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Herpes Simplex Virus Type 2 Infection of Human Epithelial Cells Induces CXCL9 Expression and CD4+ T Cell Migration via Activation of p38-CCAAT/Enhancer-Binding Protein-β Pathway

Wenjie Huang,* Kai Hu,*† Sukun Luo,*† Mudan Zhang,*‡ Chang Li,*† Wei Jin,*† Yalan Liu,* George E. Griffin,‡ Robin J. Shattock,§ and Qinxue Hu*‡

Recruitment of CD4+ T cells to infection areas after HSV-2 infection may be one of the mechanisms that account for increased HIV-1 sexual transmission. Lymphocytes recruited by chemokine CXCL9 are known to be important in control of HSV-2 infection in mice, although the underlying mechanism remains to be addressed. Based on our observation that CXCL9 expression is augmented in the cervical mucus of HSV-2–positive women, in this study we demonstrate that HSV-2 infection directly induces CXCL9 expression in primary cervical epithelial cells and cell lines, the principal targets of HSV-2, at both mRNA and protein levels. Further studies reveal that the induction of CXCL9 expression by HSV-2 is dependent upon a binding site for C/EBP-β within CXCL9 promoter sequence. Furthermore, CXCL9 expression is promoted at the transcriptional level through phosphorylating C/EBP-β via p38 MAPK pathway, leading to binding of C/EBP-β to the CXCL9 promoter. Chemotaxis assays indicate that upregulation of CXCL9 expression at the protein level by HSV-2 infection enhances the migration of PBLs and CD4+ T cells, whereas neutralization of CXCL9 or inhibition of p38-C/EBP-β pathway can significantly decrease the migration. Our data together demonstrate that HSV-2 induces CXCL9 expression in human cervical epithelial cells by activation of p38-C/EBP-β pathway through promoting the binding of C/EBP-β to CXCL9 promoter, which may recruit activated CD4+ T cells to mucosal HSV-2 infection sites and potentially increase the risk of HIV-1 sexual transmission. The Journal of Immunology, 2012, 188: 000–000.

Herpes simplex virus type 2 (HSV-2) is a large dsDNA virus belonging to the herpesviridae family (1, 2). HSV-2 is the main cause of genital herpes, and its infection is common in the lower genital tract (3, 4). Although neuronal and immune cells can be infected, epithelial cells and keratinocytes are the primary HSV-2 target cells (5). It has been well documented from epidemiological studies that HSV-2 infection increases the risk of HIV-1 acquisition and transmission (6–9). Several mechanisms for this increased susceptibility have been proposed. For instance, the formation of pustules and ulcers by genital herpes may facilitate HIV-1 entry into mucosal tissues (10, 11). Analyses of biopsies of HSV-2 lesions from patients revealed that HSV-2 reactivation resulted in an influx of activated CD4+ T cells, which may facilitate HIV-1 infection and subsequent dissemination (12, 13). Immune activation of HIV-1 target cells, including dendritic cells and CD4+ T cells by HSV-2 infection, also likely enhances HIV-1 susceptibility (14). However, because HSV-2 preferentially infects epithelial cells, the molecular mechanisms underlying immune cell activation and migration after HSV-2 infection remain to be determined.

Based on previous findings that intact human cervicovaginal epithelium provides a significant barrier to HIV-1 infection (15–17), we hypothesized that HSV-2 infection of mucosal epithelium might recruit CD4+ T cells, the main target cells in early HIV-1 infection (18, 19), to infection areas and facilitate HIV-1 transmission via perturbation of epithelial integrity. Chemokines constitute a family of small, secretory proteins that are expressed constitutively or in an inducible manner. Their main functions are to recruit leukocytes to sites of infection and inflammation and to contribute to the homeostatic circulation of leukocytes (20, 21). Most chemokines share four cysteine residues that are thought to be crucial for the tertiary structure of the proteins. The four subfamilies of chemokines, CXC, CC, C, and CX3C, are characterized by the position of the N-terminal cysteine residues (20, 21). Recent study indicates that chemokine CXCL9 can be induced in mice after HSV-2 infection and its expression is crucial for control of genital HSV-2 infection (22), although the mechanism remains to be addressed. The receptor for CXCL9, CXCR3, is functionally expressed on activated T and B cells, NK cells, and endothelial cells (23, 24). CXCL9, mainly induced by IFN-γ in macrophages, is implicated in chronic inflammation and viral infections as well as in T cell trafficking, chemotaxis, and activation (25). Because the infiltration of inflammatory cells in genitai tissues in turn may

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Abbreviations used in this article: HSV-2, herpesvirus type 2; ISRE, IFN-stimulated response element; MOI, multiplicity of infection; siRNA, small interfering RNA.

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increase the frequency of HIV-1 transmission, it is important to understand whether HSV-2 can promote CXCL9 expression in human epithelial cells.

In the current study, we observed that CXCL9 expression was elevated in cervical mucus samples of HSV-2–positive women. By using human cervical epithelial cells as a model, we demonstrated a direct induction of CXCL9 expression induced by HSV-2 infection. We further explored the molecular mechanisms underlying HSV-2–induced CXCL9 expression and CD4+ T cell migration. Our findings indicate that HSV-2 infection directly induces the expression of CXCL9, and that a C/EBP-β binding site in CXCL9 core promoter region is critical for transcriptional activation of CXCL9 by HSV-2. Further study identified a critical role played by p38-C/EBP-β pathway in the HSV-2–induced CXCL9 expression and CD4+ T cell migration. Our study provides a potential new mechanism underlying the enhancement of HIV-1 acquisition and transmission by HSV-2 infection.

Materials and Methods
Cervical mucus, primary epithelial cells, PBMCs, and CD4+ T cells
Cervical mucus samples of women aged 20–59 y were obtained from Renmin Hospital at Wuhan, with 45 samples serologically HSV-2 positive and 12 HSV-2 negative confirmed by ELISA and PCR.

Human cervical tissues were obtained from women undergoing planned therapeutic hysterecscopy in the absence of any cervical pathology at Hubei Hospital of Traditional Chinese Medicine. Primary human cervical epithelial cells were isolated, as described previously (26, 27). Briefly, tissues were cut into 3-mm × 3-mm size and digested by collagenase for 30 min. The separated cells were filtered through a stainless steel strainer (0.5–1.0 mm) and cultured in complete medium for 3 h, and then the inflammatory cells were discarded.

PBMCs were isolated from healthy donors by using a Ficoll-Hypaque density gradient. CD4+ T cells were separated from PBMCs using CD4+ T Cell Negative Isolation Kit, according to the manufacturer’s protocol (Miltenyi Biotec). PBMCs and CD4+ T cells were activated by stimulating with 1 μg/ml PHA (Sigma-Aldrich) for 3 d. Subsequent cultures were in the presence of 20 U/ml IL-2 (R&D Systems).

The approach for constructing CXCL9 promoter mutants (−495/+33)CXCL9, (−185/+33)CXCL9, and (−120/+33)CXCL9, and for site-directed mutagenesis of the NF-κB and C/EBP sites was described previously (28). Construct (−495/+33)CXCL9 in pgI3-Basic was used as a template to generate (−67/+33)CXCL9 using primers 5'-TTGGAGGCTAGAATACTATCTAAAT-3' (forward) and 5'-TTGGAGGCTAGAATACTATCTAAAT-3' (reverse). The full open reading frame of C/EBP-β was purchased from Origene and amplified using primers 5'-TGGTGAATTCTAGAATACTATCTAAAT-3' (forward) and 5'-TGTTGTGGCAGATGAGAATACTATCTAAAT-3' (reverse). The PCR products were subcloned into pcDNA3.1(+)- between the EcoRI and XhoI sites.

HSV-2 infection
Human cervical epithelial cells were cultured in 24-well plates to 90–95% confluence monolayer. Prior to HSV-2 infection, culture was replaced with fresh medium. One to five multiplicities of infection (MOI) of virus stocks were added to culture medium and incubated for 1 h in a 5% CO2 incubator. Culture medium was changed, and cells were cultured for the indicated time before next assay. UV-inactivated virus was obtained by exposure of HSV-2 to UV irradiation for 15 min, as described previously (29). To separate virus particles from the cytokines secreted by Vero cells, virus stocks were added to 100-kDa centrifugal filter unit (Millipore) and centrifuged for 30 min at 1000 × g. Membrane-retained HSV-2 was diluted and used to infect ME-180 cells.

RNA extraction and semiquantitative RT-PCR
Total RNA was extracted using TRIzol (Invitrogen), according to the manufacturer’s protocol. RNase-free DNase (Promega) was used to eliminate the contamination of genomic DNA. cDNA was synthesized, as described previously (30). The newly synthesized cDNA was amplified by PCR. The primer pairs for CXCL9 were 5'-GGCATCATCTTGTCTGTTCT-3' and 5'-TCACATCTCTCTTGATCGC-3'. β-actin was used as an internal control amplified with primers 5'-CTGGGGGCTCCCAAGGCACCA-3' and 5'-CTCCTTAATGTCACGACGATTC-3'.

ELISA
Cervical mucus samples and culture supernatants of HSV-2–infected epithelial cells were assayed for CXCL9 expression with a commercial ELISA kit, according to the manufacturer’s instructions (R&D Systems). For HSV-2–infected epithelial cells, cells were infected with HSV-2 (1–5 MOI) for the indicated time and supernatants were collected. The concentration of CXCL9 was determined using a standard curve obtained with rCXCL9 (R&D Systems).

Transfection and luciferase reporter gene activity assay
Each promoter construct together with the pRL-TK plasmid were cotransfected into subconfluent (80–90%) monolayer cells expressing Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Five hours posttransfection, cells were cultured alone or infected with HSV-2 (1–5 MOI) in fresh medium. Twenty-four hours after infection, cells were lysed and luciferase activity was assayed using the Dual Luciferase Assay Kit (Promega), according to the manufacturer’s instructions. The relative luciferase activity was determined in a Modulus Microplate Luminometer (Turner Biosystems), and transfection efficiency was normalized by Renilla activity. Transfection of primary cervical epithelial cells was performed by using Amaxa Nucleofector Kit for primary epithelial cells, according to the manufacturer’s instructions (Amaza).

Western blotting
Cells were lysed with lysis buffer (50 mM Tris [pH 7.2], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl2 with protease inhibitor) (Roche). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Nonspecific binding sites were blocked with 5% nonfat milk in TBST (120 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. Blots were incubated with Ab against C/EBP-β, p38, p-p38, and β-actin, respectively, with overnight at 4˚C. Membranes were then washed three times, incubated with HRP-conjugated secondary Ab, and visualized with Dura SuperSignal Substrate (Pierce).

EMSA
Nuclear extracts from ME-180 cells were prepared, as described previously (31). Biotin end-labeled oligonucleotides were synthesized and annealed to obtain dsDNA fragments. The oligonucleotide sequences were as follows: 5'-GCTAAAGATACATTCCAGGATTTGA-3' and 5'-GTTCAATTCTGGCAGATGTCTA-3'. EMSA was performed using LightShift Chemiluminescent EMSA Kit (Pierce), according to the manufacturer’s protocol. Briefly, DNA-binding reactions were performed in 20 μl samples containing 20 fmol biotin-labeled oligonucleotides, 4 mg nuclear extracts, 2 mg dI-LC, 2 μl 10× binding buffer, 0.1 mM EDTA, and 10% glycerol at room temperature for 20 min. For supershift assay, 2 μl polyclonal Ab against C/EBP-β (Santa Cruz Biotechnology) was also added. For competition EMSA, 100-fold unlabeled oligonucleotides were added into the first reaction prior to the addition of labeled probe. The mixture was incubated for 20 min at room temperature. Samples were run on 6% nondenaturing polyacrylamide gels with 0.5× TBE buffer (25 mM Tris [pH 8.3], 190 mM glycine, 1 mM EDTA) and transferred onto a nylon membrane.
membrane (Amersham Biosciences, Pharmacia) at 380 mA (∼100 V) for 30 min. A chemiluminescent detection method containing luminol/enhancer solution and stable peroxide solution was used, as described by the manufacturer. Membranes were scanned by Multimage Light Cabinet (Alpha Innotech).

Chromatin immunoprecipitation assay

Cells growing in T25 flasks alone or infected with HSV-2 were cross-linked using 1% formaldehyde at 37˚C for 10 min. After washes with PBS, cells were resuspended in 300 µl lysis buffer (50 mM Tris [pH 8.1], 10 mM EDTA, 1% SDS) containing protease inhibitor. DNA was sheared to small fragments by sonication. Supernatants were precleared using herring sperm DNA/protein G-Sepharose slurry (Sigma-Aldrich). The recovered supernatants were incubated with either anti-C/EBP-β Ab (Santa Cruz) or an isotype control IgG for 2 h in the presence of herring sperm DNA and protein G-Sepharose beads. The immunoprecipitated DNA was retrieved from the beads with 1% SDS and 1.1 M NaHCO3 solution at 65˚C for 6 h. The extracted DNA was used for PCR with CXCL9 promoter-specific primers, as follows: 5'-CAAGTTTGTGGTCAATTTAG-3' and 5'-CTTTAGAAGAACACATTGG-3'.

Migration assay

Migration assay was performed using 24-well Transwell plates (Costar). A total of 600 µl supernatants from human cervical epithelial cells alone or infected with HSV-2 was added to the lower chamber. Activated PBLs (5 × 105) or CD4+ T cells (5 × 105) in 100 µl RPMI 1640 medium were placed in the upper chamber. Chambers were separated by a 3-µm pore-size polycarbonate membrane. The chambers were incubated for 2 h at 37˚C in 5% CO2 incubator, and the Transwell inserts were then removed.

Results

CXCL9 expression is elevated in the cervical mucus of HSV-2–positive women

CXCL9 is a chemokine that recruits activated T cells to infection area. Recently, it has been reported that the expression of CXCL9 was enhanced in HSV-2–infected mice (22), although the underlying mechanism remains to be addressed. CXCL9 levels in cervical mucus from HSV-2–negative (n = 41) and HSV-2–positive (n = 45) women were determined by ELISA. We observed that CXCL9 levels in cervical mucus from HSV-2–positive women
were significantly higher than those from HSV-2–negative women (Fig. 1A), suggesting that CXCL9 may play an important role during HSV-2 infection.

**HSV-2 induces CXCL9 expression in human cervical epithelial cells**

The primary targets for HSV-2 are epithelial cells. To investigate whether HSV-2 infection can promote CXCL9 expression in human cervical epithelial cells, HeLa and ME-180 cells were infected with HSV-2. The protein level of CXCL9 in supernatants collected at different time points postinfection was measured. The level of CXCL9 protein significantly increased in cells infected with HSV-2, and its expression was elevated as the time of infection increased (Fig. 1B). Because HSV-2 significantly induced CXCL9 expression at 24 h postinfection and there were barely dead cells at this time point, subsequent experiments were performed at 24 h postinfection. We next investigated the mRNA level of CXCL9 after HSV-2 infection. Semiquantitative RT-PCR assay demonstrated that HSV-2 infection also enhanced the level of CXCL9 mRNA in infected cells (Fig. 1D).

To further confirm the effect of HSV-2 on the induction of CXCL9, primary epithelial cells were isolated from human cervical...
To determine whether HSV-2–induced CXCL9 expression is transactivation of CXCL9 promoter at both mRNA and protein levels (Fig. 1C, 1D). Human cervical epithelial cell lines, HSV-2 infection significantly increased CXCL9 expression in tissues and infected with HSV-2, and the mRNA and protein levels of CXCL9 were examined. In agreement with the results from human cervical epithelial cell lines, HSV-2 infection significantly increased CXCL9 expression in primary human cervical epithelial cells at both mRNA and protein levels (Fig. 1C, 1D).

**HSV-2–induced CXCL9 expression is mediated through transactivation of CXCL9 promoter**

To determine whether HSV-2–induced CXCL9 expression is mediated through transactivation of CXCL9 promoter, a reporter plasmid containing the sequence from −495 to +33 (relative to the transcriptional start site) of 5′-flanking region of human CXCL9 gene was transfected into ME-180 or HeLa cells prior to HSV-2 infection. CXCL9 promoter activity was significantly enhanced after HSV-2 infection (Fig. 2A, 2B). Furthermore, the level of luciferase activity correlated with the concentration of HSV-2 used for infection, indicating that the activation of CXCL9 promoter by HSV-2 infection is concentration dependent.

To exclude the possibility that CXCL9 induction was due to transactivation of CXCL9 promoter caused by cytokines from HSV-2–infected Vero cells, HSV-2 stocks were filtered using 100-kDa centrifugal filter unit. ME-180 cells were transfected with (−495/+33)CXCL9 and then infected with the retained HSV-2 on membrane. Membrane-retained HSV-2 significantly promoted CXCL9 expression, whereas virus-free supernatants had no effect (Fig. 2C). Furthermore, HSV-2 or UV-inactivated HSV-2 was used to infect cells transfected with (−495/+33)CXCL9, showing that UV-inactivated virus had little effect on transactivation of CXCL9 promoter (Fig. 2D). These data together indicate that productive HSV-2 infection directly induces CXCL9 expression by transactivation of CXCL9 promoter in human cervical epithelial cells.

**C/EBP-binding element is involved in HSV-2–mediated CXCL9 induction**

Bioinformatic analysis indicated the presence of several consensus cis elements, including NF-κB, AP-1, IFN-stimulated response element (ISRE), and C/EBP binding sites in the CXCL9 promoter (Fig. 2E). Because findings in the current study demonstrated a transcriptional activation of CXCL9 promoter by HSV-2, we therefore investigated the roles of these cis elements in the regulation of HSV-2–induced CXCL9 gene transcription. The reporter constructs containing serial 5′ deletions of CXCL9 promoter were transfected into ME-180 cells. Five hours posttransfection, cells were cultured alone or infected with HSV-2. Deletion from nt −495 to −129 did not affect HSV-2–induced luciferase activity. Further deletion to nt −67 completely eliminated HSV-2–induced luciferase activity (Fig. 2F). These data indicate that the sequence between nt −129 and −67 is essential for the activation of CXCL9 promoter by HSV-2.

Analysis of CXCL9 promoter revealed that there is a potential C/EBP binding site within nt −129 to nt −67 (Fig. 2E). To investigate the role of C/EBP binding site on CXCL9 activation, plasmids with mutation of the C/EBP binding or two neighboring NF-κB binding sites were constructed. Mutation of the two NF-κB binding sites did not affect HSV-2–induced promoter activity, whereas mutation of C/EBP binding site significantly abolished HSV-2–induced CXCL9 activation (Fig. 2G). These results suggest that C/EBP binding site is essential for the HSV-2–induced activation of CXCL9 promoter.

**C/EBP-β binds to CXCL9 promoter after HSV-2 infection**

Six members of C/EBPs, including C/EBP-α, C/EBP-β, C/EBP-γ, C/EBP-δ, C/EBP-ε, and C/EBP-ζ, have been characterized, whereas C/EBP-β is an important transcriptional activator in the regulation of genes involved in immune and inflammatory responses (32). To determine whether C/EBP-β binds to CXCL9 promoter after HSV-2 infection, EMSA was performed using biotin-labeled C/EBP-β consensus oligonucleotides in CXCL9 promoter (−99 to −70) as probes. ME-180 cells were cultured alone or infected with HSV-2, and the nuclear extracts were incubated with biotin-labeled probes at room temperature. The binding activity of C/EBP-β significantly increased in cells infected with HSV-2, compared with uninfected cells (Fig. 3A).

To determine the specificity of C/EBP-β–binding activity, nuclear extracts were incubated with the labeled C/EBP-β consensus oligonucleotides in the presence of either an unlabeled wild-type C/EBP-β–binding probe or a mutated probe. Wild-type C/EBP-β consensus oligonucleotides rather than the mutated oligonucleotides significantly abrogated C/EBP-β complexes (Fig. 3A). We confirmed the authenticity of C/EBP-β band using supershift assay by incubation of nuclear extracts with Ab against C/EBP-β, showing that incubation of the nuclear extracts with anti-C/EBP-β Ab supershifted the specific retardation signal (Fig. 3A).

Chromatin immunoprecipitation assay was also performed to determine whether C/EBP-β binds to CXCL9 promoter in vivo. Chromatin fragments were prepared from ME-180 cells infected with HSV-2 and immunoprecipitated with Ab against C/EBP-β. A 110-bp DNA fragment containing the C/EBP-β binding site in CXCL9 promoter was amplified by PCR using specific primers. Results showed that PCR products were only produced from DNA isolated from cells infected with HSV-2 in the presence of anti–C/EBP-β Ab, but not detectable in the presence of control Ab or in uninfected cells (Fig. 3B). These data demonstrate that C/EBP-β binds to the predicted C/EBP binding site in CXCL9 promoter.

**C/EBP-β regulates HSV-2–induced CXCL9 expression**

To confirm the essential roles of C/EBP-β in HSV-2–induced CXCL9 expression, specific siRNA was used to knock down the expression of C/EBP-β. ME-180 cells were cotransfected with (−495/+33)CXCL9 and C/EBP-β siRNA or control siRNA.
Twenty-four hours posttransfection, cells were cultured alone or infected with HSV-2 and subsequently assayed for luciferase activity. The transcriptional activation of CXCL9 promoter induced by HSV-2 was significantly reduced by knockdown of C/EBP-β (Fig. 4A). The impact of C/EBP-β on HSV-2–induced CXCL9 expression was further confirmed by ELISA, showing that C/EBP-β knockdown significantly decreased the protein level of CXCL9 in HSV-2–infected cells (Fig. 4B).

We further determined the effect of C/EBP-β on the transcriptional activation of CXCL9. ME-180 cells were cotransfected with plasmid expressing C/EBP-β and reporter plasmid (−495/+33)CXCL9. The transcriptional activity of CXCL9 was significantly higher in cells transfected with C/EBP-β-expressing constructs compared with that of the control cells (Fig. 4C). The protein level of CXCL9 was also measured in ME-180 cells transfected with plasmids expressing C/EBP-β. Overexpression of C/EBP-β significantly upregulated the expression of CXCL9 protein as assessed by ELISA (Fig. 4D). These results together indicate that C/EBP-β is crucial for HSV-2–induced CXCL9 expression.

**FIGURE 4.** C/EBP-β regulates CXCL9 expression. (A) ME-180 cells were cotransfected with (−495/+33)CXCL9 and C/EBP-β siRNA/control siRNA. Twenty-four hours posttransfection, cells were cultured alone or infected with HSV-2. Luciferase activity was assayed 24 h postinfection. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. (B) ME-180 cells were transfected with C/EBP-β siRNA or control siRNA, and then cultured alone or infected with HSV-2. Twenty-four hours postinfection, the protein level of CXCL9 in supernatants was measured by ELISA. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. (C) ME-180 cells were transfected with (−495/+33)CXCL9 and pcDNA-C/EBP-β or pcDNA3.1. The luciferase activity was measured 24 h posttransfection. The relative luciferase activity was normalized by that of cells transfected with pcDNA3.1. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. (D) ME-180 cells were transfected with pcDNA-C/EBP-β or pcDNA3.1. Twenty-four hours after transfection, the protein level of CXCL9 in supernatants was measured by ELISA. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. (E) ME-180 cells were infected with HSV-2 for 24 h, and the protein levels of C/EBP-β, p-C/EBP-β, and β-actin were determined by Western blot. One representative experiment of three is shown. (F) ME-180 cells were infected with HSV-2 for 24 h, and cytosol and nucleus fractions were prepared. The protein level of C/EBP-β from cytosol and nucleus was determined by Western blot using anti-C/EBP-β Ab. One representative experiment of three is shown. The C/EBP-β gene encodes three in-frame translational isoforms. The Abs against C/EBP-β and phosphor-C/EBP-β used in the current study recognize all three isoforms.

**HSV-2 infection induces phosphorylation of C/EBP-β and translocation of C/EBP-β to nucleus**

To investigate the effect of HSV-2 on C/EBP-β expression, ME-180 cells were cultured alone or infected with HSV-2, and the phosphorylated C/EBP-β and total C/EBP-β protein were analyzed, respectively. HSV-2 had no effect on the level of total C/EBP-β protein, but significantly increased the phosphorylation of C/EBP-β protein on Thr235 (Fig. 4E). We next analyzed the cellular location of C/EBP-β after HSV-2 infection, showing that C/EBP-β persisted in the cytoplasm and nucleus in uninfected cells. After HSV-2 infection, C/EBP-β completely translocated from cytoplasm to nucleus (Fig. 4F). These data indicate that HSV-2 infection induces phosphorylation and nuclear translocation of C/EBP-β.

**HSV-2–induced CXCL9 expression is mediated by activation of p38 MAPK pathway**

It is known that HSV-2 can regulate the expression of downstream genes by activating MAPK pathways (33); however, the underlying mechanisms remain unknown. Therefore, we investigated whether HSV-2-induced CXCL9 expression is mediated by activation of p38 MAPK. We first determined whether HSV-2 infection induced the phosphorylation of p38 MAPK. ME-180 cells were cultured alone or infected with HSV-2, and the phosphorylated p38 MAPK and total p38 MAPK protein were analyzed. HSV-2 had no effect on the level of total p38 MAPK protein, but significantly increased the phosphorylation of p38 MAPK on Thr180 and Tyr182 (Fig. 5A). These results suggest that HSV-2 infection induces phosphorylation of p38 MAPK.

**FIGURE 5.** HSV-2 infection induces phosphorylation of p38 MAPK. (A) ME-180 cells were cultured alone or infected with HSV-2 for 24 h. The protein levels of p38 MAPK, p-p38 MAPK, and β-actin were determined by Western blot. One representative experiment of three is shown.
mechanisms remain elusive. To investigate whether MAPK pathway was involved in transcriptional activation of CXCL9 promoter by HSV-2, ME-180 cells were transfected with (−495/+33) CXCL9 reporter plasmid; pretreated with PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), or SB203580 (p38 inhibitor); and then infected with HSV-2. Luciferase reporter assay showed that pretreatment of cells with SB203580, but not with PD98059 or SP600125, significantly decreased HSV-2–induced CXCL9 promoter activity (Fig. 5A).

We then determined the impact of HSV-2 infection on p38 MAPK pathway activation. ME-180 cells were infected with HSV-2, and the phosphorylation level and total protein level of p38 were examined by Western blot. HSV-2 infection increased the phosphorylation level of p38, but did not affect the total protein level of p38 (Fig. 5B). We also found that inhibition of p38 activation significantly decreased the phosphorylation of C/EBP-β by Western blot (Fig. 5C). Taken together, these results suggest that HSV-2–induced CXCL9 expression in human cervical epithelial cells is mediated through the activation of p38 MAPK pathway.

**FIGURE 5.** HSV-2–induced CXCL9 expression is mediated by p38 MAPK signaling pathway. (A) ME-180 cells were pretreated with inhibitor specifically against p38, ERK, or JNK for 1 h and transfected with reporter plasmid (−495/+33)CXCL9 before infection with HSV-2. The relative luciferase activity was normalized by that of cells without HSV-2 infection. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. (B) ME-180 cells were infected with HSV-2, and the levels of phosphorylation and total p38 protein were analyzed by Western blot. One representative experiment of three is shown. (C) ME-180 cells were pretreated with inhibitors specifically against p38 before HSV-2 infection, and total protein were isolated. Western blot was performed to measure the phosphorylation of C/EBP-β. One representative experiment of three is shown.

**Discussion**

HSV-2 infection is associated with an increased risk of HIV-1 acquisition. The infectious HSV-2 ulceration is considered to be one of the major reasons that increase HIV-1 acquisition. Antiviral drugs, such as acyclovir, have been shown to reduce the frequency of both clinical and subclinical ulcers (34, 35). However, in clinical trials, suppressive therapy with acyclovir does not decrease the rates of HIV-1 acquisition (36, 37). Furthermore, acyclovir appears to have a smaller effect on the frequency of genital ulcer disease as well as a smaller effect on the frequency and quantity of lesion HSV DNA in African women and Peruvian men (38), compared with its effects in men in the United States. The observed regional variation in the clinical and virologic efficacy of acyclovir brings into question the lack of impact on HIV-1 acquisition and may reflect a lower efficacy or lack of adherence in the HIV-1 prevention trial. Moreover, the observation that recruited CD4+ T cells persist at sites of HSV-2 reactivation for months after healing, even with daily antiviral therapy, has been proposed as an alternative explanation of the inability of anti-HSV-2 therapy to reduce HIV-1 acquisition (13). In the current study, based on our observation that CXCL9 is significantly elevated in HSV-2–positive women, we conducted experiments using both primary human cervical epithelial cells and cell lines. We have demonstrated that HSV-2 directly induces CXCL9 expression in human cervical epithelial cells and that HSV-2–induced transcription of CXCL9 is mediated by activation of p38/C/EBP-β pathway. We have further revealed that CXCL9 induced by HSV-2 can recruit CD4+ T cells.
CXCL9 was found to be inducible and critical for control of genital HSV-2 infection in mice (22). However, whether HSV-2 can induce the expression of CXCL9 in human cells and the molecular mechanisms underlying the induction of CXCL9 after HSV-2 infection has not been previously characterized. In the current study, using human cervical epithelial cells as a model, we found that HSV-2 activated the CXCL9 promoter and induced its expression in a dose-dependent manner, indicating that HSV-2 is capable of directly inducing CXCL9 expression in infected human cervical epithelial cells. Such a HSV-2–induced CXCL9 expression was confirmed at both mRNA and protein levels. Furthermore, we found that UV-inactivated HSV-2 was unable to induce CXCL9 expression, suggesting that the induction of CXCL9 requires productive HSV-2 infection. After a 2-h incubation, cells migrated to lower chambers were collected and counted using hemocytometer. Cell migration was expressed as percentage of input. Input cells in the upper chamber were $5 \times 10^5$. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. C and D) Primary cervical epithelial cells were isolated from human cervical tissues. Prior to infection with HSV-2, primary cervical epithelial cells were treated with or without C/EBP-β siRNA or SB203580. Twenty-four hours postinfection, supernatants from infected cells were added to the lower chamber of Transwell plates in the absence (C) or presence (D) of anti-CXCL9 neutralizing Ab or control Ab, and activated CD4+ T cells were placed in the upper chamber. After a 2-h incubation, cells migrated to lower chambers were collected and counted using hemocytometer. Cell migration was expressed as percentage of input. Input cells in the upper chamber were $5 \times 10^5$. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate.

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Transcriptional activation of CXCL9 is ultimately dependent on the activation of the cis elements in its promoter. IFN-γ–induced expression of CXCL9 in macrophages was reported to be mediated by the transcriptional factor, STAT1α, which binds to the ISRE site in the CXCL9 promoter (39), whereas several viral proteins were found to induce CXCL9 (monokine induced by IFN-γ) expression in vitro through binding to the NF-κB site (28, 40). By constructing and analyzing a series of deletion mutants of the CXCL9 promoter, we demonstrate that the promoter region between −129 and −67 bp is critical for transcriptional activation.
of CXCL9 by HSV-2. Bioinformatic analysis of CXCL9 promoter predicted a C/EBP binding site, and this was confirmed by site-directed mutagenesis, showing that the C/EBP binding site in CXCL9 promoter is responsible for the transcriptional activation of the CXCL9 gene by HSV-2.

Many genes encoding cytokines and chemokines have C/EBP binding sites within their promoters, and transcriptional factor C/EBP-β has been shown to affect the expression of IL-6, IL-12, CCL5, and CCL4 through the C/EBP binding sites on the target gene promoters (41). To determine whether C/EBP-β is indeed involved in HSV-2-induced CXCL9 expression, we used siRNA to knock down the expression of C/EBP-β in human cervical epithelial cells prior to HSV-2 infection. Our results revealed a direct correlation between the levels of C/EBP-β and CXCL9. Moreover, overexpression of C/EBP-β was also found to stimulate CXCL9 expression. These data indicate that C/EBP-β is a critical mediator of HSV-2-induced CXCL9 expression.

C/EBP-β has been proven to be important for the development, differentiation, and proliferation of mammary epithelial cells (42, 43). Upon stimulation, C/EBP-β can regulate the expression of target genes by increasing their protein levels (44, 45). In addition, phosphorylation of C/EBP-β protein on Thr235 increases the binding of coactivator p300 and CBP to C/EBP-β, which can regulate its transcriptional activity (46, 47). We found that HSV-2 infection did not increase the protein level of C/EBP-β, but rather enhanced the phosphorylation of C/EBP-β on Thr235 and induced the translocation of C/EBP-β to nucleus. We further demonstrated that HSV-2 infection increased the binding of C/EBP-β to the C/EBP binding site in the proximal promoter region of CXCL9. Taken together, our data indicate that HSV-2 activates C/EBP-β by phosphorylation of C/EBP-β on Thr235, promoting the binding of C/EBP-β to the CXCL9 promoter and activating CXCL9 expression.

It has been previously demonstrated that MAPKs phosphorylate C/EBP-β protein on Thr235 and regulate its transcriptional activity (48). MAPKs are proline-directed serine/threonine-specific protein kinases that regulate cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis. In mammalian cells, three principal MAPK pathways have been identified, including ERK, JNK, and p38 (49). We used specific inhibitors to block the activation of MAPK pathways prior to HSV-2 infection and revealed that inhibition of p38 pathway significantly decreased the promoter activity of CXCL9 induced by HSV-2, indicating that p38 activation is required for HSV-2–induced CXCL9 expression.

CXCL9 attracts leukocytes to sites of infection and inflammation, and shares the receptor CXCR3 with CXCL10 (50). It was reported that elevated expression levels of CXCL9 and CXCL10 were found in mice after HSV-2 infection, and the levels were associated with the mobilization of HSV-2–specific CTL and NK cells (22). We observed that CXCL9 level was significantly higher in the mucus from HSV-2–positive women than that from HSV-2–negative women. We further demonstrated that the chemotactic activity was substantially increased in supernatants harvested from both primary human cervical epithelial cells and cell lines infected with HSV-2, and blockade of CXCL9 by a neutralizing Ab significantly decreased the migration of activated PBLs and CD4+ T cells. Our data imply that CXCL9 expression induced by HSV-2 may play an important role in the recruitment of CD4+ T cells to the infection area after HSV-2 infection.

C/EBP-β is known to be an important mediator in CCL3, CCL4, and CCL2 expression and leukocyte recruitment induced by IL-1β (51, 52), whereas p38 MAPK pathway plays a critical role in endothelial chemokine production and leukocyte recruitment upon stimulation (53, 54). In the current study, we showed that p38-C/EBP-β pathway was critical for HSV-2–induced CXCL9 expression. By using specific siRNA or inhibitors to block the activation of p38-C/EBP-β pathway, we further demonstrated that knockdown of C/EBP-β or inhibition of p38 MAPK pathway significantly decreased HSV-2–induced CD4+ T cell migration. Taken together, it is reasonable to conclude that HSV-2–induced CXCL9 is capable of regulating the migration of CD4+ T cells, and that p38-C/EBP-β pathway is critical for CXCL9-mediated chemotaxis.

In conclusion, based on our finding that CXCL9 is augmented in the cervical mucus of HSV-2–positive women, we demonstrate that HSV-2 can directly induce CXCL9 expression in human cervical epithelial cells, and that binding of C/EBP-β to CXCL9 promoter is important for HSV-2–mediated transcriptional activation of CXCL9. Our results also reveal that p38-C/EBP-β pathway activated by HSV-2 is critical for CXCL9 induction and the recruitment of CD4+ T cells. We propose a model as described in Fig. 7. HSV-2 infection activates p38 MAPK pathway and phosphorylates C/EBP-β at Thr235 site. C/EBP-β then translocates to the nucleus. We further demonstrated that knockdown of C/EBP-β or inhibition of p38 MAPK pathway significantly decreased HSV-2–induced CD4+ T cell migration. Taken together, it is reasonable to conclude that HSV-2–induced CXCL9 is capable of regulating the migration of CD4+ T cells, and that p38-C/EBP-β pathway is critical for CXCL9-mediated chemotaxis.
nucleus and binds to CXCL9 promoter, resulting in transcriptional activation of CXCL9. Secreted CXCL9 protein in turn recruits CD4+ T cells to HSV-2-infected mucosal sites. When HIV-1 reaches mucosal tissues disrupted by HSV-2 infection, the recruited CD4+ T cells may be targeted by HIV-1. Although other mechanisms also likely exist, findings in our study provide one molecular explanation for HSV-2-induced CXCL9 expression, which may recruit CD4+ T cells to the infection area and potentially increase the risk of HIV-1 sexual transmission.

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Disclosures
The authors have no financial conflicts of interest.

References