Published in final edited form as:

Am J Reprod Immunol. 2009 July ; 62(1): . doi:10.1111/j.1600-0897.2009.00713.x.

CCL20/MIP3a is a Novel Anti-HIV-1 Molecule of the Human Female Reproductive Tract

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Abstract

Problem—CCL20/MIP3 is a chemokine for immature dendritic cells as well as an antibacterial against gram-positive and gram-negative bacteria. The role of CCL20/MIP3 as an antiviral is unknown. In this study, we have examined the production of CCL20/MIP3 by epithelial cells from the upper female reproductive tract as well as its activity as an antiviral molecule.

Method of study—Primary uterine and Fallopian tube epithelial cells were treated with Poly(I:C) and CCL20/MIP3 mRNA and protein was measured by Real-time RT-PCR and ELISA assays. Anti-HIV activity was determined using an indicator cell line TZM-bl and quantified by using a luminometer.

Results—Primary uterine and Fallopian tube epithelial cells produce CCL20/MIP3 constitutively and the production is enhanced following stimulation with viral double-stranded RNA mimic Poly(I:C). Recombinant CCL20/MIP3 was able to inhibit both T-cell-tropic X4/IIIB and macrophage-tropic R5/BaL HIV-1 when virus was directly incubated with CCL20/MIP3 but not when CCL20/MIP3 was added to cells either prior to infection or post-infection. This suggests that the mechanism of inhibition is likely to be a direct interaction between HIV-1 and CCL20/MIP3.

Conclusion—This study demonstrates that CCL20/MIP3 is an important endogenous anti-HIV-1 microbicide of the female reproductive tract.

Keywords

Antiviral; CCL20/MIP3 ; female reproductive tract; HIV; Poly(I:C)

Introduction

In recent years, the face of the HIV/AIDS epidemic has become increasingly female. The numbers of women and girls infected have increased disproportionately both in developed and developing countries.¹ Heterosexual transmission currently accounts for 80% of new infections² and in some studies women were found at least twice as likely as men to be infected with HIV during vaginal intercourse.³ Paradoxically, the transmission rate of HIV from a man to a woman per act of sexual intercourse is relatively low, 1:122 to 1:1000.⁴⁻⁶

One reason for this is that the cells of the female reproductive tract produce and secrete a spectrum of endogenous antimicrobials that are protective against HIV.^{7–11}

It is now known that the epithelial cells at mucosal surfaces perform functions at multiple levels more intricate than simply providing barrier protection. Innate anti-bacterial and anti-viral (including anti-HIV) activity by mucosal secretions have been described in oral, nasal, lung, skin, and intestinal epithelial cells.^{12–17} We and others have demonstrated the presence of antimicrobials in epithelial cell secretions of the female reproductive tract.^{7,9,18,19} In cervico-vaginal secretions the presence of cationic polypeptides has been linked to anti-HSV and anti-HIV activity.^{11,20}

Chemokines are small cationic molecules that link the innate and the adaptive immune responses by attracting effector cells to the site of infection or pathology. In addition to their chemotactic activity, most chemokines have also demonstrated antimicrobial activity particularly against gram-positive and gram-negative bacteria.^{21–23} CCL20/MIP3 is an important immune effector molecule that is chemotactic for immature dendritic cells and lymphocytes.^{24,25} In mouse, rat, and human, the expression and secretion of constitutive CCL20/MIP3 has been demonstrated in a number of tissues and cell-lines including intestinal, lung, skin, and uterine epithelial cell line.^{26–29} The secretion of CCL20/MIP3 has been shown to be enhanced further by the presence of pathogens or pathogen associated molecular patterns (PAMP).^{30–33} CCL20/MIP3 secretion by human vaginal epithelial cells has been shown to be enhanced in the presence of semen resulting in chemoattraction of Langerhans cells which are permissive to HIV infection.³⁴

CCL20/MIP3 has been shown to have anti-bacterial activity.²¹ Despite this finding, few studies have addressed this molecule's potential as an antiviral factor. For example, in HPV-mediated cervical cancer, viral proteins E6 and E7 reduce the levels of CCL20/MIP3 in order to decrease recruitment of immune cells to the mucosal site.³⁵ CCL20/MIP3 protein was also found to be significantly reduced in the skin of patients with vaccinia virus mediated atopic dermatitis and was found to be directly inhibitory to the virus.³⁶ CCL20/MIP3 has structural homology with the family of defensins, molecules with demonstrated anti-HIV activity, and share the receptor CCR6.^{34,37} We, therefore, wanted to ask the question as to whether this molecule might have direct anti-HIV activity.

The upper female reproductive tract, uterine endometrium (EM) and Fallopian tube (FT) have not been well-characterized in terms of innate and adaptive immune responses. We and others have previously demonstrated the potential of epithelial cells of the upper tract to be infected by and to respond to HIV.^{9,18,38} It has been demonstrated that the upper tract is not sterile and that pathogens and sperms deposited at the vagina can make their way up the tract within minutes of deposition.³⁹ Keeping this in mind, our goal was to test the hypothesis that the upper FRT epithelial cells from the uterus (UTEC) and Fallopian tube (FTEC) produce CCL20/MIP3 both constitutively and in response to TLR3-mediated stimuli. Further, we tested the hypothesis that CCL20/MIP3 secreted by the epithelial cells had direct anti-HIV activity.

Materials and methods

Source of Tissues

Human uterine and Fallopian tube tissues were obtained from women undergoing hysterectomy at Dartmouth-Hitchcock Medical Center (Lebanon, NH). Tissues used in this study were collected from patients with benign conditions such as fibroids distal from the site of pathology. The sections were examined by a pathologist and proclaimed to be free of pathological lesions. A total of nine different patients were used to obtain uterine epithelial

cells and a total of seven patients were used to obtain Fallopian tube epithelial cells. All human subjects work was carried out with the approval of the Dartmouth College institutional review board. Approval to use tissues was previously obtained from the Committee for the Protection of Human Subjects (CPHS).

Isolation of Primary Uterine and Fallopian Tube Epithelial Cells

Epithelial cells were isolated as previously described elsewhere.^{40,41} Briefly, tissues were rinsed with 1x PBS and minced into 1–2 mm fragments prior to subjecting them to enzymatic digestion for 2 hr at 37°C. The enzyme mixture contained 3.4 mg/mL pancreatin (Nitrogen Life Technologies, Carlsbad, CA, USA), 0.1 mg/mL hyaluronidase (Worthington Biochemical, Lakewood, NJ, USA), 1.6 mg/mL collagenase (Worthington Biochemical), and 2 mg/mL d-glucose, in 1x HBSS (Invitrogen Life Technologies). After digestion, cells were dispersed through a 250- μ m mesh screen, washed, and resuspended in DMEM/F12 complete medium without phenol red, supplemented with 20 mM HEPES, 2 mM L-glutamine (all from Invitrogen Life Technologies), 50 μ g/mL primocin (Invivogen, San Diego, CA, USA), and 10% defined FBS (Hyclone, Logan, UT, USA). Epithelial sheets were separated from stromal cells by filtration through a 20- μ m nylon mesh filter (Small Parts, Miami Lakes, FL, USA). Epithelial cell sheets retained on the filter were recovered by rinsing and backwashing, centrifuged at 500 $\times g$ for 5 min and analyzed for cell number and viability. By this procedure, we have isolated epithelial cells that stain positive for the epithelial antigens Ber-EP4 and cytokeratin and negative for CD4, CD45, and vimentin.⁴²

Cell Culture

To establish a cell culture system of polarized human UTEC and FTEC with both apical and basolateral compartments, primary human UTEC and FTEC were cultured in Human Extracellular Matrix (Becton Dickinson, Franklin Lakes, NJ, USA) coated Falcon cell culture inserts in 24-well culture plates (Fisher Scientific, Pittsburgh, PA, USA). For these experiments, apical and basolateral compartments contained 300 and 850 μ L of complete medium, respectively. The medium was changed every 2 days. Cells were treated with the following TLR agonists for 24 hr: ultrapure LPS from *Escherichia coli* (List Biological Laboratories, Campbell, CA, USA), 1 μ g/mL; Poly(I:C) (Invivogen), 25 μ g/mL; zymosan from *Saccharomyces cerevisiae* (Invivogen), 100 ng/mL and Flagellin from *E. coli* (Inotek Pharmaceuticals, Lexington, MA, USA), 100 ng/mL. After 24 hr incubation, apical and basolateral conditioned media were collected and centrifuged for 5 min at 10,000 $\times g$ and stored at –80°C until use.

Measurement of Transepithelial Resistance

Tight junction formation of cultured epithelial cell monolayers was assessed by periodically measuring transepithelial resistance (TER) using an EVOM electrode and Volt ohmmeter (World Precision Instruments, Sarasota, FL, USA), as described previously.⁴³ The TER is a functional measurement of the integrity of tight junctions in an epithelial cell monolayer. Since the presence of non-epithelial cells in the culture interferes with the formation of tight junctions and therefore prevents an increase in TER, the TER is also an indicator for the purity of the epithelial monolayer.

Measurement of CCL20/MIP3 α Protein Secretion

Concentrations of CCL20/MIP3 in the apical and basolateral supernatants from human primary UTEC and FTEC were determined with an ELISA DuoSet kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Amounts of CCL20/MIP3 were quantified based on a standard curve after OD measurements at 450 nm on an ELISA reader (Dynex, Chantilly, VA, USA).

Taqman Real-time Polymerase Chain Reaction

Real-time RT-PCR was done with a two-step protocol as described previously.⁴⁴ Total RNA was isolated from cells using TRIzol Reagent according to the manufacturer's recommendations (Invitrogen Life Technologies) and purified with RNeasy columns (Qiagen, Valencia, CA, USA). Coincident with RNA purification was on-column DNase digestion using the RNase-Free DNase set (Qiagen). For each specimen, 400 ng of total RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations in a 20 μ L volume. Relative mRNA expression levels of CCL20/MIP3 were measured using the 5' fluorogenic nuclease assay in real-time quantitative PCR using TaqMan chemistry on the ABI 7300 Prism real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). The CCL20/MIP3 and β -actin primer/MGB probe sets were obtained from Applied Biosystems assay-on-demand (ID nos. Hs00171125 and 4333762T, respectively). PCR was conducted using the following cycle parameters: 95°C, 12 min for 1 cycle (95°C, 20 s; 60°C, 1 min), for 40 cycles. Analysis was conducted using the sequence detection software supplied with the ABI 7300. The software calculates the threshold cycle (Ct) for each reaction which was used to quantify the amount of starting template in the reaction. The Ct values for each set of duplicate reactions were averaged for all subsequent calculations. A difference in Ct values (Δ Ct) was calculated for each gene by taking the mean Ct of each gene of interest and subtracting the mean Ct for the housekeeping gene β -actin for each cDNA sample. Assuming that each reaction functions at 100% PCR efficiency, a difference of one Ct represents a twofold difference. Relative expression levels were expressed as a fold-increase in mRNA expression and calculated using the formula $2^{-\Delta \text{Ct}}$.

CCL20/MIP3 α Inhibition of HIV-1: TZM-bl Assay

The TZM indicator cell line (kindly donated by Dr Phalguni Gupta, University of Pittsburgh) is a HeLa cell derivative that expresses high levels of CD4, CCR5 and CXCR4. The cells contain HIV long terminal repeat (LTR)-driven β -galactosidase and luciferase reporter cassettes that are activated by HIV *tat* expression.⁴⁵ TZM cells were routinely subcultured every 3–4 days by trypsinization and were maintained in TZM media consisting of DMEM (Invitrogen Life Technologies) supplemented with 10% defined FBS (HyClone), 2 mM L-glutamine (Invitrogen Life Technologies), and 50 μ g/mL primocin (Invivogen) and did not contain phenol red.

TZM cells were seeded at 2×10^4 cells per well in a 96-well microtiter plate and allowed to adhere overnight at 37°C. Varying doses of recombinant human CCL20/MIP3 (Peprotech, Rocky Hill, NJ, USA) were incubated with HIV-1 IIIB and HIV-1 BaL at multiplicity of infection (MOI) 1 for 1 hr at 37°C in a final volume of 100 μ L. Following incubation, the media was aspirated from TZM cells and the virus plus CCL20/MIP3 was added to the cells along with 100 μ L of TZM media. Luciferase activity was measured after 48 hr at 37°C with 5% CO₂ in a humidified incubator. Briefly, the supernatants were aspirated, and the cells were lysed with a beta Glo luciferase assay substrate. The light intensity of each well was measured using a luminometer. Uninfected cells were used to determine background luminescence. All infectivity assays were performed in quadruplicate.

Other experiments were conducted in order to determine whether the inhibitory effects of CCL20/MIP3 were at the cell surface level such as the blocking of a co-receptor. For these experiments, 200 ng/mL of CCL20/MIP3 were directly added to TZM cells and incubated for 1 hr at 37°C prior to washing out with 1xPBS and addition of IIIB or BaL viruses. In additional experiments to determine possible post-infection mechanisms of inhibition by CCL20/MIP3, TZM cells were infected with HIV-1 IIIB and BaL at MOI 1 and were washed out at 6 and 24 hr post-infection followed by the addition of 20 ng/mL CCL20/

MIP3. Assays were developed at 48 hr by addition of the beta-Glo substrate and measuring relative light units using a luminometer.

Statistics

A two-tailed paired *t*-test or a one-way ANOVA with Bonferonni's post-test was performed using Graph-Pad InStat version 3.0a (GraphPad Software, San Diego, CA, USA). A *P*-value of <0.05 was taken as indicative of statistical significance.

Results

Endometrial and Fallopian Tube Epithelial Cells Upregulate CCL20/MIP3 α mRNA Production Upon Treatment with TLR3 Agonist Poly(I:C)

Epithelial cells were isolated from EM and FT and grown to confluence and high TER on inserts in transwell plates. The cells were then treated with the following PAMP: LPS (TLR 4 agonist), Poly(I:C) (TLR 3 agonist), Zymosan (TLR1/2 or 2/6 agonist) and Flagellin (TLR 5 agonist) for 24 hr. Following treatment, RNA was isolated from the cells and analyzed by Taqman real-time RT-PCR. As shown in Fig. 1 with one representative patient, only TLR3 agonist Poly(I:C) treatment enhanced CCL20/MIP3 mRNA production by the cells by greater than twofold. Since Poly(I:C) treatment was the only treatment that upregulated CCL20/MIP3 mRNA, we looked at multiple patient samples to see if the upregulation was consistent among patients. As shown in Fig. 2a, in five out of five patients, we observed enhancement of CCL20/MIP3 mRNA production upon Poly(I:C) treatment. Although there was variability among patients, in all five, CCL20/MIP3 mRNA upregulation was greater than twofold which we used as our cut-off for significance.

To observe whether epithelial cells isolated from Fallopian tubes (FTEC) produce CCL20/MIP3, we cultured and treated these cells with Poly(I:C) for 24 hr. Although four out of four patient samples expressed CCL20/MIP3 mRNA, upregulation upon Poly(I:C) treatment was observed in only two out of four samples (Fig. 2b).

Constitutive and Poly(I:C) Induced CCL20/MIP3 α Protein Secretion by Endometrial and Fallopian Tube Epithelial Cells

Observing that CCL20/MIP3 mRNA expression was induced upon Poly(I:C) treatment, we next quantified CCL20/MIP3 protein secretion from both EM and FT epithelial cells. Epithelial cells were isolated and cultured on inserts in transwell plates until confluent with high TER, as described above, and treated with Poly(I:C). After 24 hr treatment, both the apical and the basolateral conditioned media were harvested, centrifuged to remove debris and assayed for CCL20/MIP3 protein by ELISA. Fig. 3 indicates that the EM and FT epithelial cells constitutively secrete CCL20/MIP3 protein into the apical compartment with FT samples generally secreting a higher quantity. The levels ranged from 0.24 to 12.48 ng/mL. When cells were treated with Poly(I:C) for 24 hr (Fig. 4), there was significant increase in both the EM and FT CCL20/MIP3 secretion. In other studies comparing apical with basolateral secretion, we found that most if not all CCL20/MIP3 was secreted apically, in the direction of the FRT lumen (data not shown).

Anti-HIV Activity of CCL20/MIP3 α

Our data showed that CCL20/MIP3 could be induced by the viral stimuli Poly(I:C). As CCL20/MIP3 is a known anti-bacterial molecule that has been shown to be effective against both gram-positive and gram-negative bacteria,²¹ we then asked whether CCL20/MIP3 could be virucidal as well. We tested the activity of recombinant CCL20/MIP3 (rCCL20/MIP3) against HIV-1 X4/T-tropic IIIB and R5/M-tropic BaL. HIV-1 IIIB and BaL were incubated with rMIP3 at concentrations of 2, 20, and 200 ng/mL for 1 hr at

37°C. The virus-rCCL20/MIP3 mixture was added to the TZM cells and incubated for 48 hr at 37°C. At the end of the incubation period, beta-glo substrate was added to the cells and viral infection was quantified as relative light units (RLU) using a luminometer. As shown in Fig. 5, rCCL20/MIP3 inhibited both X4/IIIB and R5/BaL in multiple doses that were in the same range of CCL20/MIP3 measured in EM and FT secretions (Fig. 3). Specifically we found that constitutive CCL20/MIP3 secreted by epithelial cells ranged between 0.2–7 ng/mL in EM and 5–12 ng/mL in the FT. When stimulated with Poly(I:C), the levels increased to 5–12 ng/mL by EM cells and 13–20 ng/mL by the FT cells (Fig. 4). The inhibitory activity was observed as a result of pre-incubation of HIV-1 and rCCL20/MIP3 which suggests that the effect of CCL20/MIP3 on viral replication was likely to be direct. To our knowledge, this is the first time anti-HIV activity of CCL20/MIP3 has been demonstrated.

Anti-HIV factors have been shown to inhibit HIV by multiple mechanisms, including direct interaction with HIV, by blocking cell surface receptors (CXCR4, CCR5), and by affecting post-infection steps. For example, macrophage inflammatory protein 1 alpha (MIP1 α), beta (MIP1 β), and Regulated on Activation, Normal T Expressed and Secreted (RANTES) are natural ligands for CCR5 that can therefore block entry of HIV into the cell. In addition, these molecules also function at a post-infection level by decreasing levels of intracellular cyclic AMP (cAMP) which is required for early steps in viral replication.⁴⁶ Multiple mechanisms of HIV inhibition has also been observed for the well-characterized anti-HIV molecule SLPI.⁴⁷

After observing direct inhibitory effects of CCL20/MIP3 on HIV-1 infection, we wanted to determine whether CCL20/MIP3 might also have indirect effects on viral infection. To determine whether CCL20/MIP3 acts by blocking HIV-1 receptors or co-receptors, TZM cells were pre-incubated with increasing concentrations of rCCL20/MIP3 for 1 hr at 37°C. After incubation cells were washed with media to remove CCL20/MIP3 prior to the addition of HIV-1 IIIB and BaL viruses. Unlike what was observed with CCL20/MIP3 preincubation with HIV-1, we found no significant inhibition of either the X4/IIIB or the R5/BaL viruses (Fig. 6a).

To determine whether CCL20/MIP3 acts through post-infection mechanisms, TZM cells were infected with IIIB and BaL, washed out at 6 and 24 hr post-infection, after which CCL20/MIP3 was added to TZM cells. Other than a slight inhibition observed at 6 hr post-infection with the BaL virus, we observed no significant post-infection inhibition (Fig. 6b). In combination, these data indicate that the inhibitory activity of CCL20/MIP3 is likely through a direct interaction with the virus rather than at the level of the cell surface, or through disabling a later post-infection step.

Because these experiments suggested that antiviral activity might be due to epithelial cell production of CCL20/MIP3, studies were undertaken to remove CCL20/MIP3 by antibody neutralization. To ensure that the antibody used was sufficient to remove CCL20/MIP3, we first attempted to neutralize known amounts of rCCL20/MIP3. We found that neutralization of rCCL20/MIP3 (20 ng/mL) was possible with complete reversal of anti-HIV-1 activity. However, we could not reverse the anti-HIV-1 activity in secretions containing CCL20/MIP3 (<2 ng/mL) by using the same neutralization antibody at the same concentrations (data not shown). We and others have previously shown FRT secretions to contain a family of antimicrobials, some of which have anti-HIV-1 activity.^{9,11,18,20,48} As CCL20/MIP3 is one of the many molecules present in these secretions, neutralization would not be measurable as a separate entity.

Discussion

This study demonstrates that human polarized epithelial cells from the uterus and the Fallopian tube produce and secrete CCL20/MIP3 both constitutively as well as upon stimulation with TLR3 agonist Poly(I:C). We have further demonstrated that CCL20/MIP3 has direct anti-HIV activity against both T-tropic/X4 HIV-1 IIIB and M-tropic/R5 HIV-1 BaL. To the best of our knowledge this is the first demonstration of production of CCL20/MIP3 by primary human EM and FT epithelial cells as well as the significant anti-HIV-1 activity of rCCL20/MIP3.

We have previously demonstrated that the epithelial cells of the upper FRT are capable of responding to viral infections at several different levels. These include upregulation of intracellular anti-viral pathways involving interferon-beta, MxA, OAS and PKR; secretion of antimicrobials such as SLPI and defensins; and secretion of cytokines and chemokines that recruit immune cells to the sites of infection thereby linking the initial innate immune response to the later adaptive immune response.^{7,9,18,48} The present studies extend our understanding of the upper FRT immune protection by the secretion of antimicrobials which, in addition to contributing to local protection, may also provide protection downstream in the ectocervix and the vagina as fluids pass from the upper to lower tract. The presence of antimicrobial molecules in the secretions from the upper FRT takes on added importance in the light of recent findings that challenge the conventional concept that the upper FRT is sterile. When macrospheres or radio-opaque dyes were deposited at the cervix and vagina, localization was observed in the uterus, Fallopian tubes and ovaries within minutes.^{39,49} It is also well-documented that sperm and associated ejaculate can be a carrier for pathogenic viruses as they enter the upper tract within minutes of deposition in the vagina.^{50–52} Others have shown that the HIV-1 envelope glycoprotein gp120 binds to spermatozoa, indicating that sperm may be a vehicle for viral dissemination to the upper FRT.^{53,54}

Our data demonstrate that CCL20/MIP3 is secreted by uterine and some Fallopian tube epithelial cells of the FRT and that secretion is enhanced upon stimulation with Poly(I:C), a synthetic mimic for viral dsRNA. Sun et al. and Cremel et al. have demonstrated CCL20/MIP3 production in human endometrial epithelial cell lines^{29,55} but, to our knowledge, secretion of CCL20/MIP3 by primary human uterine and Fallopian tube epithelial cells has not been described before. CCL20/MIP3 is secreted both apically and basolaterally with a preferential apical release from cells. Others have shown that CCL20/MIP3 is chemotactic for lymphocytes and immature dendritic cells.^{24,25} Our findings that CCL20/MIP3 is secreted both apically and basolaterally suggests that it may function to both recruit immune cells as well as provide a first line of defense. The more significant apical secretion of CCL20/MIP3 by the upper FRT epithelial cells suggests that it might be functioning as an antimicrobial in this case. Specifically, the apical secretion of CCL20/MIP3 can potentially aid in blocking HIV-1 as it comes in contact with the epithelial cells. The doses at which inhibition was demonstrated corresponded to the physiological and/or Poly(I:C) stimulated levels of CCL20/MIP3 secreted by our uterine and Fallopian tube epithelial cells.

All the EM samples we tested showed both constitutive and Poly(I:C) induced upregulation of CCL20/MIP3. However, in case of the FT samples, although all showed constitutive production of CCL20/MIP3, 50% of the samples were not induced upon Poly(I:C) stimulation. One explanation is that the constitutive production of CCL20/MIP3 was generally higher in FTEC than UTEC. The FTEC might therefore seem unresponsive to Poly(I:C) because they might already be producing CCL20/MIP3 at maximal levels.

An unexpected finding in this study was that neutralization of CCL20/MIP3 in epithelial cell secretions had no effect on anti-HIV activity (data not shown). One explanation is that CCL20/MIP3 is only one of a number of antimicrobials secreted by FRT epithelial cells,^{9,18,48} some of which like SLPI and defensins are anti-HIV.^{47,56} Venkataraman et al.²⁰ have shown anti-HIV activity of vaginal lavage samples and that the removal of all cationic components from these samples resulted in the loss of activity. These findings along with ours suggest that multiple factors in the FRT secretions act in synergy to inhibit pathogens, a phenomenon that has been demonstrated with cationic peptides in the lung and the skin.^{57,58} Beyond the absolute level of these molecules in CVL, the biological activity of each antimicrobial must also be considered. For example, others have recently reported that kallikreins, a family of serine proteases known for their ability to enhance innate antimicrobial peptide function, are present in FRT secretions.⁵⁹ As kallikreins vary with stage of the menstrual cycle,⁶⁰ these findings suggest that conversion of inactive molecules to biologically active ones may be as important as the levels of antimicrobials present in FRT secretions. Another processing molecule is the serine protease CD26 which is important for activating chemokines that bind to the cell surface receptors required by HIV-1 for entry.^{61–63}

That CCL20/MIP3 may be an endogenous antiviral is suggested from a study just completed in which cervical vaginal lavages (CVL) were analyzed for anti-HIV activity. We found a correlation between CCL20/MIP3 levels and anti-HIV-1 activity in CVL from HIV positive women (Ghosh M, Fahey J, Shen Z, Cu-Uuin S, Wu Zhijan, Mayer K, Wright P, Ochsenbaur-Jambar C, Wira C, Submitted). In these studies, CVL with high anti-HIV activity contained approximately 10-fold higher CCL20/MIP3 than those with low anti-HIV activity. Studies are currently underway to define the role of CCL20/MIP3 in CVL secretions as they exert protective effects.

Although several chemokines as well as CCL20/MIP3 have been shown to have antibacterial activity,²¹ the present study demonstrates a new function for CCL20/MIP3 in that it has potent antiviral activity against HIV-1. Whether it has broad-spectrum antiviral activity or specific to HIV or retroviruses remains to be determined. We have found that CCL20/MIP3 is effective in inhibiting both X4/IIIB and R5/BaL HIV-1. Moreover, these studies indicate that CCL20/MIP3 likely functions by direct interaction with the virus and not through blocking of receptors. Direct inhibition of HIV-1 has also been observed for defensins which share structural homology as well as the receptor CCR6 with CCL20/MIP3.⁶⁴ Our conclusion is based on the findings that preincubation of TZM cells with rCCL20/MIP3 followed by infection, did not show any inhibition whereas direct incubation of CCL20/MIP3 and virus showed anti-HIV-1 inhibitory activity. Additionally, 6 and 24 hr post-infection treatment of TZM cells with rCCL20/MIP3 also did not show any effects. The 6 hr time point was chosen based on our preliminary studies showing that 6 hr post-infection was the earliest time point when we could observe detectable viral infection above background levels as defined by the TZM-bl assay. We however have not eliminated the possibilities that CCL20/MIP3 might inhibit early post-fusion events in the viral replication cycle.

In conclusion, our studies have identified CCL20/MIP3 as a novel endogenous anti-HIV-1 factor of the female reproductive tract. We have established that CCL20/MIP3 is secreted by upper FRT epithelial cells both constitutively and upon stimulation with Poly(I:C) at levels that are inhibitory to HIV-1. Similar studies are likely to identify other such endogenous molecules which can act in a complex synergy to protect the FRT from harmful pathogens.

Acknowledgments

This work was supported by AI51877 and AI071761 (awarded to Dr. Charles Wira) from National Institute of Health.

The authors thank Richard Rossoll, M.S., Dartmouth Medical School and Deena Ratner, B.S., University of Pittsburgh, for excellent technical assistance in the preparation of cells and virus stocks. The authors also thank Vincent Memoli, M.D., Section Chief of Anatomical Pathology, for procuring tissues; other members of the Department of Pathology for inspecting and dissecting tissue specimens: Jorge Gonzalez, M.D., Alan Schned, M.D., Peter Seery, Shannon Schutz, Elizabeth Rizzo, Richard Merrill, Charles-Robert Moultry, Patricia Larkin, Aimee Larson, Jennifer Simonton and Dawn Madda-line; for clinical support and scheduling: Laura Wolfe, Linda Hallock, Kathleen Pilchman, Karen Carter, Kris Ramsey, Tamara Krivit, and Joanne Lavin; surgeons: Barry Smith, Joan Barthold, Jackson Beecham, John Currie, Leslie Demars, Paul Hanissian, John Ketterer, Benjamin Mahlab, Paul Manganiello, Misty Porter, Karen George, William Young, Kris Strohbehn, Roger Young, Stephen Andrews, and Eric Sailer; OR nurses: Jeanette Sawyer, Tracy Stokes, Fran Reinfrank, and Jaclyn Logan.

References

1. UNAIDS. AIDS epidemic update. 2007. http://data.unaids.org/pub/EPISlides/2007/2007_epiupdate_en.pdf
2. CDC. HIV/AIDS among women. 2007. <http://www.cdc.gov/hiv/topics/women/resources/factsheets/pdf/women.pdf>
3. Ndesendo VM, Pillay V, Choonara YE, Buchmann E, Bayever DN, Meyer LC. A review of current intravaginal drug delivery approaches employed for the prophylaxis of HIV/AIDS and prevention of sexually transmitted infections. *AAPS PharmSciTech*. 2008; 9:505–520. [PubMed: 18431651]
4. Gray RH, Wawer MJ, Brookmeyer R, Sewankambo NK, Serwadda D, Wabwire-Mangen F, Lutalo T, Li X, vanCott T, Quinn TC. Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. *Lancet*. 2001; 357:1149–1153. [PubMed: 11323041]
5. Wawer MJ, Gray RH, Sewankambo NK, Serwadda D, Li X, Laeyendecker O, Kiwanuka N, Kigozi G, Kiddugavu M, Lutalo T, Nalugoda F, Wabwire-Mangen F, Meehan MP, Quinn TC. Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection, in Rakai, Uganda. *J Infect Dis*. 2005; 191:1403–1409. [PubMed: 15809897]
6. Johnston MI, Fauci AS. An HIV vaccine – evolving concepts. *N Engl J Med*. 2007; 356:2073–2081. [PubMed: 17507706]
7. Fahey JV, Wira CR. Effect of menstrual status on antibacterial activity and secretory leukocyte protease inhibitor production by human uterine epithelial cells in culture. *J Infect Dis*. 2002; 185:1606–1613. [PubMed: 12023766]
8. Novak RM, Donoval BA, Graham PJ, Boksa LA, Spear G, Hershow RC, Chen HY, Landay A. Cervicovaginal levels of lactoferrin, secretory leukocyte protease inhibitor, and RANTES and the effects of coexisting vaginosis in human immunodeficiency virus (HIV)-seronegative women with a high risk of heterosexual acquisition of HIV infection. *Clin Vaccine Immunol*. 2007; 14:1102–1107. [PubMed: 17671228]
9. Schaefer TM, Fahey JV, Wright JA, Wira CR. Innate immunity in the human female reproductive tract: antiviral response of uterine epithelial cells to the TLR3 agonist poly(I:C). *J Immunol*. 2005; 174:992–1002. [PubMed: 15634923]
10. Cole AM. Innate host defense of human vaginal and cervical mucosae. *Curr Top Microbiol Immunol*. 2006; 306:199–230. [PubMed: 16909923]
11. Keller MJ, Guzman E, Hazrati E, Kasowitz A, Cheshenko N, Wallenstein S, Cole AL, Cole AM, Profy AT, Wira CR, Hogarty K, Herold BC. PRO 2000 elicits a decline in genital tract immune mediators without compromising intrinsic antimicrobial activity. *Aids*. 2007; 21:467–476. [PubMed: 17301565]
12. Moutsopoulos NM, Greenwell-Wild T, Wahl SM. Differential mucosal susceptibility in HIV-1 transmission and infection. *Adv Dent Res*. 2006; 19:52–56. [PubMed: 16672550]
13. Abiko Y, Saitoh M, Nishimura M, Yamazaki M, Sawamura D, Kaku T. Role of beta-defensins in oral epithelial health and disease. *Med Mol Morphol*. 2007; 40:179–184. [PubMed: 18085375]

14. Cole AM, Liao HI, Stuchlik O, Tilan J, Pohl J, Ganz T. Cationic polypeptides are required for antibacterial activity of human airway fluid. *J Immunol.* 2002; 169:6985–6991. [PubMed: 12471133]
15. Hiemstra PS. The role of epithelial beta-defensins and cathelicidins in host defense of the lung. *Exp Lung Res.* 2007; 33:537–542. [PubMed: 18075828]
16. Wollenberg A, Klein E. Current aspects of innate and adaptive immunity in atopic dermatitis. *Clin Rev Allergy Immunol.* 2007; 33:35–44. [PubMed: 18094945]
17. Wehkamp J, Schmid M, Stange EF. Defensins and other antimicrobial peptides in inflammatory bowel disease. *Curr Opin Gastroenterol.* 2007; 23:370–378. [PubMed: 17545771]
18. Ghosh M, Schaefer TM, Fahey JV, Wright JA, Wira CR. Antiviral responses of human Fallopian tube epithelial cells to toll-like receptor 3 agonist poly(I:C). *Fertil Steril.* 2008; 89(5 Suppl.):1497–1506. [PubMed: 17669408]
19. King AE, Critchley HO, Kelly RW. Innate immune defences in the human endometrium. *Reprod Biol Endocrinol.* 2003; 1:116. [PubMed: 14641912]
20. Venkataraman N, Cole AL, Svoboda P, Pohl J, Cole AM. Cationic polypeptides are required for anti-HIV-1 activity of human vaginal fluid. *J Immunol.* 2005; 175:7560–7567. [PubMed: 16301665]
21. Yang D, Chen Q, Hoover DM, Staley P, Tucker KD, Lubkowski J, Oppenheim JJ. Many chemokines including CCL20/MIP-3alpha display antimicrobial activity. *J Leukoc Biol.* 2003; 74:448–455. [PubMed: 12949249]
22. Linge HM, Collin M, Nordenfelt P, Morgelin M, Malmsten M, Egesten A. The human CXC chemokine granulocyte chemotactic protein 2 (GCP-2)/CXCL6 possesses membrane-disrupting properties and is antibacterial. *Antimicrob Agents Chemother.* 2008; 52:2599–2607. [PubMed: 18443119]
23. Linge HM, Collin M, Giwercman A, Malm J, Bjartell A, Egesten A. The antibacterial chemokine MIG/CXCL9 is constitutively expressed in epithelial cells of the male urogenital tract and is present in seminal plasma. *J Interferon Cytokine Res.* 2008; 28:191–196. [PubMed: 18338951]
24. Liao F, Rabin RL, Smith CS, Sharma G, Nutman TB, Farber JM. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. *J Immunol.* 1999; 162:186–194. [PubMed: 9886385]
25. Schutyser E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev.* 2003; 14:409–426. [PubMed: 12948524]
26. Kwon JH, Keates S, Bassani L, Mayer LF, Keates AC. Colonic epithelial cells are a major site of macrophage inflammatory protein 3alpha (MIP-3alpha) production in normal colon and inflammatory bowel disease. *Gut.* 2002; 51:818–826. [PubMed: 12427784]
27. Reibman J, Hsu Y, Chen LC, Bleck B, Gordon T. Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. *Am J Respir Cell Mol Biol.* 2003; 28:648–654. [PubMed: 12760962]
28. Schmuth M, Neyer S, Rainer C, Grassegger A, Fritsch P, Romani N, Heufler C. Expression of the C-C chemokine MIP-3 alpha/CCL20 in human epidermis with impaired permeability barrier function. *Exp Dermatol.* 2002; 11:135–142. [PubMed: 11994140]
29. Sun B, Nasu K, Fukuda J, Mine S, Nishida M, Miyakawa I. Expression of macrophage inflammatory protein-3alpha in an endometrial epithelial cell line, HHUA, and cultured human endometrial stromal cells. *Mol Hum Reprod.* 2002; 8:930–933. [PubMed: 12356943]
30. Soboll G, Crane-Godreau MA, Lyimo MA, Wira CR. Effect of oestradiol on PAMP-mediated CCL20/MIP-3 alpha production by mouse uterine epithelial cells in culture. *Immunology.* 2006; 118:185–194. [PubMed: 16771853]
31. Crane-Godreau MA, Wira CR. CCL20/macrophage inflammatory protein 3alpha and tumor necrosis factor alpha production by primary uterine epithelial cells in response to treatment with lipopolysaccharide or Pam3Cys. *Infect Immun.* 2005; 73:476–484. [PubMed: 15618187]
32. Roxstrom-Lindquist K, Ringqvist E, Palm D, Svard S. Giardia lamblia-induced changes in gene expression in differentiated Caco-2 human intestinal epithelial cells. *Infect Immun.* 2005; 73:8204–8208. [PubMed: 16299316]

33. Demedts IK, Bracke KR, Van Pottelberge G, Testelmans D, Verleden GM, Vermassen FE, Joos GF, Brusselle GG. Accumulation of dendritic cells and increased CCL20 levels in the airways of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2007; 175:998–1005. [PubMed: 17332482]
34. Berlier W, Cremel M, Hamzeh H, Levy R, Lucht F, Bourlet T, Pozzetto B, Delezay O. Seminal plasma promotes the attraction of Langerhans cells via the secretion of CCL20 by vaginal epithelial cells: involvement in the sexual transmission of HIV. *Hum Reprod.* 2006; 21:1135–1142. [PubMed: 16531471]
35. Guess JC, McCance DJ. Decreased migration of Langerhans precursor-like cells in response to human keratinocytes expressing human papillomavirus type 16 E6/E7 is related to reduced macrophage inflammatory protein-3 α production. *J Virol.* 2005; 79:14852–14862. [PubMed: 16282485]
36. Kim BE, Leung DY, Streib JE, Boguniewicz M, Hamid QA, Howell MD. Macrophage inflammatory protein 3 α deficiency in atopic dermatitis skin and role in innate immune response to vaccinia virus. *J Allergy Clin Immunol.* 2007; 119:457–463. [PubMed: 17141855]
37. Hoover DM, Boulegue C, Yang D, Oppenheim JJ, Tucker K, Lu W, Lubkowski J. The structure of human macrophage inflammatory protein-3 α /CCL20. Linking antimicrobial and CC chemokine receptor-6-binding activities with human beta-defensins. *J Biol Chem.* 2002; 277:37647–37654. [PubMed: 12149255]
38. Asin SN, Fanger MW, Wildt-Perinic D, Ware PL, Wira CR, Howell AL. Transmission of HIV-1 by primary human uterine epithelial cells and stromal fibroblasts. *J Infect Dis.* 2004; 190:236–245. [PubMed: 15216456]
39. Zervomanolakis I, Ott HW, Hadziomerovic D, Mattle V, Seeber BE, Virgolini I, Heute D, Kissler S, Leyendecker G, Wildt L. Physiology of upward transport in the human female genital tract. *Ann NY Acad Sci.* 2007; 1101:1–20. [PubMed: 17416925]
40. Fahey JV, Prabhala RH, Guyre PM, Wira CR. Antigen-presenting cells in the human female reproductive tract: analysis of antigen presentation in pre- and post-menopausal women. *Am J Reprod Immunol.* 1999; 42:49–57. [PubMed: 10429767]
41. Fahey JV, Humphrey SL, Stern JE, Wira CR. Secretory component production by polarized epithelial cells from the human female reproductive tract. *Immunol Invest.* 1998; 27:167–180. [PubMed: 9653665]
42. Wallace PK, Yeaman GR, Johnson K, Collins JE, Guyre PM, Wira CR. MHC class II expression and antigen presentation by human endometrial cells. *J Steroid Biochem Mol Biol.* 2001; 76:203–211. [PubMed: 11384879]
43. Richardson JM, Kaushic C, Wira CR. Polymeric immunoglobulin (Ig) receptor production and IgA transcytosis in polarized primary cultures of mature rat uterine epithelial cells. *Biol Reprod.* 1995; 53:488–498. [PubMed: 7578671]
44. Godfrey TE, Kim SH, Chavira M, Ruff DW, Warren RS, Gray JW, Jensen RH. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5' nuclease quantitative reverse transcription-polymerase chain reaction. *J Mol Diagn.* 2000; 2:84–91. [PubMed: 11272893]
45. Wei X, Decker JM, Liu H, Zhang Z, Arani RB, Kilby JM, Saag MS, Wu X, Shaw GM, Kappes JC. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob Agents Chemother.* 2002; 46:1896–1905. [PubMed: 12019106]
46. Amella CA, Sherry B, Shepp DH, Schmidtmayerova H. Macrophage inflammatory protein 1 α inhibits postentry steps of human immunodeficiency virus type 1 infection via suppression of intracellular cyclic AMP. *J Virol.* 2005; 79:5625–5631. [PubMed: 15827177]
47. Moreau T, Baranger K, Dade S, Dallet-Choisy S, Guyot N, Zani ML. Multifaceted roles of human elafin and secretory leukocyte proteinase inhibitor (SLPI), two serine protease inhibitors of the chelonianin family. *Biochimie.* 2008; 90:284–295. [PubMed: 17964057]
48. Fahey JV, Schaefer TM, Channon JY, Wira CR. Secretion of cytokines and chemokines by polarized human epithelial cells from the female reproductive tract. *Hum Reprod.* 2005; 20:1439–1446. [PubMed: 15734755]

49. Kunz G, Noe M, Herbertz M, Leyendecker G. Uterine peristalsis during the follicular phase of the menstrual cycle: effects of oestrogen, antioestrogen and oxytocin. *Hum Reprod Update*. 1998; 4:647–654. [PubMed: 10027618]
50. Kunz G, Beil D, Deiniger H, Einspanier A, Mall G, Leyendecker G. The uterine peristaltic pump. Normal and impeded sperm transport within the female genital tract. *Adv Exp Med Biol*. 1997; 424:267–277. [PubMed: 9361805]
51. Kotronias D, Kapranos N. Detection of herpes simplex virus DNA in human spermatozoa by in situ hybridization technique. *In Vivo*. 1998; 12:391–394. [PubMed: 9706490]
52. Hadchouel M, Scotto J, Huret JL, Molinie C, Villa E, Degos F, Brechot C. Presence of HBV DNA in spermatozoa: a possible vertical transmission of HBV via the germ line. *J Med Virol*. 1985; 16:61–66. [PubMed: 3840197]
53. Piomboni P, Baccetti B. Spermatozoon as a vehicle for HIV-1 and other viruses: a review. *Mol Reprod Dev*. 2000; 56(2 Suppl.):238–242. [PubMed: 10824975]
54. Bandivdekar AH, Velhal SM, Raghavan VP. Identification of CD4-independent HIV receptors on spermatozoa. *Am J Reprod Immunol*. 2003; 50:322–327. [PubMed: 14672335]
55. Cremel M, Hamzeh-Cognasse H, Genin C, Delezay O. Female genital tract immunization: evaluation of candidate immunoadjuvants on epithelial cell secretion of CCL20 and dendritic/Langerhans cell maturation. *Vaccine*. 2006; 24:5744–5754. [PubMed: 16730865]
56. Garzino-Demo A. Chemokines and defensins as HIV suppressive factors: an evolving story. *Curr Pharm Des*. 2007; 13:163–172. [PubMed: 17269925]
57. Singh PK, Tack BF, McCray PB Jr, Welsh MJ. Synergistic and additive killing by antimicrobial factors found in human airway surface liquid. *Am J Physiol Lung Cell Mol Physiol*. 2000; 279:L799–L805. [PubMed: 11053013]
58. Chen X, Niyonsaba F, Ushio H, Okuda D, Nagaoka I, Ikeda S, Okumura K, Ogawa H. Synergistic effect of antibacterial agents human beta-defensins, cathelicidin LL-37 and lysozyme against *Staphylococcus aureus* and *Escherichia coli*. *J Dermatol Sci*. 2005; 40:123–132. [PubMed: 15963694]
59. Yamasaki K, Schaubert J, Coda A, Lin H, Dorschner RA, Schechter NM, Bonnart C, Descargues P, Hovnanian A, Gallo RL. Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. *FASEB J*. 2006; 20:2068–2080. [PubMed: 17012259]
60. Shaw JL, Diamandis EP. A potential role for tissue kallikrein-related peptidases in human cervico-vaginal physiology. *Biol Chem*. 2008; 389:681–688. [PubMed: 18627298]
61. Ajami K, Pitman MR, Wilson CH, Park J, Menz RI, Starr AE, Cox JH, Abbott Ca, Overall CM, Gorrell MD. Stromal cell-derived factors 1alpha and 1beta, inflammatory protein-10 and interferon-inducible T cell chemo-attractant are novel substrates of dipeptidyl peptidase 8. *FEBS Lett*. 2008; 582:819–825. [PubMed: 18275857]
62. Wolf M, Albrecht S, Marki C. Proteolytic processing of chemokines: implications in physiological and pathological conditions. *Int J Biochem Cell Biol*. 2008; 40:1185–1198. [PubMed: 18243768]
63. Guan E, Wang J, Norcross MA. Amino-terminal processing of MIP-1beta/CCL4 by CD26/dipeptidylpeptidase IV. *J Cell Biochem*. 2004; 92:53–64. [PubMed: 15095403]
64. Weinberg A, Quinones-Mateu ME, Lederman MM. Role of human beta-defensins in HIV infection. *Adv Dent Res*. 2006; 19:42–48. [PubMed: 16672548]

Fig. 1.

Induction of CCL20/MIP3 mRNA by uterine epithelial cells upon PAMP stimulation. Primary uterine epithelial cells were treated with PAMPS LPS, Zymosan, Poly(I:C), and Flagellin for 24 hr, the cells were harvested and RNA was extracted. Real-time RT-PCR was used to determine the relative levels of expression of CCL20/MIP3. After normalization to endogenous control β -actin and calibration to the untreated control, Results show one representative patient sample.

Fig. 2.

Induction of CCL20/MIP3 mRNA by uterine epithelial cells upon treatment with TLR3 agonist Poly(I:C). Primary uterine epithelial cells from five different patients (a) and primary Fallopian tube epithelial cells from four different patients (b) were treated with Poly(I:C) for 24 hr prior to harvesting cells and extracting RNA. Real-time RT-PCR was used to determine the relative expression levels of CCL20/MIP3. After normalization to endogenous control β -actin, each patient sample was further calibrated to its own untreated control.

Fig. 3. Constitutive CCL20/MIP3 secretion by uterine and Fallopian tube epithelial cells. Twenty-four hour accumulation of constitutive apical and basolateral conditioned media was collected from uterine and Fallopian tube epithelial cells and assayed for CCL20/MIP3 protein secretion by ELISA. Five UTEC and four FTEC samples are shown.

Fig. 4.

Poly(I:C) induced CCL20/MIP3 by uterine and Fallopian tube epithelial cells. Twenty-four hour accumulation of Poly(I:C) treated apical and basolateral conditioned media was collected from uterine (dark bars) and Fallopian tube (light bars) epithelial cells and assayed for CCL20/MIP3 protein secretion by ELISA. Figure shows 2 representative UTEC and FTEC samples. The P values were calculated using a two-tailed paired t -test. Significantly greater than control, ** $P < 0.01$, * $P < 0.05$.

Fig. 5.

Direct anti-HIV-1 activity of recombinant CCL20/MIP3. Human recombinant CCL20/MIP3 at 2, 20, and 200 ng/mL was incubated with HIV-1 IIIB and BaL at MOI 1 for 1 hr at 37°C prior to adding onto TZM-bl cells. Substrate -Glo was added 48 hr post-infection and level of viral infection was quantified by using a luminometer. Data was expressed as 'percent of control' with the 'virus only' samples set at 100%. The *P*-values were calculated using a two-tailed paired *t*-test. Significantly greater than control, ***P* < 0.01, **P* < 0.05.

Fig. 6.

Anti-HIV-1 activity of CCL20/MIP3 was not through interaction with TZM-bl cells or through post-infection mechanisms. (a) Recombinant CCL20/MIP3 at 200 ng/mL was added to TZM-bl cells for 1 hr at 37°C followed by washing and adding HIV-1 IIIB and BaL at MOI 1. Substrate -Glo was added 48 hr post-infection and viral infection was quantified by using a luminometer. Data were expressed as 'percent of control' with the 'virus only' samples set at 100%. (b) TZM-bl cells were infected with HIV-1 IIIB and BaL at MOI 1. At 6 and 24 hr post-infection, cells were washed and 20 ng/mL recombinant CCL20/MIP3 was added.