literature. To further understand if the CCL28 and its receptor have any role in humoral immunity during genital herpes infection, we studied the expression of CCR10 on B cells in VM. Interestingly, a majority of memory B cells in the VM of these mice expressed CCR10. There was also a decreased frequency of $CD27^+B220^+$ memory B cells in these $CCL28^{(-/-)}$ mice. Increased frequency of CCR10 expressing $CD8^+$ T cells in ASYMP herpes may suggest an association of mucosal chemokine CCL28 with protection in herpes infection. Thus, the mucosal chemokine CCL28 mediates protection from disease severity through the mobilization of both $CCR10^+CD44^+$ memory $CD8^+$ T cells and $CCR10^+B220^+CD27^+$ memory B cells to the VM. Recent studies have shown that low-dose CCL28 act as a molecular adjuvant when combined with the immunogen HSV-2 gB or HSV-2 gD with increased levels of virus-specific serum IgG and vaginal fluid IgA (66). This suggests that, in addition to the infiltration of memory T cells. CCL28 may also play a key role in the infiltration of memory B cells into the VM compartment.

During the last 20 years only a single vaccine strategy—adjuvanted recombinant HSV glycoprotein D (gD), with or without gB—has been tested and retested in clinical trials (67). Despite inducing strong HSV-specific neutralizing antibodies, this strategy failed to reach the primary endpoint of reducing herpes disease (68). These failures emphasize the need to induce T cell-mediated immunity (69). Following the resolution of viral infections, a long-lived memory $CD8^+$ T cell subset that protects secondary (2°) infections is generated (18–22). This memory $CD8^+$ T cell subset is heterogeneous but can be

divided into three major subsets: (1) effector memory $CD8^+$ T cells ($CD8^+$ T_{EM} cells); (2) central memory $CD8^+$ T cells ($CD8^+$ T_{CM} cells); and (3) tissue-resident memory $CD8^+$ T cells ($CD8^+$ T_{RM} cells) (70). The three major sub-populations of memory T cells differ in their phenotype, function, and anatomic distribution. T_{CM} cells are $CD62L^{high}CCR7^{high}CD103^{low}$. T_{EM} cells are $CD62L^{low}CCR7^{low}CD103^{low}$. T_{RM} cells are $CD62L^{low}CCR7^{low}CD103^{high}CD11a^{high}CD69^{high}$ (70–73). $CD8^+$ T_{RM} cells are found in the vaginal mucosa and offer protection in mouse models of genital herpes (74). $CD8^+$ T_{EM} cells are also found in the dermal-epidermal junction in women's vaginal mucosa (75, 76). Once formed, T_{RM} cells do not re-enter the circulation and play an essential role in locally guarding mucosal tissues against secondary (2°) infections. However, the precise mechanisms by which non-circulating mucosa-resident memory $CD8^+$ T_{RM} cells are formed, maintained, and expanded remain to be fully elucidated. In the present study, we found that a high frequency of $CD8^+$ T_{RM} cells is retained in the vaginal mucosa of HSV-infected asymptomatic mice compared to symptomatic mice and that this is associated with CCL28 mucosal chemokine production. Specifically, we demonstrated that higher frequencies of vaginal mucosa tissue-resident antiviral memory $CD8^+$ T cells ($CD8^+$ T_{RM} cells) are a key mediator of protection against genital herpes, supporting previous reports (27, 75, 77–81). Since the primary cell target of HSV-2 is vaginal epithelial cells (VEC), the key to achieving anti-herpes mucosal immunity likely is to boost the frequencies of HSV-specific $CD8^+$ T_{RM} cells in the vaginal mucosa that can expand locally and persist long-term. $CD8^+$ T_{RM} cells persist long-term in tissues and are often embedded in the epithelial borders of mucosal tissues (82–86). However, little information exists on the mechanisms regulating the formation, retention, and expansion of vaginal-mucosa-resident $CD8^+$ T_{RM} cells. To our knowledge, this report is the first to show CCL28/CCR10 chemokine axis mediated signals may be required for high frequencies of vaginal mucosa tissue-resident antiviral memory $CD8^+$ T_{RM} cells. It remains to determine the mechanism of expansion and long-term retention of these $CD8^+$ T_{RM} cells within the vaginal mucosa. Such knowledge will help design innovative vaccines to induce $CD8^+$ T_{RM} cell-mediated protection from genital herpes. Collectively, this knowledge could greatly enhance our understanding of mucosal immunity and represents a unique opportunity to develop a powerful and long-lasting genital herpes vaccine that would have a significant impact on this disease's epidemiology.

To our knowledge, our study represents the first in-depth analysis of the role of the CCL28/CCR10 chemokine axis in anti-herpes T and B cell responses in the VM during HSV-2 infection. We demonstrated that following intravaginal HSV-2 re-infection of B6 mice, high production of CCL28 chemokine in the VM was associated with increased infiltration of $CCR10^+CD44^+$ memory $CD8^+$ T cells and $CD27^+B220^+$ memory B cells in the VM. Our findings could further aid in future innovative immunotherapeutic approaches for genital herpes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work is supported by Public Health Service Research R01 Grants EY026103, EY019896, and EY024618 from the National Eye Institute (NEI), AI158060, AI150091, AI143348, AI147499, AI143326, AI138764, AI124911, and AI110902 from the National Institutes of Allergy and Infectious Diseases (NIAID) to LBM, and in part by The Discovery Center for Eye Research (DCER) and the Research to Prevent Blindness (RPB) grant. 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. We thank the NIH Tetramer Facility (Emory University, Atlanta, GA) for providing the Tetramers used in this study.

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- CCL28/CCR10 axe protects the vaginal mucosal from herpes infection and disease.
- CCL28/CCR10 mobilizes memory $CCR10^{+}CD44^{+}CD8^{+}$ T cells into the vaginal mucosa.
- CCL28/CCR10 mobilizes memory $CCR10^{+}B220^{+}CD27^{+}$ B cells into the vaginal mucosa.

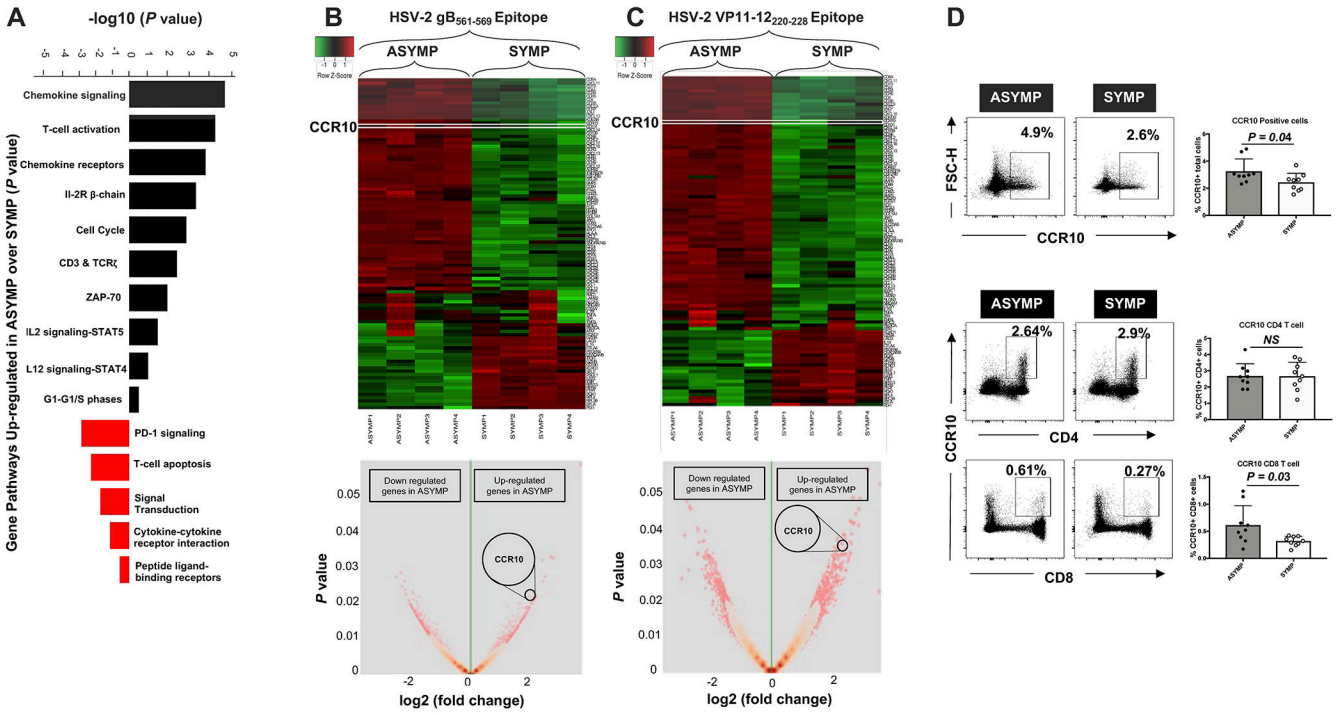

Figure 1. CCR10 expression level in CD8$^+$ T cells from PBMC of herpes-infected SYMP compared to ASYMP patients.

(**A**) Major gene-specific pathways detected in CD8$^+$ T cells from PBMC of herpes-infected SYMP compared to ASYMP patients. (**B**) Differential gene expression (DGE) analysis using bulk RNA sequencing for HSV-2 $gB_{561-569}$ epitope-specific CD8$^+$ T cells from SYMP (n = 4) vs. ASYMP patients (n = 4) shown as a heatmap (*top panel*) and a volcano plot (*bottom panel*). (**C**) Differential gene expression (DGE) analysis using bulk RNA sequencing for $VP11\text{-}12_{220\text{-}228}$ epitope-specific CD8$^+$ T cells from SYMP vs. ASYMP patients heatmap (*top panel*) and a volcano plot (*bottom panel*). (**D**) Representative dot plots showing the frequency of CCR10 in total lymphocytes from SYMP compared to ASYMP patients (*top left panel*). Average frequencies of CCR10 in total lymphocytes from PBMC of SYMP (n = 9) and ASYMP (n = 9) HSV-1 infected patients (*top right panel*). Representative dot plots showing the frequency of CCR10$^+$CD4$^+$ T cells and CCR10$^+$CD8$^+$ T cells from SYMP compared to ASYMP patients (*bottom left panels*). Average frequencies of CCR10$^+$CD4$^+$ T cells and CCR10$^+$CD8$^+$ T cells from PBMCs of SYMP (n = 9) and ASYMP (n = 9) HSV-1 infected patients (*bottom right panels*). The results are representative of two independent experiments. The indicated P values are calculated using the unpaired t-test, comparing results obtained from SYMP vs. ASYMP patients.

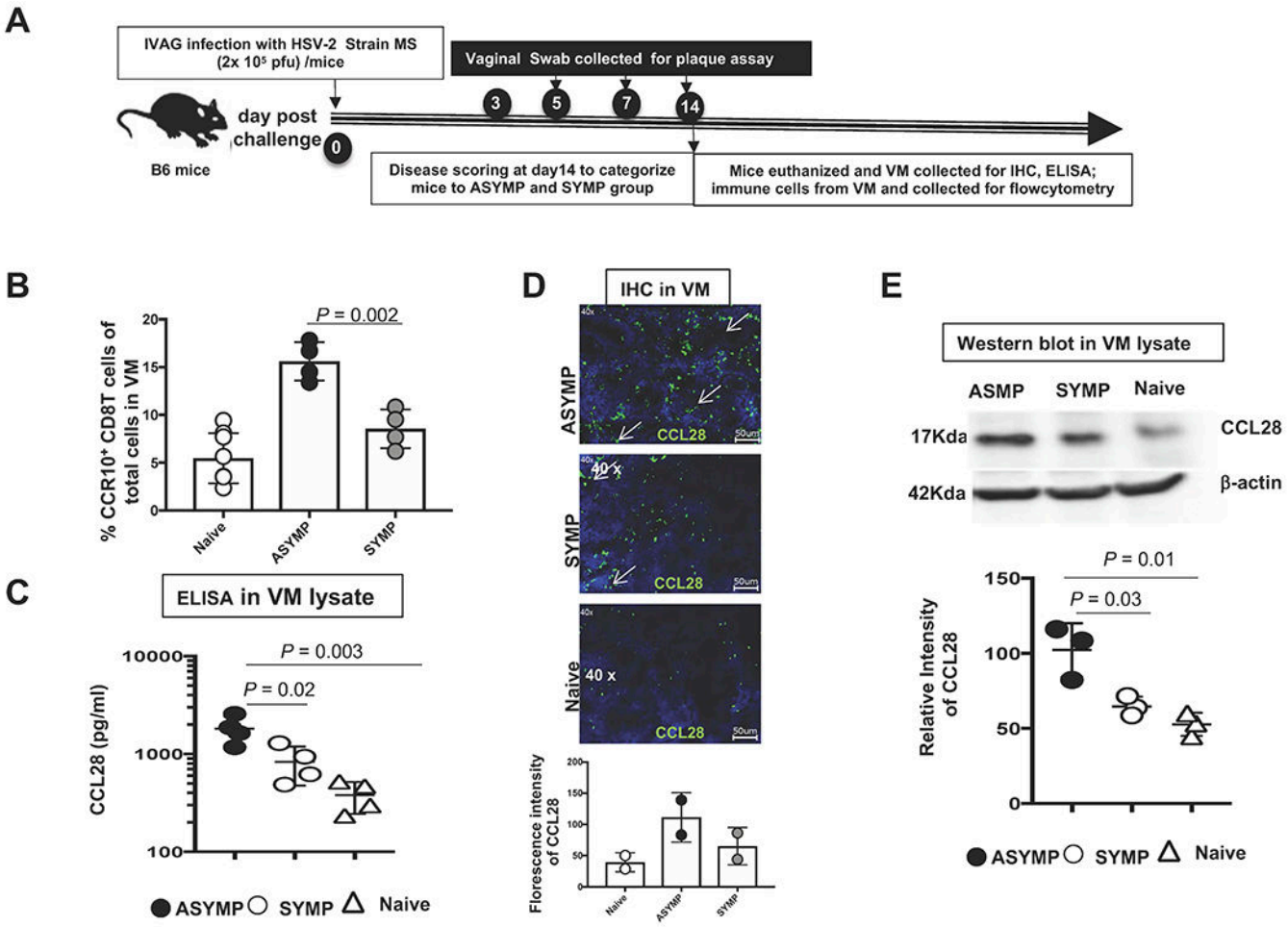

Figure 2. Production of CCL28 chemokines in the vaginal mucosa of HSV-2-infected SYMP and ASYMP B6 mice.

(**A**) Experimental plan showing B6 mice (*n* = 20) were infected intra-vaginally (IVAG) with 2 x 10^5 pfu of HSV-2 (strain MS). The severity of genital herpes disease was scored for 14 days to segregate mice into SYMP or ASYMP groups, as described in the *Material and Methods.* On day 14 post-infection (dpi), SYMP and ASYMP mice and non-infected naïve mice (controls were euthanized and the vaginal mucosae were harvested and cell extracts were assayed by flow cytometry for frequencies of $CD8^+$ T cells expressing CCR10, the receptor of CCL28 (i.e., $CCR10^+CD8^+$ T cells), and for CCL28 chemokine using IHC and ELISA. (**B**) Frequency of $CCR10^+CD8^+$ cells among total VM cells determined by flow cytometry in individual HSV-infected ASYMP (n = 4), SYMP (*n* = 4), and control non-infected (*naïve*) (*n* = 8) B6 mice. (**C**) The level of CCL28 chemokine quantified by ELISA (Abcam kit: *ab210578*) in the VM lysates of HSV-infected symptomatic (*SYMP*) B6 mice, HSV-infected asymptomatic B6 mice (*ASYMP*), and non-infected control B6 mice (*Naïve*). VM lysates from each mouse (*n* = 3) were pooled for this experiment. (**D**) Immunohistochemical staining of CCL28 (green) and DAPI (blue) in VM sections harvested on day 8 post-infection (dpi), from ASYMP, SYMP, and Naïve B6 mice. The lower panel shows a graph summarizing the fluorescence intensity (quantitated using Fiji) for CCL28 in the VM of mice. (**E**) Immunoblot of VM lysates from ASYMP, SYMP, and Naïve B8 mice

(*n* = 3) probed using western blot for CCL28 (Abcam mAb clone ab23155) (*top panel*). The relative intensity of CCL28 normalized to b-actin is shown in the *bottom panel*. The results are representative of two independent experiments. The indicated *P* values are calculated using the unpaired t-test, comparing results obtained in SYMP vs. ASYMP and results obtained in ASYMP vs. Naïve mice.

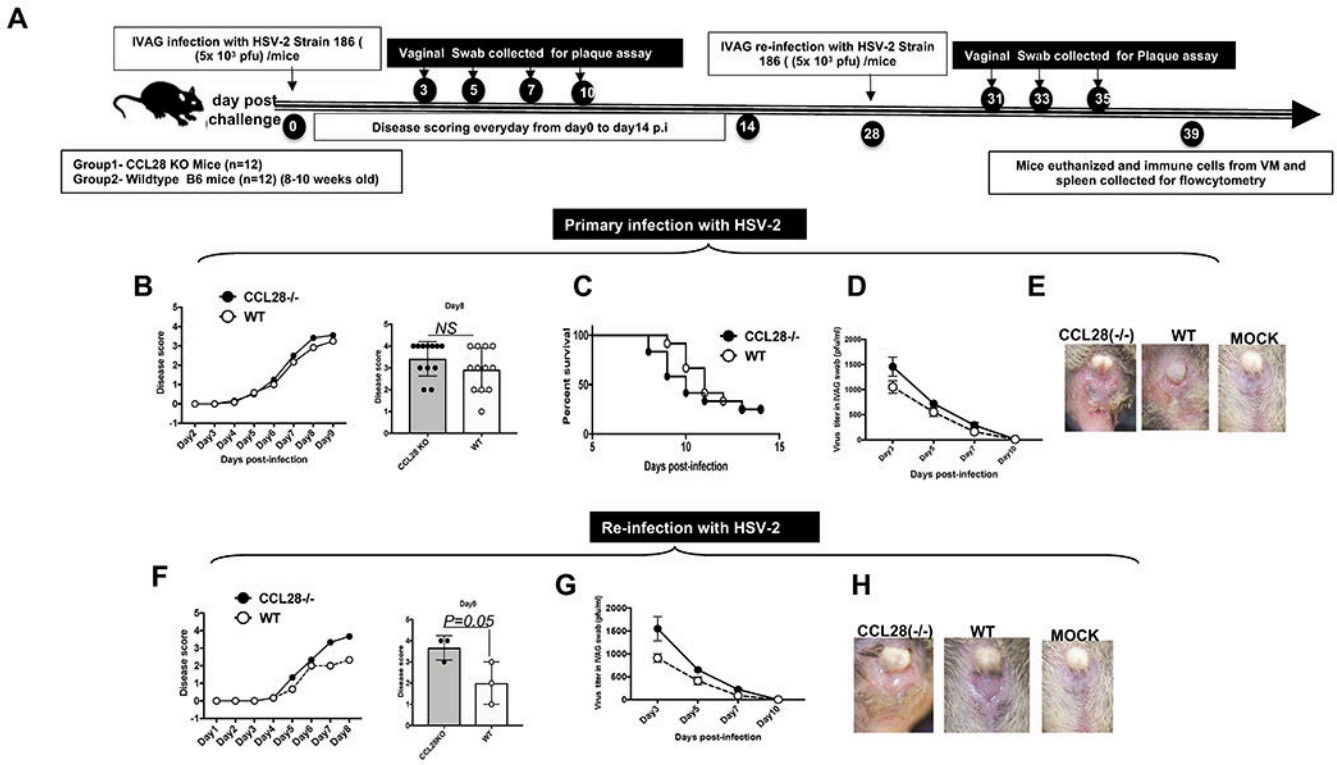

Figure 3. Susceptibility of CCL28$^{(-/-)}$ knockout mice and B6 wild-type mice to genital herpes infection and disease following intravaginal infection and re-infection with HSV-2.

(**A**) CCL28 KO mice (n = 12) and WT B6 mice (n = 12) were infected with IVAG with 5 x 10^3 pfu of HSV-2 (strain 186). CCL28 KO and WT B6 mice were scored every day for 8 to 9 days p. I for symptoms of genital herpes and severity of genital herpes scored, as described in *Material and Methods.* The disease was scored as 0- no disease, 2- swelling and redness of external vagina, 3- severe swelling and redness of vagina and surrounding tissue and hair loss in the genital area, 4- ulceration and hair loss in the genital and surrounding tissue. The vaginal swabs were collected on days 3, 5, and 7 p. I to determine virus titers. (**B**) Disease scoring in CCL28 KO mice (*CCL28*$^{-/-}$) (n = 12) and WT B6 mice (*WT*) (n = 12) was determined for 9 days after primary infection with HSV-2 strain 186 (*left panel).* The maximal disease severity in CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (*WT*) was determined 8 days after primary infection with HSV-2 strain 186(*right panel).* (**C**) Survival graph of in CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (*WT*) determined for 14 days after primary infection with HSV-2. (**D**) The graph shows the virus titers detected in the vaginal swabs of CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (*WT*) collected on 3-, 5-, 7-, and 10-days post-primary infection with HSV-2. (**E**) Representative pictures of genital disease in CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (*WT*) taken on day 8 post-primary infection with HSV-2. (**F**) CCL28 KO mice (n = 3) and WT B6 mice (n = 3) were re-infected with IVAG with 5 x 10^3 pfu of HSV-2 (strain 186) on day 28 post-primary infection. Disease scoring in CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (*WT*) was determined for 9 days after secondary re-infection with HSV-2 strain 186 (*left panel).* The maximal disease severity in CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (*WT*) was determined 8 days after secondary re-infection with HSV-2 (*right panel).* (**G**) The graph

shows the virus titers detected in the vaginal swabs of CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (*WT*) collected 5-, 7-, and 10-day post-secondary infection with HSV-2. (**H**) Representative pictures of genital disease in CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (*WT*) taken on day 8 post-secondary infection with HSV-2. The results are representative of two independent experiments. The indicated *P* values were calculated using the unpaired t-test and compared results obtained from CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (*WT*).

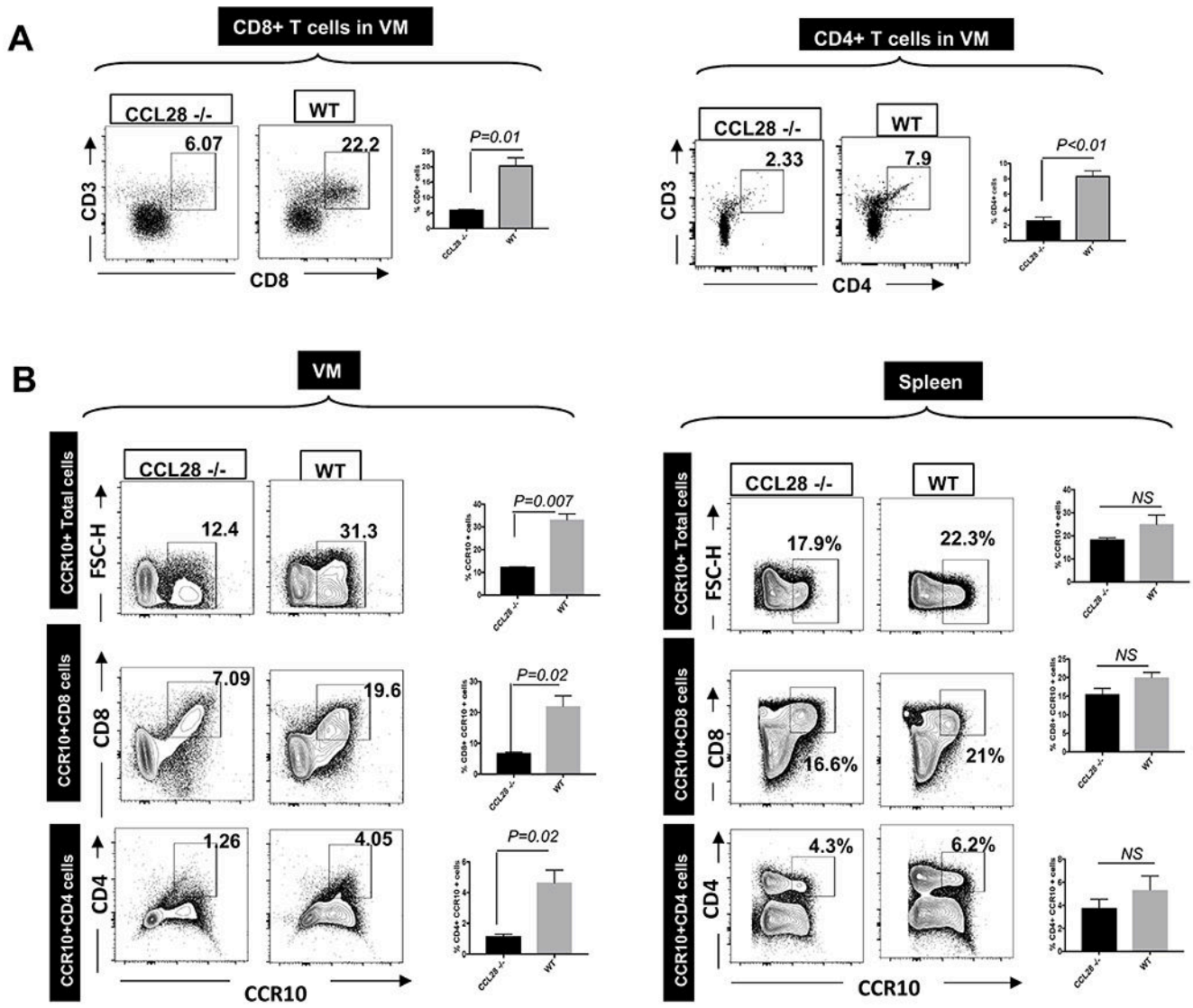

Figure 4. Frequencies of CD8$^+$ and CD4$^+$ T cells expressing CCR10, the receptor of CCL28, in the vaginal mucosa of CCL28$^{(-/-)}$ knockout mice and B6 wild-type mice following intravaginal infection and re-infection with HSV-2.

CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (n = 20) were IVAG infected with 5 x 10^3 pfu of HSV-2 strain 186 and then re-infected with 5 x 10^3 pfu of the same strain of HSV-2 on day 28 p.i. On day 10 post-final and secondary infection, mice were euthanized, and cell suspension from the vaginal mucosa (VM) and spleen was analyzed by flow cytometry for frequencies of CD8$^+$ and CD4$^+$ T cells expressing CCR10, the receptor of CCL28. (**A**) Representative and average frequencies of total CD8$^+$ T cells (*left panels*) and total CD4$^+$ T cells (*right panels*) in the VM of CCL28 KO mice (*CCL28*$^{-/-}$) (n = 3) and WT B6 mice (n = 3) 10 days following re-infection with HSV-2. (**B**) Average frequencies of total CCR10$^+$ T cells (*top panels*), CCR10$^+$CD8$^+$ T cells (*middle panels*), and CCR10$^+$CD4$^+$ T cells (*bottom panels*) detected in the VM (*right panels*) and spleen (*left panels*) of CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice 10 days following re-infection with HSV-2. The results are

representative of two independent experiments. The indicated *P* values were calculated using the unpaired *t*-test and compared results obtained from CCL28$^{(-/-)}$ and WT mice.

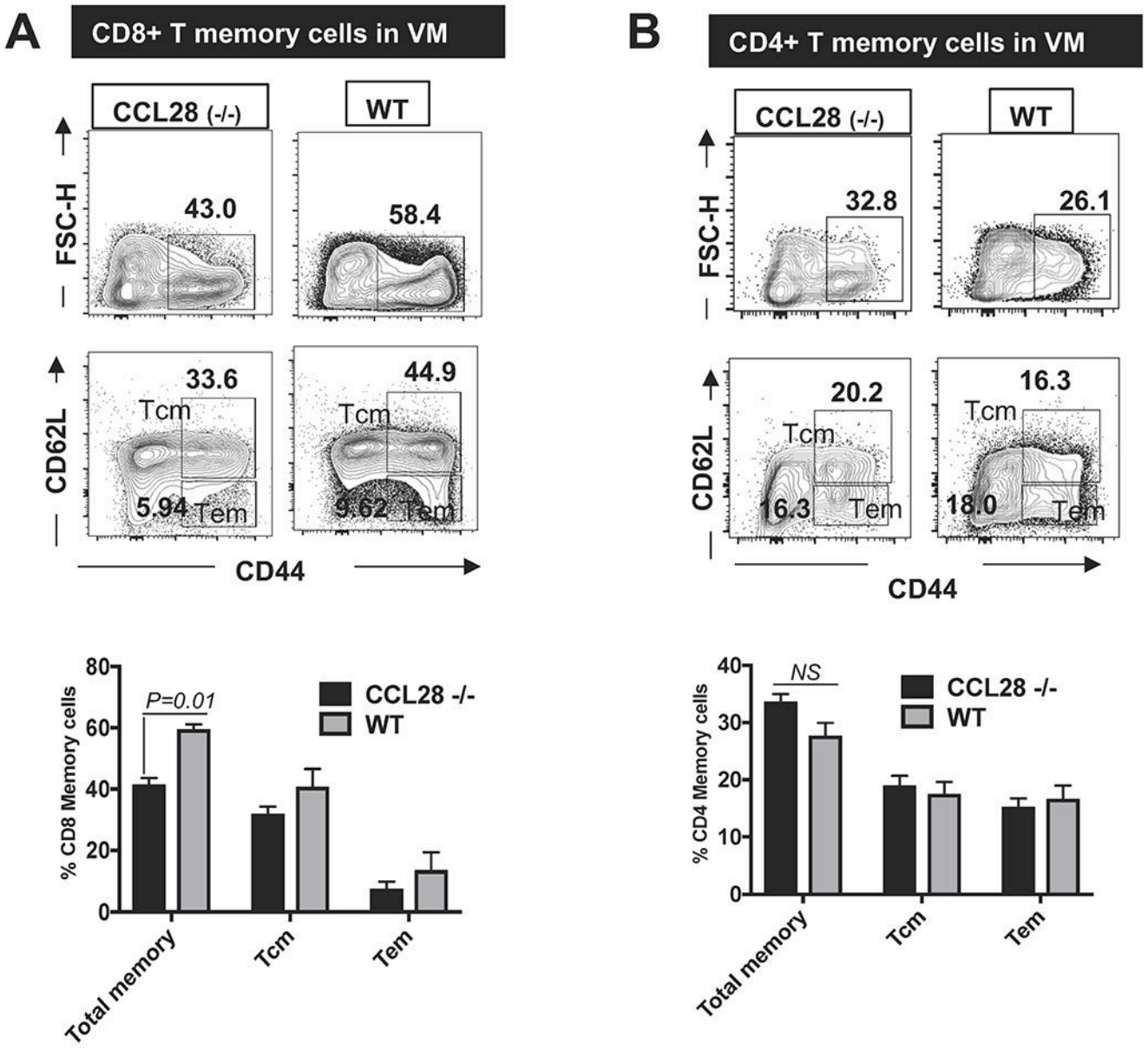

Figure 5. Frequencies of central and effector memory CD44$^+$CD8$^+$ and CD44$^+$CD4$^+$ T cells in the vaginal mucosa of CCL28$^{(-/-)}$ knockout mice and B6 wild-type mice following intravaginal infection and re-infection with HSV-2.

CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (n = 20) were IVAG infected with 5 x 10^3 pfu of HSV-2 strain 186 and then re-infected with 5 x 10^3 pfu of the same strain of HSV-2 on day 28 p.i. On day 10 post-re-infection, mice were euthanized and the frequencies of central memory CD44$^+$CD62L$^+$CD8$^+$ T_{CM} cells and CD44$^+$CD62L$^+$CD4$^+$ T_{CM} cells and of effector memory CD44$^+$CD62L$^-$CD8$^+$ T_{EM} cells and CD44$^+$CD62L$^-$CD4$^+$ T_{EM} cells were compared in the vaginal mucosa of CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice using flow cytometry. (**A**) Representative data of the frequencies of total memory CD8$^+$ T cells (*top 2 panels*) and central memory CD44$^+$CD62L$^+$CD8$^+$ and effector memory CD44$^+$CD62L$^-$CD8$^+$ T cells (*middle 2 panels*) in VM of CCL28 KO

mice (*CCL28*$^{-/-}$) and WT B6 mice re-infected with HSV-2. Average frequencies of total memory CD8$^+$ T cells and central memory CD44$^+$CD62L$^+$CD8$^+$T$_{CM}$ cells and effector memory CD44$^+$CD62L$^-$CD8$^+$ T$_{EM}$ cells (*bottom panel*) in the VM of CCL28$^{-/-}$ and WT B6 mice are re-infected with HSV-2. (**B**) Representative data of the frequencies of total memory CD4$^+$ T cells (*top 2 panels*) and central memory CD44$^+$CD62L$^+$CD4$^+$ and effector memory CD44$^+$CD62L$^-$CD4$^+$ T cells (*middle 2 panels*) in VM of CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice re-infected with HSV-2. Average frequencies of total memory CD4$^+$ T cells and central memory CD44$^+$CD62L$^+$CD4$^+$T$_{CM}$ cells and effector memory CD44$^+$CD62L$^-$CD4$^+$ T$_{EM}$ cells (*bottom panel*) in the VM of CCL28$^{-/-}$ and WT B6 mice are re-infected with HSV-2. The indicated *P* values were calculated using the unpaired *t*-test and compared results obtained from CCL28$^{(-/-)}$ (n = 3) and WT mice (n = 3) and the results are representative of two independent experiments.

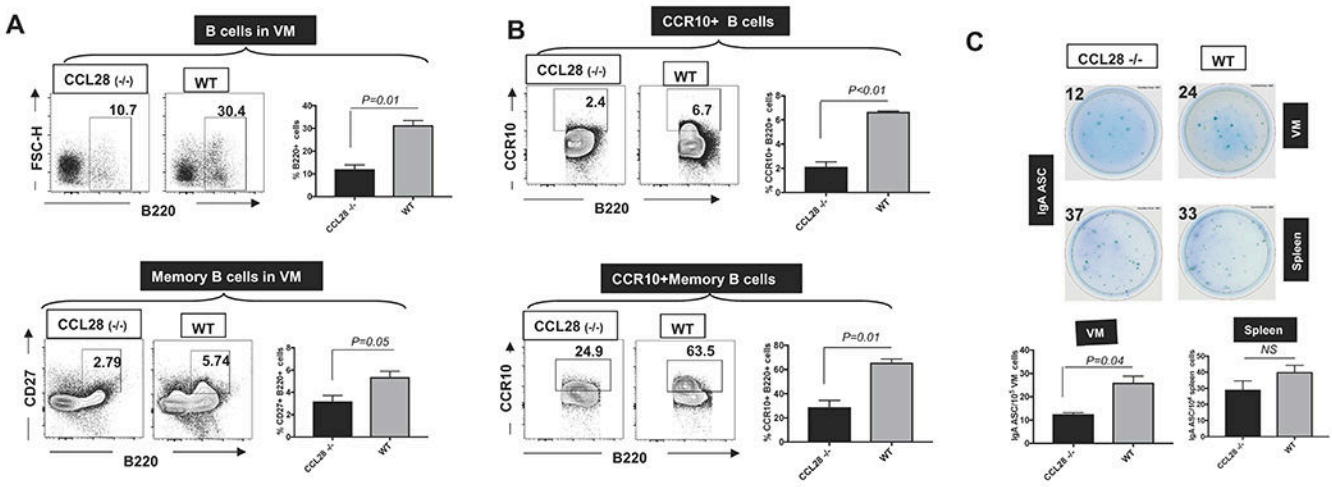

Figure 6. Frequencies of total B cells and memory B cells in the vaginal mucosa of CCL28$^{(-/-)}$ knockout mice and B6 wild-type mice following intravaginal infection and re-infection with HSV-2.

CCL28 KO mice (*CCL28*$^{-/-}$) and WT B6 mice (n = 20) were IVAG infected with 5 x 10^3 pfu of HSV-2 strain 186 and then re-infected with 5 x 10^3 pfu of the same strain of HSV-2 on day 28 p.i. On day 10 post-re-infection, mice were euthanized and the frequencies of total B220$^+$B cells and memory B220$^+$B cells, expressing the expressing CCR10, the receptor of CCL28, were determined for flow cytometry in the VM and spleen of CCL28 KO mice and WT B6 mice. (**A**) Representative (*left 4 panels*) and average (*right 2 panels*) frequencies of total B220$^+$B cells (*top 3 panels*) and memory B cells (*bottom 3 panels*) in VM of CCL28 KO mice (n = 3) and WT B6 mice (n = 3) , 10 days following re-infection with HSV-2. (**B**) Representative (*left 4 panels*) and average (*right 2 panels*) frequencies of total B cells expressing CCR10, the receptor of CCL28, (CCR10$^+$B220$^+$ B cells *top 3 panels*), and of memory B cells expressing CCR10 (CCR10$^+$B220$^+$CD27$^+$ memory B cells, *bottom 3 panels*) were determined in the VM of CCL28 KO mice and WT B6 mice 10 days following re-infection with HSV-2. (**C**) The ELISPOT images show IgA ASC in the VM (*top*) and spleen (*middle*) of CCL28 KO mice and WT B6 mice 10 days following re-infection with HSV-2. Corresponding average SFU for IgA ASC in the VM and Spleen are shown in the 2 *bottom panels*. The results are representative of two independent experiments. *P* values were calculated using the unpaired *t*-test and compared with results obtained in CCL28 KO mice and WT B6 mice.