CCR6 REGULATION OF THE ACTIN CYTOSKELETON ORCHESTRATES HUMAN BETA DEFENSIN-2 AND CCL20-MEDIATED RESTITUTION OF COLONIC EPITHELIAL CELLS

Rebecca A. Vongsa¹, Noah P. Zimmerman¹, and Michael B. Dwinell¹
¹Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI, 53226

Running head: CCR6 activates Rho, calcium and PI3K in restitution
Address correspondence to: Michael B. Dwinell, PhD, Bobbie Nick Voss Laboratory, Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA, Email: mdwinell@mcw.edu

Intestinal inflammation is exacerbated by defects in the epithelial barrier and subsequent infiltration of microbes and toxins into the underlying mucosa. Production of chemokines and antimicrobial peptides by an intact epithelium provide the first line of defense against invading organisms. In addition to its antimicrobial actions, human beta defensin-2 (HBD2) may also stimulate the migration of dendritic cells through binding the chemokine receptor CCR6. As human colonic epithelium expresses CCR6, we investigated the potential of HBD2 to stimulate intestinal epithelial migration. HBD2 was equipotent to CCL20 in stimulating migration of polarized human intestinal Caco2 and T84 cells and non-transformed IEC6 cells. Neutralizing antibodies confirmed HBD2 and CCL20 engagement to CCR6 were sufficient to induce epithelial cell migration. Consistent with restitution, motogenic concentrations of HBD2 and CCL20 did not induce proliferation. Stimulation with those CCR6 ligands leads to calcium mobilization and elevated active RhoA, phosphorylated myosin light chain and F-actin accumulation. HBD2 and CCL20 were unable to stimulate migration in the presence of either Rho-kinase or PI3K inhibitors or an intracellular calcium chelator. Together, these data indicate that the canonical wound healing regulatory pathway, along with calcium mobilization regulate CCR6-directed epithelial cell migration. These findings expand the mechanistic role for chemokines and HBD2 in mucosal inflammation to include immunocyte trafficking and killing of microbes with the concomitant activation of restitutive migration and barrier repair.

Intestinal epithelial cells actively modulate the innate immune system through regulated production of cytokines, bioactive amines, chemokines and antimicrobial peptides (1;2). Chemokines are important innate immune molecules that are prototypic mediators of cell migration and regulate the trafficking of leukocytes through binding G-protein coupled chemokine receptors (3;4). Chemokines have also been implicated in several cell biological processes including cancer metastasis, angiogenesis, and stem cell recruitment (3;5;6). These chemoattractant molecules can be subdivided into two distinct subsets, inducible chemokines are upregulated by inflammatory stimuli and constitutive chemokines are minimally regulated by proinflammatory cytokine stimulation (4).

Defensins, like chemokines, are highly conserved key host defense molecules that participate in host defense through the direct killing of microbes (7). Unlike alpha defensins which are produced by Paneth cells at the base of intestinal crypts, beta defensins are produced by epithelial cells including the colonic mucosa. Phylogenetic studies show that beta defensins are evolutionarily conserved in mammals (7-9), and are characterized by pairing of specific cysteine residues (Cys1-Cys5, Cys2-Cys4, Cys3-Cys6). Of the four characterized human beta defensins (HBD), HBD1 is constitutively expressed while HBD2, HBD3, and HBD4 are inducibly expressed (10). Structurally, HBD1-4 share 6 conserved cysteine residues and tertiary structure that is key to their biologic activity (10). HBD2 is upregulated in mucosal inflammatory disorders (11-13).

The current, restricted, model states that chemokines direct the trafficking of damage-provoking or damage-exacerbating immune cells...
to the gut mucosa (1;14-17). This model is limited in that it ignores the physiologic contribution of chemokine signaling through their cognate receptors expressed by the cells of the intestinal epithelium. Expression of an array of chemokine receptors by human intestinal epithelial cells makes them robust targets for innate immune mediators produced in host defense responses (17-21). The studies herein support the significant ongoing expansion of the current model and indicate that chemokines upregulated in human inflammatory disorders enhance barrier repair. Like the homeostatic chemokine receptor CXCR4, the inducible chemokine receptor CCR6 is expressed by immature dendritic cells and circulating T cells and directs their trafficking to sites of inflammation following binding by the chemokine ligand CCL20 (22-24). CCL20 is prominently expressed by intestinal epithelial cells and upregulated during mucosal inflammatory disorders, including the inflammatory bowel diseases (IBD) (17;25;26). CCR6 is constitutively expressed by the human colonic epithelium and like its cognate ligand is upregulated during inflammation (17;18;26;27). The conserved tertiary structure of HBDs facilitate binding and activating G-protein coupled receptors, with human HBD1-4 shown to variably regulate chemotactic migration via the chemokine receptors CCR6 and CXCR4 (28-30).

Epithelial expression of CCR6 and production of its ligands, HBD2 and CCL20, are markedly upregulated in the course of inflammatory diseases when the innate epithelial barrier is compromised. Using epithelial cell model systems we demonstrate for the first time that HBD2 and CCL20 stimulate restitutive intestinal cell migration through mobilization of intracellular calcium, activation of phosphoinositide-3-kinase (PI3K), monomeric RhoGTPase, and myosin light chain (MLC) signaling pathways. Those distinct, co-regulated pathways converge upon and regulate reorganization of the F-actin cytoskeleton to increase epithelial sheet migration. These results significantly expand the mechanistic role for chemokines and defensins and are consistent with the notion that HBD2 and CCL20 have dual benefits as frontline defense molecules through the concomitant killing of microbes and leukocyte recruitment with activation of epithelial wound repair mechanisms.

Experimental Procedures

Materials- Recombinant HBD2 and human CCL20 was purchased from Peprotech (Rocky Hill, NJ), and were 96% and 99% pure as defined by the manufacturer. Pertussis toxin was purchased from EMD Biosciences (La Jolla, CA). Recombinant human CXCL12 and transforming growth factor-beta 1 (TGFβ1) were purchased from R&D Systems (Minneapolis, MN). The Rho kinase (ROCK) inhibitor Y27632 (K_i = 140mM) and the specific PI3K inhibitor LY294002 (IC50 = 1.4μM) was purchased from Sigma (St. Louis, MO). Alexafluor-488 phallloidin was from Invitrogen (Carlsbad, CA). Antibodies for phospho-myoosin light chain (pMLC) and total-myoosin light chain (MLC) were obtained from Cell Signaling Technologies (Danvers, MA). The antibody used to detect CCR6 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Neutralizing anti-CCR6 antibody was from R&D Systems. Monoclonal antibody to total RhoA was purchased from Cytoskeleton (Denver, CO).

Cell culture- The human intestinal carcinoma cell line Caco2 was cultured in DMEM (4g/L glucose) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Omega Scientific, Tarzana, CA), 2mM L-glutamine, and 1.5g/L NaHCO3. Human T84 colonic carcinoma cells (31) were cultured in DMEM/Ham’s F-12 medium (1:1) supplemented with 5% (v/v) newborn calf serum (Invitrogen) and 2mM L-glutamine as described previously (20;21). The normal, non-transformed rat small intestinal (IEC-6) cell line (CRL-1592) were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2mM L-glutamine, 1.5g/L NaHCO3 and 0.1 U/ml bovine insulin (Invitrogen).

IEC-6 wounding assay- Confluent IEC-6 cell monolayers grown in 60-mm dishes were incubated for 24 hours in serum-free medium, wounded with a sterile razor blade and incubated in medium alone or in the presence of defined concentrations of HBD2 or CCL20 for 18-hours at 37°C in 5% CO2. To assess cell migration signaling mechanisms, monolayers were pre-treated for 30 minutes with Y27632 (10μM),
LY294002 (2-50μM) or BAPTA-AM (10μM) and stimulated in the presence or absence of HBD2 and CCL20. Photomicrographs were taken using 100X magnification at 4–5 locations per wound and the number of migrated cells determined by counting nucleated cells that crossed the wound edge.

**T84 and Caco2 wounding assay**—Polarized T84 and Caco2 cells were grown to confluence in 6-well Transwell inserts (pore size 0.4μm Corning, Danvers, MA) and transepithelial resistance (TER) measured using a hand-held Millicell-ERS volt ohmmeter (Millipore, Billerica, MA). Cells were serum starved 24-hours and wounded with a 0.1-10μL plastic pipette tip (USA Scientific, Ocala, FL) connected to a bench top vacuum aspirator. In our hands, this apparatus consistently established wounds of between 800-1000μm in diameter. Medium on wounded polarized monolayers was replaced with serum-free medium, or serum-free medium containing HBD2 or CCL20 every 24 hours throughout the duration of the experiment. CXCL12 (20ng/ml) or TGFβ1 (5ng/ml) were assessed as positive controls. Photomicrographs were taken of the circular wounds using the 4X objective after wounding and each day thereafter and the area of each wound defined using MetaMorph software (Molecular Devices, Downingtown, PA). The TER of wounded monolayers was monitored immediately before wounds were photographed.

**Cell proliferation assay**—Cell proliferation was measured using propidium iodide staining and cell cycle analysis. Cells were stimulated with either HBD2 or CCL20 for 4, 8, 12, and 24 hours. Ten percent serum was assessed as a positive control. Ethanol-fixed cells were stained in 50μg/ml propidium iodide (EMD Biosciences) and 10μg/ml RNase A (Promega, Madison, WI,) and analyzed by flow cytometry.

**F-actin formation**—To quantify cellular F-actin content, IEC-6 cells were grown to 80% confluence and serum starved 24-hours prior to stimulation. Cells were pre-treated for 30 minutes with 10μM Y27632 to assess the involvement of ROCK in HBD2 and CCL20-mediated activation of F-actin. Cells were permeabilized with 1% (w/v) saponin in PBS and stained with Alexafluor-488 phalloidin for 20 minutes at 37°C 5% CO2. To facilitate release of the cells from the dish the cells were incubated at 37°C for 20 minutes in 50mM EDTA/PBS. The cells were transferred to FACS tubes (BD Biosciences, San Jose, CA), washed in PBS, fixed in 2% (w/v) paraformaldehyde/PBS and fluorescence measured using flow cytometry (BD Biosciences).

**Immunoblot analysis**—IEC-6 cells were grown to 80% confluence and serum starved 24 hours before stimulation with titrated doses of HBD2 or CCL20. Cells were solubilized in hypotonic lysis buffer [50mM Tris-HCl, pH 7.4, 10mM MgCl2, and Protease Inhibitor Cocktail Set III (EMD Biosciences)]. Lysates were passed through a pipette tip several times and centrifuged at 8000 rpm for 10 minutes at 4°C. Protein concentrations were determined using a Bradford protein assay kit (BCA kit, Pierce Biotechnology, Rockford IL) and 10 μg of protein were size-separated using reducing SDS-PAGE. Proteins were electrophoretically transferred to PVDF (Immobilon-P, Millipore) for immunoblot analyses as detailed previously (21).

**Fluorescence microscopy**—IEC-6 cell were grown to 30% confluence, washed in PBS, and fixed in 4% (w/v) paraformaldehyde for 15 minutes. After a wash step in PBS, the cells were incubated in 1% (w/v) BSA/PBS for 30 minutes, followed by an overnight incubation with a 1:50 dilution of rabbit polyclonal anti-CKR6 antibody (Santa Cruz) that specifically binds CCR6. Cells were washed and cell surface CCR6 was detected by incubation with an anti-rabbit FITC conjugated antibody. Cells were counterstained with DAPI and visualized using a fluorescence microscope at 200X.

**RhoA activation**—Activated RhoA was detected using the solid-phase G-LISA™ RhoA Activation Assay Biochem Kit™ from Cytoskeleton according to the manufacturer’s instructions. Briefly, IEC-6 cells were grown to 95% confluence and serum starved overnight. Monolayers were stimulated with optimal doses of HBD2 and CCL20, and 1μM LPA for 5 minutes. The cells were solubilized and RhoA-GTP was detected according to the manufacturer’s instructions. Data were analyzed by measuring light emission in counts per second for 0.1 seconds using Victor2 Wallac (Perkin-Elmer, Waltham, MA). Total RhoA and actin was detected from the same cell lysates using immunoblot analysis as described above.
**RhoGTP immunofluorescence**- Wounded Caco2 monolayers were stimulated with 20ng/ml HBD2 or 20ng/ml CCL20 for 20 minutes. The cells were fixed with 4% (w/v) paraformaldehyde (Kodak Eastman Company, Rochester, NY). Autofluorescence was quenched with 50mM NH₄Cl in PBS and the cells permeabilized with 0.3% (v/v) Triton-X100 in PBS for 10 minutes. Cells were washed in 1% (w/v) BSA in PBS wash buffer and blocked 30 minutes in 5% (w/v) BSA/PBS and incubated with 40μg/ml RBD-GST or 40μg/ml recombinant GST (Upstate, Charlottesville, VA) overnight at 4°C. The cells were washed in buffer and incubated 1 hour with 1μg/ml mouse-anti-GST (Cell Signaling) or mouse IgG (Molecular Probes) in 1% (w/v) BSA/PBS at room temperature. Cells were washed and incubated with 2μg/ml Alexafluor-488 goat-anti-mouse antibody (Molecular Probes). Cells were then stained for F-actin using Alexafluor-595 phallolidin according to the manufacturer's directions. Cells were visualized using confocal or fluorescence microscopy.

**Calcium mobilization assay**- Intracellular calcium mobilization was measured using the Fluo-4NW Assay as we defined previously (32). IEC-6 cells were plated in 96-well white walled plates (BD, Franklin Lakes, NJ) and grown to 90% confluence. Cells were serum starved overnight and loaded with the cell permeant Fluo-4 AM. HBD2 and CCL20 were added at indicated concentrations and intracellular calcium flux was measured by fluorescence spectroscopy every 5 seconds for 220 seconds (Victor Wallac). Background fluorescence for each well was measured for 30 seconds before addition of ligand and the average background was subtracted from each value.

**Statistical analysis**- Differences between unstimulated controls and experimental samples were analyzed using an unpaired Student's t-test using SigmaStat (Jandel Scientific Software, San Rafael, CA).

**RESULTS**

**HBD2 and CCL20 stimulate cellular migration of model intestinal epithelium.** To ascertain the role for inflammation-induced CCR6 ligands on enterocyte restitution, a conventional wound healing model was employed. Cells stimulated with either CCL20 (Fig. 1A) and HBD2 (Fig. 1B) migrated more than the unstimulated control monolayers and equal to the TGFβ1 positive control. Furthermore, stimulation of migration was specific for the inducible defensin HBD2 as IEC-6 monolayers treated with 20ng/ml of the constitutively expressed defensin HBD1 did not significantly increase migration (Fig. 1C). HBD2 and CCL20 dose dependently stimulated migration of non-transformed IEC-6 cells (Fig. 1E) consistent with previously published chemotaxis of CCR6-transfected HEK293 cells (33).

Migration in the absence of proliferation, defined as restitution, governs the early processes of barrier repair (34). Therefore, we sought to determine if cellular proliferation contributed to the migratory phenotype observed in monolayers stimulated with HBD2 and CCL20. To this end, serum starved cells were stained with propidium iodide and cell cycle analysis performed. As shown in Figure 1D, IEC-6 monolayers stimulated with either HBD2 or CCL20 did not have an increased percentage of cells in S-phase compared to unstimulated controls after 24 hours. However, cells stimulated with the positive control, 10% fetal bovine serum, had a significant increase in cells undergoing DNA synthesis. The lack of proliferation observed with the migration optimal dose of 20ng/ml was mirrored at 100ng/ml or 1000ng/ml of either HBD2 or CCL20 at 4, 8, 12 or 24 hours (data not shown). These results indicate that HBD2 and CCL20 specifically induce restitutive migration of model intestinal epithelium.

**HBD2 and CCL20 induce cell migration of human polarized monolayers.** We next confirmed restitution of those migrating IEC-6 epithelial sheets using two complimentary human polarized model epithelial cell lines. For this, Caco2 and T84 cells were grown until the TER was greater than 300Ωcm² or 700Ωcm², respectively. Cells were wounded and closure calculated by measuring the area of the denuded surface. Human Caco2 epithelial monolayers stimulated with either 20ng/ml CCL20 (Fig. 2A) or 20ng/ml HBD2 (Fig. 2B) had increased wound closure after 24 hours compared to unstimulated controls. Moreover, HBD2 and CCL20 stimulated wound closure of polarized Caco2 monolayers was equal to TGFβ1 (Fig. 2).
To further strengthen the notion that inflammatory mediators regulate epithelial migration, wounded human T84 monolayers were stimulated with either HBD2 or CCL20. In agreement with our data from the IEC-6 and Caco2 model epithelia, the motogenic 20ng/ml concentration increased wound closure above the unstimulated controls, an increase paralleled by CXCL12 (Table 1) assessed as a positive control. Consistent with those data, barrier integrity, defined as a measure of TER, demonstrated that polarized model epithelium stimulated with HBD2 or CCL20 increased resistance more rapidly than unstimulated controls (Table 1). In sum, results from three model epithelia indicate that HBD2 and CCL20 regulate intestinal barrier homeostasis.

Neutralization of CCR6 blocks HBD2 and CCL20 stimulated IEC-6 cell migration. HBD2 and CCL20 evoke cellular migration of dendritic cells and neutrophils specifically by binding and activating the chemokine receptor CCR6 (35;36). Human intestinal epithelial cells in vitro and in vivo express CCR6 (17). Therefore, we next sought to determine if HBD2 and CCL20 utilize CCR6 for cell migration. Since chemokine receptor expression in IEC-6 cells was incomplete, we first confirmed CCR6 expression in that particular model intestinal epithelial cell line. Immunoblot analysis defined expression of CCR6 in cell lysates of IEC-6 cells and Caco2 cells (Fig. 3A). Immunofluorescence microscopy of non-permeablized IEC-6 cells verified CCR6 localization to the cell surface in a pattern consistent with published reports (Fig. 3B) (37). These results indicate that IEC-6 cells express CCR6 at the cell surface where it is available to bind extracellular ligand.

In order to define specificity of HBD2 for CCR6 in cell migration, we used a specific neutralizing antibody to block activation of CCR6. Since CCL20 is the cognate ligand for CCR6 we also assessed the ability of the neutralizing antibody to block CCL20-mediated intestinal cell migration as a control (22). IEC-6 monolayers were pre-incubated with 5μg/ml of CCR6 neutralizing antibody or 5μg/ml isotype control antibody. Wounded cells were stimulated with 20ng/ml HBD2 or CCL20 and cellular migration quantified. Pretreatment with the CCR6 neutralizing antibody inhibited HBD2 and CCL20 mediated cell migration, while the isotype control antibody did not block migration (Figs. 3C and D). Migration assays showed that pre-treatment with CCR6 neutralizing antibody or the non-specific isotype control did not affect TGFβ1 migration (Fig. 3E). These data indicate that HBD2 and CCL20 activate cellular migration specifically through CCR6.

HBD2 and CCL20 stimulate accumulation of F-actin, phosphorylation of MLC, and RhoGTP. We next sought to define signaling molecules involved in HBD2 and CCL20 mediated cell migration. Previous work from our laboratory suggests chemokine receptors activate a canonical wound healing pathway consisting of RhoGTP, Rho-kinase, phospho-myosin light chain, and F-actin accumulation in model intestinal epithelium (21). Moreover, work by others has defined components of this pathway to be key regulators of migration in a variety of cell types (38-42). Therefore, we initially focused on defining the activation of these key cell migration molecules in IEC-6 cells treated with HBD2 and CCL20. Since F-actin accumulation at the leading edge of a migrating monolayer is a major hallmark of cell migration, we first quantified F-actin accumulation in IEC-6 cells using FITC-phalloidin and flow cytometry. As shown in Figure 4A and B, HBD2 and CCL20 stimulation increased F-actin ~ 25% above unstimulated cells.

Myosin light chain (MLC) is a regulatory subunit of myosin that upon activation by phosphorylation on Ser 19 facilitates the assembly of F-actin bundles (Russo et al., 2005). Therefore, we used immunoblot analyses to show that HBD2 and CCL20 induced Ser 19 phosphorylation on MLC, an upstream regulator of F-actin (Fig. 4C). We next examined RhoA as an upstream regulator of MLC phosphorylation (pMLC). Rho is activated in its GTP bound form and stimulates pMLC and F-actin bundling through Rho-kinase (ROCK) (40). Using an ELISA-based solid phase assay, we determined that HBD2 and CCL20 activate Ser 19 phosphorylation on MLC, an upstream regulator of F-actin (Fig. 4C). We next examined RhoA as an upstream regulator of MLC phosphorylation (pMLC). Rho is activated in its GTP bound form and stimulates pMLC and F-actin bundling through Rho-kinase (ROCK) (40). Using an ELISA-based solid phase assay, we determined that HBD2 and CCL20 activate RhoA in IEC-6 cells (Fig. 4D, top panel). Total RhoA and actin were subsequently assessed in those same cell lysates as a loading control (Fig. 4D, bottom panel). These data indicate that HBD2 and CCL20 activate key molecules regulating the actin cytoskeleton in migrating epithelia.

Next, we sought to verify that inflammatory mediators CCL20 and HBD2 are specifically regulating the actin cytoskeleton through CCR6
effectors. Like all chemokine receptors, CCR6 is a G-protein coupled receptor activated predominantly via the Gαi subunit (3). In fact, CCR6-mediated chemotaxis of immune cells is potently inhibited upon blockade of Gαi with pertussis toxin (33;36). Although not previously described for epithelial migration, pertussis toxin was used to assess if Gαi signaling was involved in the activation of RhoA by HBD2 and CCL20. In agreement with our data on CXCL12 (20), pretreatment of IEC-6 cells with pertussis toxin decreased RhoGTP (Fig. 4E) with the concomitant decrease in HBD2 or CCL20-stimulated migration (data not shown). Although the inhibition was not complete, the data support the notion that heterotrimeric proteins coupled to CCR6 are activated and initiate down-stream effectors of the actin cytoskeleton.

To ascertain if CCR6-regulated F-actin accumulation was simply a function of epithelial sheet migration, or a more global effector of stimulated epithelium, we examined polarized Caco2 monolayers. Fluorescence microscopy was next used to determine that both active RhoGTP and F-actin bundles increasingly localize at the leading edge of wounded Caco2 monolayers stimulated with optimal concentrations of HBD2 or CCL20 (Fig. 4F). These data indicate that the mechanisms regulating enterocyte migration in human polarized, circular wound model system parallel those activated in migrating IEC-6 epithelial sheets.

ROCK participates in HBD2 and CCL20 mediated migration and F-actin accumulation. ROCK is a direct downstream effector of RhoGTP and controls MLC by inactivating its regulatory phosphatase or directly catalyzing MLC phosphorylation (40). In order to further dissect the regulatory mechanisms in HBD2 and CCL20-mediated migration, the specific ROCK inhibitor Y27632 was used. Inhibition of ROCK abrogated IEC-6 cell migration (Fig. 5A) and F-actin accumulation stimulated by HBD2 and CCL20 (Fig. 5B). These data indicate that Rho and its immediate downstream effector ROCK participate in both F-actin accumulation and cell migration induced by HBD2 and CCL20.

Intracellular calcium flux induced by HBD2 and CCL20 contributes to cell migration. Intracellular calcium flux is an established regulator of F-actin accumulation and cell migration and is stimulated by CCR6 ligands in immune cells (22;43;44). Therefore, we first asked if HBD2 and CCL20 regulated calcium mobilization in model intestinal epithelium. The Fluo-4NW assay defined for the first time that HBD2 (Fig. 6A) and CCL20 (Fig. 6B) induce a dose dependent intracellular calcium flux in adherent IEC-6 cells. Calcium mobilization in response to 100ng/ml of the chemokine CXCL12 was shown as a positive control as it is known to induce calcium flux in human intestinal cells (19).

Calcium mobilization occurs either by the opening of channels on the plasma membrane allowing extracellular calcium to enter, or via release from internal stores located primarily in the endoplasmic reticulum (45). In order to distinguish between these two mechanisms, we first chelated extracellular calcium with 3μM EGTA and determined that the initial calcium flux, before 100 seconds, was not significantly altered following addition of those ligands (Fig. 6C). However, the sustained calcium response after 100 seconds was impaired in cells treated with EGTA indicating influx of extracellular calcium was responsible for the persistent elevation in intracellular calcium. Next, we treated cells with the intracellular calcium chelator, BAPTA-AM, and ascertained that calcium mobilization was decreased at the 3μM dose (data not shown) and abolished at the 10μM dose (Fig. 6D). These data indicate that HBD2 and CCL20 induce release of intracellular calcium stores, which, in turn, stimulate a sustained influx of extracellular calcium consistent with store-operated calcium entry.

Calcium mobilization is a critical regulator of epithelial cell migration. Upon influx, calcium binds to calmodulin which together regulates a variety of cellular kinases including myosin light chain kinase (MLCK), the kinase primarily responsible for the activation of MLC (42). Since CCR6 ligands stimulate calcium flux in leukocytes (22), we reasoned that calcium mobilization was involved in HBD2 and CCL20-directed migration of wounded epithelial cells. As shown in Fig. 7A, pre-incubation with 3μM or 10μM BAPTA-AM did not affect baseline IEC-6 migration in the wound healing assay. However, pre-treatment with 30μM or 100μM BAPTA-AM dose-dependently blocked migration, with the latter
dose abolishing cell movement. These results indicate that treatment with 10μM BAPTA-AM does not affect constitutive migration, yet this concentration was sufficient to block HBD2 and CCL20-induced calcium mobilization (Fig. 6D). Furthermore, 10μM BAPTA-AM did not affect TGFβ1 stimulated wound healing indicating chelation of intracellular calcium did not globally disrupt enterocyte migration signaling (Fig. 7A). In contrast to TGFβ1, 10μM BAPTA-AM was sufficient to block HBD2 and CCL20-stimulated migration (Fig. 7B), indicating BAPTA-AM specifically interrupts migratory signaling by those CCR6 ligands. Together these data implicate calcium mobilization as a necessary step for the induction of cell migration by that G-protein coupled receptor.

**Phosphoinositide-3-Kinase regulates HBD2 and CCL20-mediated cell migration.** Having demonstrated a role for Gαi, RhoGTP and calcium in activated CCR6 regulated epithelial cell migration we next addressed the role for phosphoinositide 3-kinase (PI3K) in epithelial cell migration. In leukocytes, chemokine receptor functions are tightly linked with PI3Kγ signaling (46). Moreover, heterotrimeric Gαi-protein coupled receptor activation of PI3Kγ has previously been shown to regulate the sustained influx of external calcium, a response we demonstrated in HBD2 and CCL20-stimulated IEC-6 cells (Fig 6) (47). Although a role for activated PI3K in epithelial restitution had not previously been demonstrated, we had previously shown that inhibition with wortmannin or LY294002 potently blocked human T84 colonic epithelial cell migration (20). IEC-6 monolayers pretreated with the specific PI3K inhibitor LY294002 were wounded, stimulated with CCR6 ligands and migration assessed. Consistent with a role for PI3K in chemokine receptor regulated migration, LY294002 dose-dependently inhibited restitution stimulated by 20ng/ml HBD2 (Fig. 7C). Further, PI3K-dependent migration was mediated in part through activation of Rho (Relative RhoGTP levels: HBD2 = 131.8±3.5; HBD2 + LY294002 = 97.1±24.6; LY294002 = 112±17.1). Based on those data, we propose that HBD2 and CCL20 signal cell migration an inter-related mechanisms consisting of calcium, PI3K, and Rho that lead to increased F-actin accumulation and localization within the migrating epithelial cells.

**Discussion**

The single layer of epithelial cells lining the mucosal surface of the gastrointestinal tract is a critical component of the mucosal innate immune system and comprises a physical barrier between the external luminal milieu and the internal environment. The intestinal epithelium is injured on a daily basis by a variety of stimuli including noxious luminal contents, normal digestion, inflammation, interactions with microbes, and pharmaceuticals (48). Therefore, maintenance of this essential innate immune barrier requires the ability of this single layer of cells to efficiently repair wounds and establish polarity in order to maintain homeostasis (49). Upon injury the intestinal epithelium undergoes a wound repair process that starts with proliferation-independent epithelial cell migration, termed restitution, into the wounded area, whereupon the migrated cells subsequently proliferate and differentiate into mature enterocytes (48-50). Pathologic intestinal inflammation is exacerbated by breakdowns in the epithelial barrier and subsequent penetration of luminal microbes and toxins into the underlying mucosa. An intact barrier, chemokine signaling, and antimicrobial peptides, provide the first line of protection against invading organisms. Together, our data are consistent with the notion that HBD2 and CCL20 are bi-functional host defense molecules that function to prevent penetration of luminal contents by directing dendritic cell trafficking or directly killing microbes and by stimulating efficient barrier repair. These findings significantly expand the model and indicate that secreted innate host defense mediators may also orchestrate epithelial wound repair to further limit entry of noxious stimuli.

Enterocyte migration induced by CCR6 ligands was demonstrated using Caco2 and T84 polarized human model epithelium. Further, both HBD2 and human CCL20 robustly stimulated cellular migration in a model of the non-transformed rat IEC-6 epithelium. It is not surprising that human ligands are functional on rat cell lines given the high degree of conservation among chemokine receptors, chemokines, and beta defensins (8;9). Structural studies on rodent CCL20 and HBD2 suggest that HBD2 is a
simplified version of CCL20 and both contain similar Asp-Leu residues considered responsible for binding CCR6 (8). Although structural studies of rat beta defensins are not available, the residues proposed to be important for HBD2 binding to CCR6 are conserved in several rat beta defensin genes (9;51). Despite the lack of those structural studies HBD2 has been shown to bind and activate both mouse and rat cells in culture (52). This is a phenomenon shared by chemokine receptors and their ligands. For example, the human chemokine CXCL12 can bind and activate its receptor CXCR4 on rodent cells (20;21). Moreover, the ability of HBD2 and human CCL20 to signal in rat cells was verified herein using calcium flux assays, a classical and well-established readout of chemokine receptor signaling.

Neutralizing antibodies verified CCR6 as the receptor regulating CCL20 and HBD2 intestinal migration. In additional studies we showed that motogenic and antimicrobial doses of either CCL20 or HBD2 failed to induce a rapid proliferative response, indicating those CCR6 ligands specifically stimulate restitutive migration. In contrast, a prior report shows that sustained incubation with supraphysiologic doses of CCL20 activates proliferation in Caco2 cells after 72 hours of treatment (37). These latter data suggest that CCL20 signaling may have an even broader role in epithelial barrier repair than rapid restitutive migration. We also assessed the ability of a constitutively expressed beta defensin, to stimulate wound healing and found that 20ng/ml HBD1 was not sufficient to induce migration. Our findings are consistent with previous reports in keratinocytes and neutrophils that show HBD2 but not HBD1 induce chemotaxis and cellular migration (36;53). These results indicate that cell migration is not a common property of all beta defensins, but specific to at least HBD2. Further studies with HBD3 and HBD4 will determine if these beta defensins can also be categorized as cell migratory.

Other investigators have recently shown that HBD2 induces epithelial migration at 1000ng/ml in the transformed HT29 intestinal cell line (54). In marked contrast, we chose to focus our studies using 20ng/ml of HBD2 as that concentration is within bactericidal range of the molecule (55) and approximates the concentration of HBD2 observed in human gastric juices and human bronchoalveolar lavage (56). Although the exact concentration of HBD2 in colonic mucosa remains unclear, studies confirm its presence in human colon and that it is upregulated during inflammation and IBD (12;13). In addition, salt concentrations are known to inhibit the antimicrobial functions of beta defensins and therefore may also affect their ability to stimulate migration (7). The 20ng/ml concentration of HBD2 has been shown to stimulate immune cell chemotaxis, however it is below the 1000ng/ml dose shown to be effective in dendritic cell chemotaxis (33) and may reflect the fundamental differences between epithelial and leukocyte migration (50). Alternatively, differences in beta-defensin-induced migratory responses may reflect lower salt concentrations at the gut mucosa or selective pressure of intestinal epithelium to be more sensitive to HBD2 in order to maintain this important immune barrier.

Intestinal permeability defects are associated with several intestinal diseases such as IBD, cancer, radiation injury, enterocolitis, and Celiac disease (57-62). Inflammatory molecules are classically thought to contribute to defects in permeability and exacerbation of disease (63;64). However, using polarized T84 and Caco2 monolayers we showed that HBD2 and CCL20 enhanced barrier integrity of epithelium as measured by transepithelial resistance. This suggests that inflammatory molecules like HBD2 and CCL20 could be beneficial in preventing or limiting disease in individuals with gut permeability defects. Likewise, these data present the possibility that dysregulation of HBD2 and CCL20 contributes to intestinal permeability defects consistent with studies showing that individuals with Crohn’s disease have impaired induction of beta defensins (2). Despite a battery of molecules having been shown to stimulate restitution the mechanisms by which they elicit their functions are not well known. Therefore, we investigated the mechanism(s) of HBD2 and CCL20 mediated restitution in model epithelium. It is important to note that the intestinal mucosa resides in a highly complex and dynamic milieu. Restitution is dependent on several factors present in vivo that are absent from our model system including mucin producing goblet cells, extracellular matrix producing fibroblasts, immune cells, and luminal
contents. However, the IEC6, Caco2, and T84 model systems have been successful at predicting cellular mechanisms important in restitution in vivo (65-67).

Previously our laboratory has shown that the constitutively expressed chemokine CXCL12 activates RhoGTP, and in turn its downstream effectors ROCK and phosphorylated MLC leading to the accumulation of F-actin (21). This pathway is classically associated with organization of contractile F-actin bundles, a hallmark of epithelial cell migration (39;41). Our studies determined that HBD2 and CCL20 similarly activated RhoA, pMLC, and accumulation of F-actin, with ROCK signaling a regulator in CCR6-driven migration and F-actin accumulation. Moreover, we built upon that foundation and determined that intracellular calcium flux was involved in HBD2 and CCL20 mediated cell migration.

Calcium is an established regulator of F-actin and an well-defined readout for chemokine receptor activation (44;45). HBD2 and CCL20 similarly induced intracellular calcium mobilization in a dose dependent manner. Elevation in intracellular calcium was maintained after chelation of external calcium, while mobilization of intracellular calcium measured after 100 seconds was decreased. Furthermore, intracellular calcium mobilization was abolished after chelation of intracellular calcium with BAPTA-AM. Together, those data suggest that intracellular calcium flux is derived primarily from internal stores of calcium (45). In support of that notion, chelation of intracellular calcium abolished HBD2 and CCL20 directed cell migration without disrupting TGFβ1-mediated migration. These data suggest that calcium chelation specifically inhibits CCR6-mediated enterocyte cell migration and does not globally disrupt epithelial cell migration. Moreover, intracellular calcium mobilization may be a mechanism of cell migration unique to chemokine receptors or other G-protein coupled receptors.

Calcium, in conjunction with calmodulin, regulates a variety of cellular kinases including MLCK, primarily responsible for phosphorylating MLC (42;68;69). Calcium also regulates F-actin formation, although the mechanism for that increase remains poorly understood (44). Therefore, it is conceivable that calcium mobilization is involved in regulating components of the Rho-directed accumulation and reorganization of the F-actin cytoskeleton needed for epithelial cell migration.

Together, our results show HBD2 and CCL20 work through the chemokine receptor CCR6 to activate Rho and PI3K, and mobilize intracellular calcium to evoke reorganization of the actin cytoskeleton. Activation of those regulatory pathways contributes to efficient epithelial migration and mucosal barrier repair. It is important to note that other signaling molecules may also contribute to the wound healing process. Recent studies have shown that molecules such as Rac, LIM-kinase, cofilin, and mDIA (70;71) are also important in cell migration and may also be involved in CCR6 mediated cell migration.

Mucosal wound healing is a treatment goal for individuals undergoing therapy for IBD (72). This concept was validated in recent studies that show mucosal wound healing is significantly associated with a low risk of colectomy and decreased inflammation (73). These studies strengthen the notion that the ability to rapidly heal wounds that afflict the intestinal epithelium is critical to maintaining homeostasis and prevention of disease. Therefore, factors that stimulate wound healing are of clinical importance as possible therapeutics for IBD. Overall, these migration studies suggest that chemokines and beta defensins are protective host defense molecules that function not only to recruit immune cells and kill microbes, but also to increase the efficiency wound healing in the gut.
References


Footnotes

We gratefully acknowledge Soonyean Hwang and Kristen Aicher for technical assistance provided in the completion of these studies. We thank Nita Salzman, MD, PhD, Department of Pediatrics, for discussion of the work and guidance on defensin biology. This work was supported in part by a grant from the NIDDK (DK062066).

Abbreviations used are: HBD, human beta defensin; IBD, inflammatory bowel disease; MLCK, myosin light chain kinase; MLC, myosin light chain; pMLC, phospho-myosin light chain; ROCK, Rho-kinase; TER, transepithelial resistance; TGFβ1, transforming growth factor beta.
Table 1. Increased wound closure and barrier integrity in T84 polarized human model epithelia stimulated with CCR6 ligands.

<table>
<thead>
<tr>
<th>stimuli</th>
<th>Wound Closure (% day 0)</th>
<th>TER (% day 0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>None</td>
<td>10±2.3</td>
<td>18±3.4</td>
</tr>
<tr>
<td>HBD2</td>
<td>14±1.8</td>
<td>27±3.2*</td>
</tr>
<tr>
<td>CCL20</td>
<td>16±3.5</td>
<td>27±4.5</td>
</tr>
<tr>
<td>CXCL12</td>
<td>27±2.4*</td>
<td>44±3.2*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n =3. TER; transepithelial resistance. Cells were stimulated with an optimal 20ng/ml concentration of HBD2, CCL20 or CXCL12 and wound closure and TER measured as defined in Experimental Procedures. Asterisks denote statistical difference (P≤0.05) between stimulated and unstimulated control monolayers.
Figure Legends

**Fig. 1.** CCL20 and HBD2 stimulate restitutive migration of IEC-6 cells. *A.* and *B.* IEC-6 monolayers were wounded and either left untreated (no stim) or stimulated with 20ng/ml CCL20 (*A*), 20ng/ml HBD2 (*B*), 5ng/ml TGFβ1, or 20ng/ml CXCL12. Representative wounds after 18 hours after wounding are shown (*A* and *B* bottom panels) and cells that migrated into the wound after 18-hours were enumerated. CCL20 and HBD2 (top panels) stimulated cellular migration was equal to two separate positive controls TGFβ1 and CXCL12. Scale bar equals 200µm. Values are mean ± SEM, n=3-5. *C.* IEC-6 monolayers left untreated (no stim), or stimulated with HBD1 (20ng/ml), HBD2 (20ng/ml), or TGFβ1 (5ng/ml) were assessed for cellular migration as in *A.* HBD1 induced minimal migration above untreated control monolayers. Values are mean ± SEM, n=3. *D.* Proliferation was assessed in IEC-6 cells left unstimulated (no stim) or treated with 20ng/ml HBD2, 20ng/ml CCL20, or 10% serum after 24 hours. The percentage of S-phase cells was calculated from flow cytometry and averages from 3 experiments are shown. *E.* IEC-6 monolayers left untreated or stimulated with increasing concentrations of HBD2 and CCL20 or TGFβ1 were assessed for cellular migration as in panel *A.* HBD2 and CCL20 stimulated migration increased with increasing concentration of stimuli. Data are representative of three experiments. Asterisks denote statistically significant difference from untreated cells (P≤0.05).

**Fig. 2.** CCL20 and HBD2 and stimulate cell migration in human polarized Caco2 monolayers. *A.* and *B.* Caco2 monolayers were wounded and % closure was calculated after 24 hours. Wound closure was increased following addition of either 20ng/ml CCL20 (*A*) or 20ng/ml HBD2 (*B*) relative to untreated controls (no stim) and similar to the positive control, 5ng/ml TGFβ1, in three separate experiments (top panel). Representative wounds after 0 hours and 24 hours of closure are shown in the bottom panels. Asterisks denote statistically significant difference from untreated cells (P≤0.05). Scale bar equals 200µm.

**Fig. 3.** Neutralization of CCR6 inhibits CCL20 and HBD2 mediated IEC-6 wound healing. *A.* IEC-6 and Caco2 cell lysates were analyzed using immunoblot and demonstrated total CCR6 protein expression in model intestinal epithelial cells. *B.* Non-permeabilized IEC-6 cells were immunostained with anti-CCR6 or IgG control antibody. CCR6 localization was visualized using immunofluorescence microscopy (*B*, top panels). Nuclei were visualized with DAPI (middle panels) and brightfield images of cells were obtained with light microscopy (bottom panels). Results in *A* and *B* are representative of 3 experiments. *C.*, *D.*, and *E.* IEC-6 monolayers were pre-treated with 5µg/ml neutralizing CCR6 antibody (anti-CCR6) or an IgG isotype control. The monolayers were wounded and stimulated with serum free medium alone (no stim), 20ng/ml CCL20 (*C*), 20ng/ml HBD2 (*D*) or 5ng/ml TGFβ1 as a positive control and cell migration assessed. Blockade of CCR6 inhibited CCL20 and HBD2 directed cell migration. Treatment with the control antibody did not affect cell migration (*C* and *D*) or TGFβ1 stimulated migration (*E*). Values in *C* and *D* are mean ± SEM of three experiments. Values in *E* are mean ± SD and representative of 3 experiments. Asterisks denote statistically significant difference from untreated cells (P≤0.05).

**Fig. 4.** HBD2 and CCL20 regulate the actin cytoskeleton in migrating epithelial cells. *A.* and *B.* IEC-6 cells were stimulated with 20ng/ml HBD2, 20ng/ml CCL20, or lysophosphatidic acid (LPA) [1µg/ml], assessed as a positive control, and F-actin stained using FITC-phalloidin. *A.* Representative flow cytometry histogram of F-actin accumulation 15 minutes after stimulation. *B.* Mean fluorescence intensity was determined and normalized to unstimulated (no stim) control values. Increased F-actin accumulation in HBD2 or CCL20 stimulated cells as a percent of control. *C.* Increased phosphorylation of myosin light chain kinase (pMLC) in HBD2 or CCL20 stimulated IEC-6 cells. LPA was assessed as a positive control. Cell lysates were analyzed by immunoblot analysis. Total myosin light chain (tMLC) and F-actin were assessed as a loading control. Representative blots from 4 experiments are shown. *D.*
IEC-6 cells stimulated with 20ng/ml HBD2, 20ng/ml CCL20 or 1μg/ml LPA as a positive control had more activated RhoA than untreated controls. Activated RhoA was analyzed using a solid-phase assay (top panel). Total RhoA and actin were assessed by immunoblot as a loading control and representative data shown (bottom panel). E. Treatment with pertussis toxin decreased HBD2 and CCL20 stimulated RhoA activation to unstimulated (no stim) levels. IEC-6 cells were pretreated with 200ng/ml pertussis toxin (PTx) and activated RhoA assessed. Representative immunobLOTS confirmed equal protein loading (bottom panel). F. Increased localization of RhoGTP (green) and F-actin (red) at the leading edge of wounded Caco2 monolayers stimulated with 20ng/ml HBD2 and CCL20 for 20 minutes. Data are representative of four separate wounds. Values in B, D, and E are mean ± SEM of 3-5 experiments. Asterisks denote statistically significant difference from untreated cells (P≤ 0.05).

Fig 5. Rho-kinase regulates HBD2 and CCL20 stimulated IEC-6 cell migration and F-actin accumulation. A. Inhibition of ROCK abrogated HBD2 and CCL20 mediated cell migration to unstimulated control (no stim) levels 18 hours after stimulation. IEC-6 monolayers were pre-treated with the specific Rho-kinase (ROCK) inhibitor Y27623 (10μM). Wounded cells were stimulated with 20ng/ml HBD2, 20ng/ml CCL20, or 5ng/ml TGFβ1 as a positive control. B. Blockade of ROCK inhibited HBD2 and CCL20 directed F-actin accumulation. IEC-6 cells were wounded and stimulated as in panel A and F-actin content quantified 15 minutes after stimulation using flow cytometry. Mean fluorescence intensity values were determined and normalized to control (no stim). LPA (1μg/ml) was used as a positive control. Values are mean ± SEM, n = 3-4 experiments. Asterisks denote statistically significant difference from untreated cells (P< 0.05).

Fig 6. HBD2 and CCL20 induce calcium mobilization. Intracellular calcium mobilization was induced in a dose dependent manner when IEC-6 cells were treated with titrated concentrations of HBD2 (A), or CCL20 (B), or 100ng/ml CXCL12 as a positive control. C. Calcium flux after 100 seconds was diminished in EGTA-treated cells. Pre-treatment with 3μM EGTA minimally regulated the initial (<100seconds) calcium flux. D. Pre-treatment with the cell permeant chelator 10μM BAPTA-AM (BAPTA) abolished calcium mobilization stimulated by 100ng/ml HBD2, 100ng/ml CCL20, or the positive control 100ng/ml CXCL12. Intracellular calcium mobilization was measured every 5 seconds for 220 seconds using a pre-loaded fluorescent indicator dye Fluo-4NW. Relative fluorescence units (RFU) were obtained using a fluorescence plate reader and background (bkgrd) was subtracted from each value. Data are representative of 3-5 independent experiments.

Fig 7. Calcium mobilization participates in HBD2 and CCL20 mediated migration of model intestinal epithelium. A. Pre-treatment with 30μM or 100μM BAPTA-AM (BAP) inhibited IEC-6 cell migration while the 3μM and 10μM doses had limited effect on basal cell motility and was equal to the untreated control (no stim). Monolayers stimulated with 5ng/ml TGFβ1 had increased migration over untreated controls and was not effected by 10μM BAPTA-AM pre-treatment. B. HBD2 and CCL20 mediated IEC-6 migration was inhibited by 10μM BAPTA-AM. Monolayers were pretreated with BAPTA-AM, wounded, and stimulated with 20ng/ml HBD2 or 20ng/ml CCL20. Values in A and B are mean ± SEM from 3-5 independent experiments. C. Specific inhibition of PI3K with LY294002 dose-dependently inhibited IEC-6 cell migration stimulated by 20ng/ml HBD2, or the positive control 5ng/ml TGFβ1. Values in C are mean ± SD from 3 independent experiments. Asterisks denote statistically significant difference from untreated cells (P< 0.05).
Figure 1
Figure 3

A

IEC-6  CaCo2

CCR6

-43kD

GAPDH

-25kD

B

IEC6 cells

CCR6

IgG cont.

DAPI

bright field

C

Cells migrated

150

125

100

75

50

25

0

100

75

50

25

0

150

125

100

75

50

25

0

D

200

150

100

50

0

E

# cells migrated

no stim  TGF-β1  IgG cont.  TGFβ1  αCCR6  TGFβ1  αCCR6

--- +  +  +  +  +  +  +

--- +  +  +  +  +  +  +

--- +  +  +  +  +  +  +

--- +  +  +  +  +  +  +

--- +  +  +  +  +  +  +
Figure 5