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Targeted intestinal epithelial deletion of the chemokine receptor CXCR4 reveals important roles for extracellular-regulated kinase-1/2 in restitution

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Barrier defects and/or alterations in the ability of the gut epithelium to repair itself are critical etiological mechanisms of gastrointestinal disease. Our ongoing studies indicate that the chemokine receptor CXCR4 and its cognate ligand CXCL12 regulate intestinal–epithelial barrier maturation and restitution in cell culture models. Gene-deficient mice lacking CXCR4 expression specifically by the cells of the intestinal epithelium were used to test the hypothesis that CXCR4 regulates mucosal barrier integrity *in vivo*. Epithelial expression of CXCR4 was assessed by RT-PCR, Southern blot, immunoblot and immunohistochemistry. *In vivo* wounding assays were performed by addition of 3% dextran sodium sulfate (DSS) in drinking water for 5 days. Intestinal damage and DAI scores were assessed by histological examination. Extracellular-regulated kinase (ERK) phosphorylation was assessed *in vivo* by immunoblot and immunofluorescence. CXCR4 knock-down cells were established using a lentiviral approach and ERK phosphorylation was assessed. Consistent with targeted roles in restitution, epithelium from patients with inflammatory bowel disease indicated that CXCR4 and CXCL12 expression was stable throughout the human colonic epithelium. Conditional CXCR4-deficient mice developed normally, with little phenotypic differences in epithelial morphology, proliferation or migration. Re-epithelialization was absent in CXCR4 conditional knockout mice following acute DSS-induced inflammation. In contrast, heterozygous CXCR4-depleted mice displayed significant improvement in epithelial ulcer healing in acute and chronic inflammation. Mucosal injury repair was correlated with ERK1/2 activity and localization along the crypt–villus axis, with heterozygous mice characterized by increased ERK1/2 activation. Lentiviral depletion of CXCR4 in IEC-6 cells similarly altered ERK1/2 activity and prevented chemokine-stimulated migration. Taken together, these data indicate that chemokine receptors participate in epithelial barrier responses through coordination of the ERK1/2 signaling pathway.
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KEYWORDS: chemokine receptor; conditional knockout; ERK1/2; intestinal barrier; restitution; wound healing

INTRODUCTION

Gastrointestinal diseases are among the most common and least understood human health problems. A common feature of these diseases is a change in the inflammatory tone of the mucosal lining of the gut. Pathologically, elevation in mucosal inflammation is reflective of increased production of immune mediators such as tumor necrosis factor (TNF), interleukin-1, and chemokines to activate and direct the trafficking of immune cells into the lamina propria.[1] Inflammatory molecules are classically thought to exacerbate disease by contributing to defects in permeability across the protective epithelium.[2,3] Intestinal permeability defects have been implicated in the pathogenesis of several intestinal diseases such as inflammatory bowel disease (IBD), cancer, radiation injury, enterocolitis, and celiac disease.[4–7] The concept of targeted therapy to limit barrier permeability in human disease is an important treatment goal[8] that was recently validated in a study showing that increased mucosal wound healing is associated with decreased risk of colectomy, inflammation,[9] and disease recurrence.[10]

The human gastrointestinal mucosa forms an active interface between the human body and the external luminal environment. The polarized epithelial cell monolayer is held together by cellular junctions and has two important tasks.

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Firstly, epithelial cells regulate the absorption of nutrients as well as the diffusion and transport of solutes and particles. Secondly, and perhaps more importantly, the cells of the epithelium are a dynamic barrier that excludes the passage of potentially noxious molecules and organisms into the body. The protective epithelium not only forms a physical barrier, but also actively orchestrates innate and adaptive immune responses. These include the luminal secretion of mucins, trefoil factors, antimicrobial peptides and the mucosal production of chemokines, which regulate the trafficking of immune cells to the site of infection or antigen entry. Repair of the intestinal epithelium is an intrinsic property of epithelial cells to rapidly migrate across the denuded surface following injury.[11] This process, termed restitution, is independent of subsequent proliferation of epithelial cells to complete repair of the injured surface. Basal, constitutive restitution may be actively increased by an array of secreted mediators within the mucosal milieu, including transforming growth factor-β (TGF-β),[12] epidermal growth factor,[13] trefoil factors,[14] and, as we have shown, chemokine receptor ligands.[15–18] Although restitution is a multifactorial process, temporal and quantitative expression analyses of those factors in human inflammatory disorders or murine models remains scant.

Chemokines have critical roles in directing immune cell infiltration and activation in mucosal inflammation.[1] Members of the chemokine family can be classified by expression patterns into inducible and constitutive subfamilies based on their regulation by proinflammatory stimuli or host stress response.[19] Previous reports variably link inducible chemokines and chemokine receptors with exacerbation of inflammation in murine models of colitis.[20–23] Previous work suggests that the mucosal barrier is a likely target for chemokine signals as intestinal epithelial cells express a battery of chemokine receptors, notably CXCR4, CCR5, CCR6, and CX_3CR1.[24,25] Chemokines are small chemotactic cytokines that function through the activation of G-protein-coupled seven-transmembrane receptors. Research in our laboratory and others has determined that CXCR4 localized to the cell surface of cultured human intestinal epithelial cells activates G proteins, modulates electrogenic ion transport, and upregulates the expression of neutrophil chemoattractants and intercellular adhesion molecule-1.[24–26] The known role of chemokines in the directed migration of cells through engagement of cognate receptors prompted us to investigate the role of CXCR4 in the maintenance of the intestinal epithelial barrier. Activation of intestinal epithelial expressed CXCR4 by its cognate ligand CXCL12 has a role in intestinal epithelial restitution, consistent with a role for this axis in the maintenance of mucosal barrier integrity.[15,16,18]

Mice genetically deficient in either CXCR4 or CXCL12 die perinatally, with significant defects in hematopoiesis, cardiogenesis, and vascularization of the gastrointestinal tract.[27,28] The embryonic lethality of CXCR4 knockout mice has, to date, precluded the study of the biological role of CXCR4–CXCL12 signaling within the adult intestinal mucosa. Thus, to determine the role of CXCR4 signaling in the intestinal mucosa, we conditionally inactivated CXCR4 specifically in cells of the intestinal epithelium. Conditional loss of CXCR4 disrupted re-epithelialization of injured mucosa. Extracellular-regulated kinase-1/2 (ERK1/2) activity was differentially regulated in conditional knockout mice consistent with roles for that key signaling pathway in epithelial restitution *in vivo*. Taken together, our data suggest that CXCR4 has a role in epithelial injury repair *in vivo* and its targeted deletion results in the specific activation of a redundant chemokine-regulated compensatory mechanism for intestinal epithelial cell migration.

METHODS

Human Colonic Epithelium

Colonic crypts and epithelial sheets were isolated from colonic resections as described previously[29] and in accordance with a Medical College of Wisconsin Institutional Review Board-approved human research review committee protocol.

Cell Culture

The normal, non-transformed rat small intestinal epithelial cell line (IEC-6; CRL-1592) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured using DMEM (4 g/l glucose) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Omega Scientific, Tarzana, CA, USA), 2 mM L-glutamine, 1.5 g/L $NaHCO_3$, and 0.1 U/ml bovine insulin (Invitrogen, Carlsbad, CA, USA). The human T84 colonic carcinoma cell line[30] was cultured in DMEM/Ham's F-12 medium (1:1) supplemented with 5% (v/v) newborn calf serum (Invitrogen) and 2 mM L-glutamine as described previously.[15]

Mouse Intestine Epithelial Isolation

Mice were killed and the large and small intestines removed, minced, and placed in cell dissociation buffer containing 3 mM EDTA and 1 μM dithiothreitol. Tissue was washed and shaken several times and filtered through the gauze to separate epithelium from lamina propria. Epithelium was then centrifuged at 1200 r.p.m. for 5 min. The pellet was resuspended in 5 ml of PBS and overlaid onto a 40 ml sucrose gradient to remove single cells, the filtrate removed and centrifuged to collect epithelial sheets and crypts.[29]

Generation of CXCR4 Conditional Knockout Mice

Conditional knockout mice were generated using a Cre-loxP approach.[31] Heterozygous C57BL/6J mice carrying a floxed CXCR4 allele ($CXCR4^{f/+}$) were obtained from Dr Daniel Littman (Howard Hughes Medical Institute, New York University, New York, NY, USA). As detailed previously,[32] *loxP* consensus sequences were inserted 791 bp upstream and 221 bp downstream of exon 2 of the *CXCR4* gene. As exon 2 encodes 98% of the CXCR4 molecule,[33] Cre recombinase-mediated deletion will abolish CXCR4 function.

Homozygous floxed CXCR4 ($CXCR4^{f/f}$) mice were generated from those founder mice by a series of backcrosses. To inactivate selectively CXCR4 in intestinal epithelial cells, $CXCR4^{f/f}$ mice were crossed with transgenic mice expressing the Cre recombinase under the transcriptional control of the murine villin promoter (Tg(Vil-cre)-997Gum; Jackson Laboratories, Bar Harbor, ME, USA).[34] Impact of targeted CXCR4 deletion will be morphometrically assessed in heterozygous and homozygous CXCR4 floxed mice expressing the Cre transgene, $CXCR4^{f/+}$-villinCre (vC) or $CXCR4^{f/f}$;vC, respectively. Wild-type mice expressing villin-Cre transgene (+/+;vC) were analyzed as controls. Experimental protocols were used following approval by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin.

Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNA was isolated from colonic crypt epithelium and cultured cell lines using TRIzol reagent (Invitrogen), DNase-treated (Ambion, Austin, TX, USA), and 2 μg of total RNA was converted to cDNA via reverse transcription using random priming in a 40 μl volume. CXCL12, CXCR4, glyceraldehyde-3-phosphate dehydrogenase, CD45, and villin mRNA transcripts were amplified using previously described polymerase chain reaction (PCR) primers and conditions.[15,29] As a negative control, RNA was excluded from the cDNA synthesis reactions. As a separate control, template nucleic acids were excluded from the PCR reaction.

Genotype Analysis

Mice were genotyped using PCR analysis of genomic tail DNA. Mouse sequence-specific primers for discerning between wild-type and floxed CXCR4 alleles and the Cre recombinase were as follows: mCXCR4, sense, 5′-CCACCCA GGACAGTGTGACTCTAA-3′ and antisense, 5′-GATGGGAT TTCTGTATGAGGATTAGC-3′; Cre sense, 5′-CCGGGCT GCCACGACCAA-3′ and antisense, 5′-GGCGCGGCAACA CCATTTTT-3′. Cycling conditions for wild-type and mutant CXCR4 were as follows: 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min, followed by a final 7-min extension at 72°C The following parameters defined Cre expression: 30 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 1 min, followed by a final 7-min extension at 72°C to yield a product of 445 bp product. A 450 bp amplicon defined wild-type CXCR4 and an amplification product of 550 bp reflected the mutant CXCR4 allele.

Immunoblot Analysis

IEC-6 cells were grown to 80% confluence and serum starved 48 h before stimulation with 20 ng/ml recombinant CXCL12.[35] T84 cells were grown on tissue culture inserts until transepithelial resistance was $\geq 800\,\Omega cm^2$ as measured using a hand-held Millicell-ERS volt-ohmmeter (Millipore, Billerica, MA, USA). IEC-6 cells were cultured as a confluent monolayer. Epithelial cells were serum-starved overnight and stimulated 6 or 12 h in the presence or absence of 20 or 50 ng/ml TNF. Small intestinal epithelial sheets, colonic crypts, and liver were dissected from experimental and control mice and CXCR4 protein expression assessed as defined previously.[15] Cells were solubilized in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% (v/v) sodium deoxycholate, 1.0% (v/v) NP-40, 0.1% (v/v) SDS and 1 mM EDTA) supplemented with Protease Inhibitor Cocktail Set III (EMD Biosciences, San Diego, CA, USA) and 10 mM sodium orthovanadate, 40 mM glycerolphosphate, 20 mM sodium fluoride, and 20 mM PNPP phosphatase inhibitors. Lysates were passed through a 25-G needle and centrifuged at 550 r.p.m. for 5 min at 4°C to pellet nuclei. Protein concentration was determined using the Bradford protein assay (BCA Kit; Pierce Biotechnology, Rockford, IL, USA) and 10 µg of protein size separated using reducing SDS-PAGE. Proteins were electrophoretically transferred to PVDF membranes (Immobilon-P; Millipore) for immunoblot analysis as detailed previously.[18] Actin served as a loading control. Equal protein loading was confirmed by Coomassie blue staining.

Southern Blot Analysis

Targeted deletion of CXCR4 was verified using Southern blot analysis of genomic DNA extracted from intestinal and colonic crypts, liver, or tail tips. DNA was isolated and digested with *Bam*H1, and then separated on agarose gel electrophoresis and blotted to nylon membrane. Southern blot probes were generated as described previously.[32]

Immunohistochemistry

Immunostaining was performed on 4 μm sections from paraffin-embedded tissue of experimental and control mice as described previously.[24,36] To immunostain for activated mitogen-activated protein kinase, tissue sections were incubated with total and phospho-specific rabbit polyclonal antibody to ERK1/2 (Cell Signaling Technologies, Danvers, MA, USA) and visualized using donkey anti-rabbit Texas Red-conjugated secondary antibodies (Jackson ImmunoLabs, West Grove, PA, USA).

Enterocyte Proliferation and Migration

Proliferation of intestinal epithelial cells was monitored by bromodeoxyuridine (BrdU) incorporation. Briefly, animals were injected with BrdU (1 ml/100 g animal weight) by intraperitoneal injection and the mice were killed at 4, 8, 12, 24, or 48 h later. The large intestine and portions of the small intestine were removed and fixed with zinc-buffered formalin, embedded in paraffin, and 4 μm sections placed on glass slides. Tissue sections were analyzed by an investigator blinded to their status and BrdU visualized using specific antibodies as defined by the manufacturer (BrdU Staining Kit; Zymed, San Francisco, CA, USA). BrdU-positive cells were counted at each time point beginning from the base of

the crypts and the number of cell positions were compared between genotypes.

Intestinal Permeability

Intestinal permeability was assessed as defined previously.[37] Animals were fasted 24 h before surgery, anesthetized by intraperitoneal injection with ketamine (75 mg/kg) and xylazine (25 mg/kg), and injected intravenously with 250 μl of 1 mg/ml Alexa 488-conjugated-bovine serum albumin (BSA). The abdomen was opened by mid-line laporatomy and a 5 cm loop of intestinal jejunum was cannulated at the proximal and distal ends with 0.8 mm internal diameter tubing. A flushing solution (140 mM NaCl, 5 mM HEPES, pH 7.4), warmed to 37°C, was perfused through the jejunal loop at 1 ml/min for 10 min using a peristaltic pump (BioRad, Hercules, CA, USA). Flushing solution was followed by recirculating perfusion with 5 ml of pre-warmed (37°C) test solution (50 mM NaCl, 5 mM HEPES, 2 mM sodium ferrocyanide, 2.5 mM KCl, 29 mM glucose, pH 7.4) at 1 ml/min for ~3 h. The jejunal loop was then excised and its length and wet-weight measured.

Induction of Acute Colitis and Mucosal Damage

Mice were kept in microisolator cages and provided free access to food and water in a specific pathogen-free facility. Acute colitis was induced by administration of 5% (w/v) DSS (~36 000–50 000 Da; MP Biomedicals, Solon, OH, USA) dissolved in acidified water *ad libitum* for 7 days. Mice were weighed every second day to assess loss of body weight. Fecal blood was also assessed using Hemocult SENSA paper, which was developed with the provided developer solution (SmithKline Diagnostics, San Jose, CA, USA). Animals were allowed to recover for 1 day, at which time mice were killed and the small and large intestine removed. Chronic, relapsing colitis was induced using three repeated 5-day administration of 3% (w/v) DSS, separated by a 5-day healing period. Tissues were removed and 'Swiss-rolled', fixed in formalin, routinely processed, and 4 μm sections were stained by hematoxylin and eosin. Damage was assessed by light microscopy, performed in a blinded manner by a board-certified pathologist observing previously defined criteria.[38] Briefly, severity of inflammation (graded 0 = none to 3 = severe), extent of injury (0 = none to 3 = transmural), regeneration (4 = no tissue repair to 0 = complete tissue repair), crypt damage (0 = none to 4 = entire crypt and epithelium lost) and percent involvement (1 = 1–25% to 4 = 100%) were graded on at least three sections from the same animals.

Statistical Analysis

Differences between unstimulated control and experimental samples were analyzed by unpaired Student's *t*-test using SigmaStat (Jandel Scientific Software, San Rafael, CA, USA). Multiple comparisons between groups were analyzed using a two-way ANOVA and a Bonferroni *post hoc* analysis used to identify pairwise differences (GraphPad Prism 4, La Jolla, CA, USA). Statistical significance was defined as $P \leq 0.05$.

RESULTS

Sustained CXCR4 and CXCL12 Epithelial Expression in Human Inflammatory Bowel Diseases

Work from our laboratory indicates that the constitutive chemokine CXCL12 is a potent inducer of migration through activation of its cognate receptor CXCR4 expressed by cells of the intestinal epithelium.[15–18,39] These reports support a model in which chemokine receptors expressed by the epithelial barrier facilitate restitution and mucosal differentiation. As a first step in unraveling *in vivo* roles for CXCL12 and CXCR4 in the gut mucosa, colonic crypts and epithelial sheets were isolated from surgical specimens. Epithelium from normal colon was compared with those from patients undergoing surgical intervention for ulcerative colitis (UC) or Crohn's disease (CD), two inflammatory diseases whose pathogenesis is known to reflect, in part, epithelial barrier defects.[7] As shown in Figure 1a, RT-PCR of purified colonic crypts indicated that transcript levels of CXCR4 and CXCL12 was sustained at variable, but consistent, levels in human epithelium. In agreement with these data, immunohistochemical analyses indicated that CXCR4 and CXCL12 protein levels in the epithelium were not significantly different between normal and inflamed colon from UC (Figure 1b) and CD patients (not shown).

To determine if the variable mRNA and protein expression reflected homeostatic regulation, disease pathogenesis, or alterations following therapeutic intervention, we sought to determine if CXCR4 or CXCL12 transcript expression was altered by the proinflammatory cytokine TNF. Human colonic T84 epithelial cells cultured as a polarized model epithelium were stimulated 6 h with either 20 or 50 ng/ml concentrations of TNF previously shown to optimally regulate chemokine gene expression.[40,41] TNF shows little, if any, change in CXCR4 transcript or protein expression (Figure 1c and d). Consistent with our previous report showing that CXCL12 expression is epigenetically silenced in colorectal cancers, CXCL12 mRNA expression in T84 cells was not stimulated by cytokine stimulation.[29,42] The observed phenotype was not restricted to the T84 carcinoma cells as CXCR4 protein (Figure 1c) and transcript (not shown) expression was similarly refractory to TNF stimulation in normal, non-transformed rat IEC-6 intestinal epithelial cells. Taken together, these data suggest that expression of the CXCL12–CXCR4 homeostatic chemokine–chemokine receptor pair is little altered between the physiologically and pathologically inflamed intestine.

Expression and Targeted Deletion of CXCR4 in the Cells of the Intestinal Epithelium

To investigate the roles for CXCR4 and CXCL12 in the regulation of intestinal functions, we sought to establish a murine model. In agreement with our analysis of human

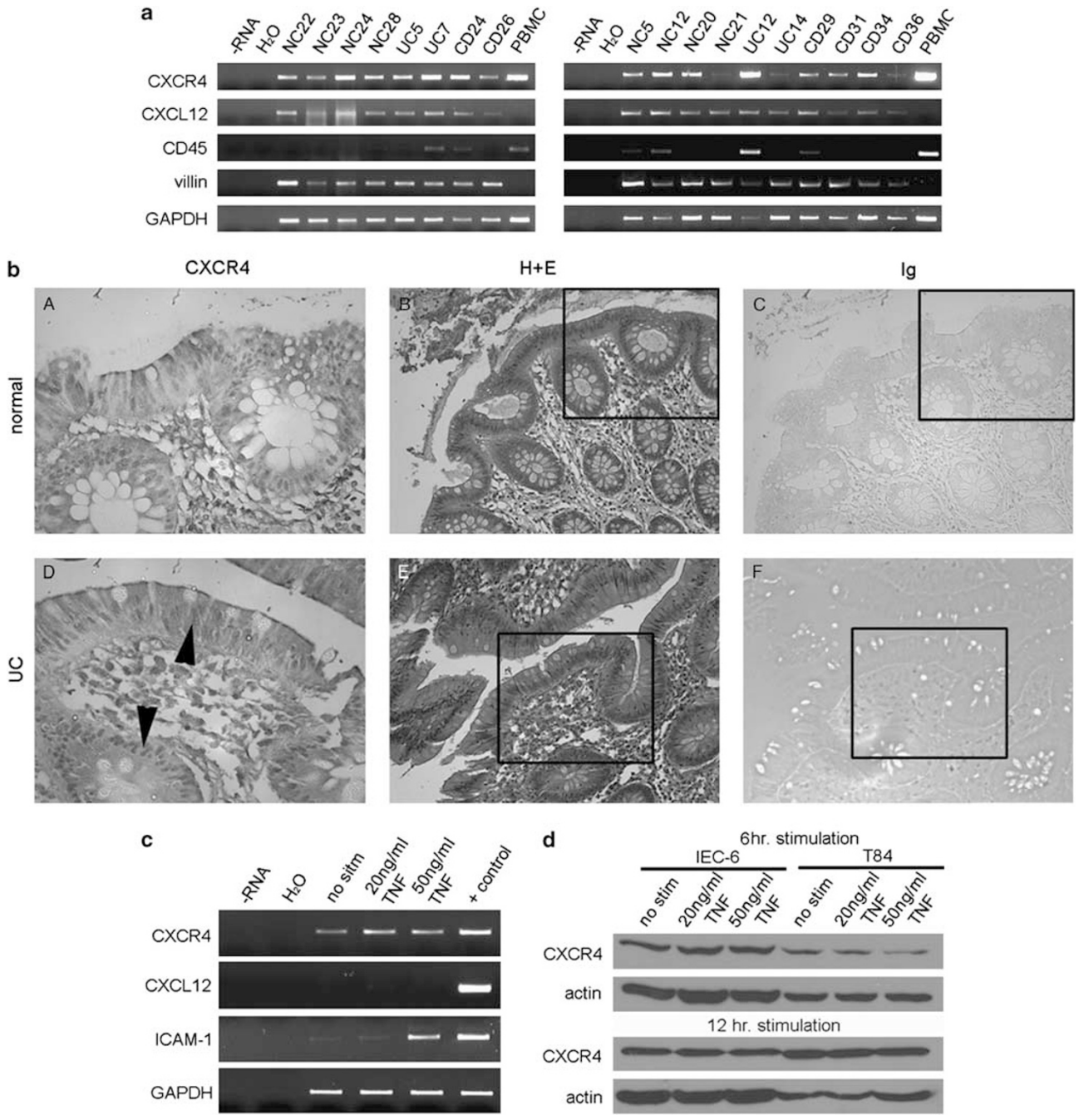


Figure 1 Maintenance of CXCR4 and CXCL12 expression in normal and inflamed human intestinal epithelium. (**a**) RT-PCR analyses indicated the consistent expression of CXCR4 and CXCL12 mRNA transcripts in human crypt epithelium. mRNA from human colonic crypts was isolated from normal colon (NC), Crohn's disease (CD), and ulcerative colitis (UC) lesions, or as a control, from peripheral blood mononuclear cells (PBMCs). Expression of the leukocyte marker CD45 demonstrated the contribution of those cells to the observed CXCR4 expression. Villin mRNA expression verified the epithelial enrichment of our crypt preparations and GAPDH transcripts were assessed as a loading control. As a negative control, RNA was excluded in cDNA synthesis reactions (−RNA) and as a control template water was excluded from the PCR reaction (H_2O). (**b**) CXCR4 protein expression is sustained in inflamed and normal colonic mucosa. Immunohistochemical analysis of parallel tissue sections indicate that epithelial CXCR4 expression levels in ulcerative colitis (UC) were similar to those in normal colon. H&E and IgG control images shown at $\times 100$ magnification with the boxed areas indicating the $\times 400$ magnification images shown for CXCR4 staining in the left-most panels. Data are representative of 4 separate RT-PCR or immunohistochemical analyses. (**c**) CXCR4 mRNA expression in TNF-stimulated T84 cells. Polarized T84 cells stimulated with 20 ng/ml TNF for 6 h modestly increased CXCR4 transcript levels. CXCL12 mRNA was not expressed, while ICAM-1 and GAPDH were analyzed as stimulation and loading controls, respectively. (**d**) Immunoblot analysis of CXCR4 protein levels. TNF stimulation for 6 h (top panels) or 12 h (bottom panels) in IEC-6 cells and polarized T84 cells resulted in minimal changes in protein expression. Actin was assayed as a loading control. Data are representative of three independent experiments.

colonic mucosa, we first characterized CXCR4 and CXCL12 expression in the mouse intestinal epithelium. Intestinal crypts and epithelial sheets were isolated from the colon and small intestine of healthy CD1 and C57BL/6J strain mice. As shown in Figure 2a, isolated murine epithelium from the small and large bowel consistently expressed CXCR4 and CXCL12 transcripts. In agreement with our data from isolated human colonocytes,[24,29] *in vivo* CXCR4 expression was paralleled by its cognate ligand CXCL12.

Functional *in vivo* studies for CXCR4 and CXCL12 are hampered by the embryonic lethality of systemic knockout mice.[27,28] To circumvent that limitation, we generated mice in which CXCR4 was conditionally inactivated in the cells of the murine intestinal epithelium. Targeted deletion of CXCR4 was iteratively defined using RT-PCR analysis of total RNA purified from isolated small or large intestinal crypts and epithelial sheets. As expected, the CXCR4 transcript was expressed in the small intestinal and colonic epithelium of wild-type (+/+) and heterozygous floxed ($CXCR4^{f/+}$) mice expressing the Cre transgene (vC). As shown in Figure 2b, the CXCR4 transcript was absent from animals in which both alleles had been floxed and the epithelial cells expressed Cre recombinase ($CXCR4^{f/f}$;vC). Total RNA from the liver of these same animals confirmed the tissue-specific deletion of CXCR4.

Southern blot analysis was next used to establish the genomic organization of the *CXCR4* gene in the cells of the small and large intestine. Genomic DNA isolated from purified intestinal crypts was radio-labeled with a probe to distinguish between the 3.8-kb fragment in wild-type mice and the Cre-recombined 9.6-kb fragment. Consistent with the transcript expression, experimental mice expressed the mutant CXCR4 alleles in the cells of the small and large bowel, but not the liver. Heterozygous animals displayed an intermediate phenotype, whereas animals lacking the Cre transgene solely expressed the native *CXCR4* gene (Figure 2c).

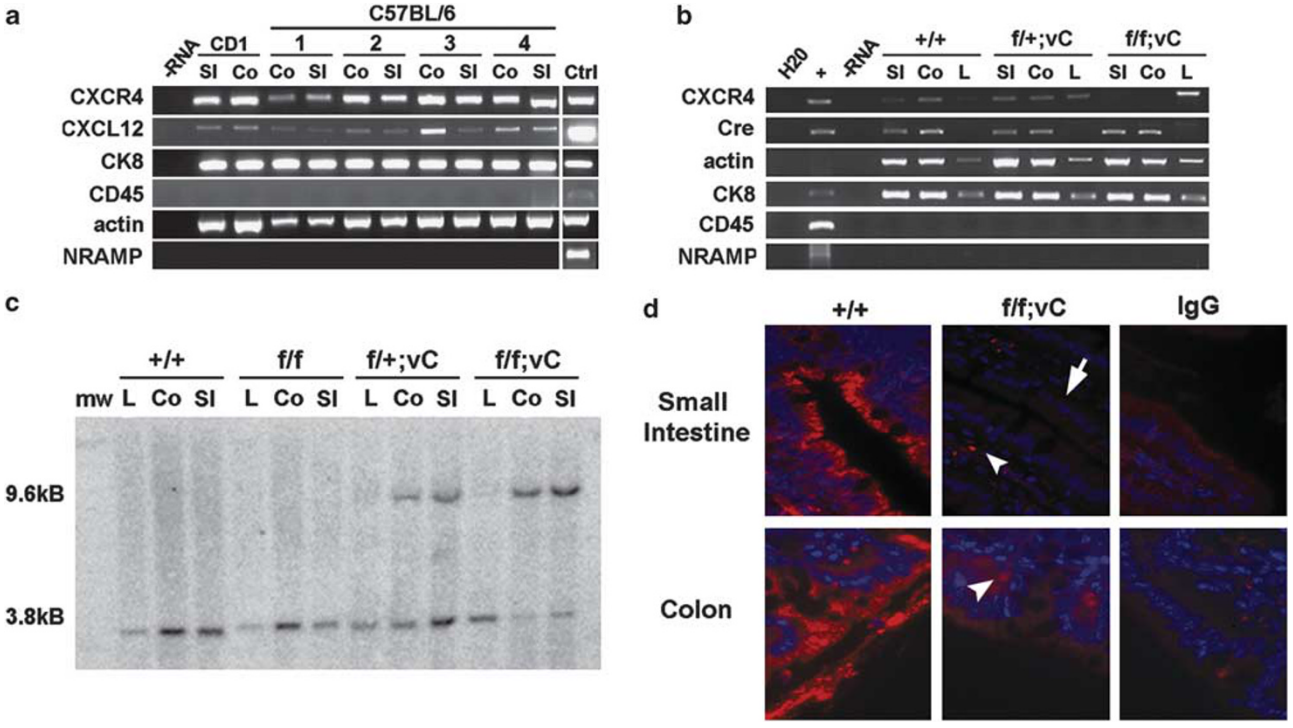


Figure 2 Targeted CXCR4 deletion in mouse intestine. (**a**) Constitutive CXCR4 and CXCL12 transcript expression in mouse intestinal crypts. Small intestinal (SI) and colonic (Co) crypts from CD1 and 4;[1–4] C57BL/6 strain mice were isolated and total cellular RNA prepared and mRNA expression assessed using murine specific primers. Primers to the NRAMP promoter assessed levels of genomic DNA contamination. (**b**) Reverse transcription-polymerase chain reaction (RT-PCR) analysis confirmed the loss of CXCR4 expression in the colon and SI of floxed CXCR4 mutant mice expressing the Cre transgene (f/f;villin-Cre (vC)). Data in panel **a** and panel **b** representative of independent analyses from six different mice. The epithelial cell marker cytokeratin 8 (CK8) showed the presence of epithelial cells. CD45 shows the purity of the intestinal epithelium preparations. NRAMP shows the absence of genomic DNA contamination. Actin levels were assessed as a loading control. (**c**) CXCR4 deletion in the intestinal epithelium. Digestion of genomic DNA by *Bam*HI resulted in a 3.8-kb fragment of the *CXCR4* gene in wild-type mice compared to a 9.6-kb fragment after probe hybridization of CXCR4 floxed alleles. Southern blot analysis shows that the recombination event occurred only in the SI and colon (C) of $CXCR4^{f/f}$;vC mice, whereas no recombination event occurred in the liver (Li) of these animals. $CXCR4^{f/+}$;vC mice showed that both alleles were present after hybridization in the SI and C, whereas only wild-type alleles were detected in the liver and all tissues of the wild-type animals. Data were representative of three separate Southern blot analyses. (**d**) Knockout of epithelial CXCR4 protein expression. CXCR4 immunoreactivity in the intestinal epithelium of wild-type (+/+) SI and C epithelium. CXCR4 immunoreactivity was absent in the intestinal epithelium of $CXCR4^{f/f}$;vC mice (arrows). Similar CXCR4 immunostaining was noted in the lamina propria cells (arrowhead) in wild-type and CXCR4 mutant mice. Immunostaining with an immunoglobulin G (IgG) antibody confirmed CXCR4 antibody specificity. Images are representative of separate analyses from 3 to 4 control and mutant animals.

Consistent with the transcript and genomic analyses, immunofluorescence microscopy confirmed the targeted deletion of the CXCR4 protein in the cells of the mouse intestine (Figure 2d). CXCR4 protein was abundantly expressed throughout the crypt–villus axis of the small intestine and colon of control wild-type mice (Figure 2d). Although CXCR4 protein was absent in the cells of the epithelium, chemokine receptor-expressing leukocytes were identified in the lamina propria of experimental CXCR4$^{f/f}$;vC-expressing mice. Taken together, these data show that CXCR4 has been specifically deleted from the cells of the intestinal epithelium.

Intestinal Morphology of Conditional Epithelial CXCR4 Knockout Mice

Having generated CXCR4 conditional knockout mice, we next sought to assess their overall health and characterize the intestinal mucosa in these animals. In strong contrast to the role of CXCR4 during development,[28] animals with intestinal epithelial deletion of CXCR4 were virtually indistinguishable from wild-type litter mates in development and overall viability. Consistent with these observations, growth rate, body weight (Figure 3a), and fecundity was similar between mutant and control mice. Moreover, morphometric analyses

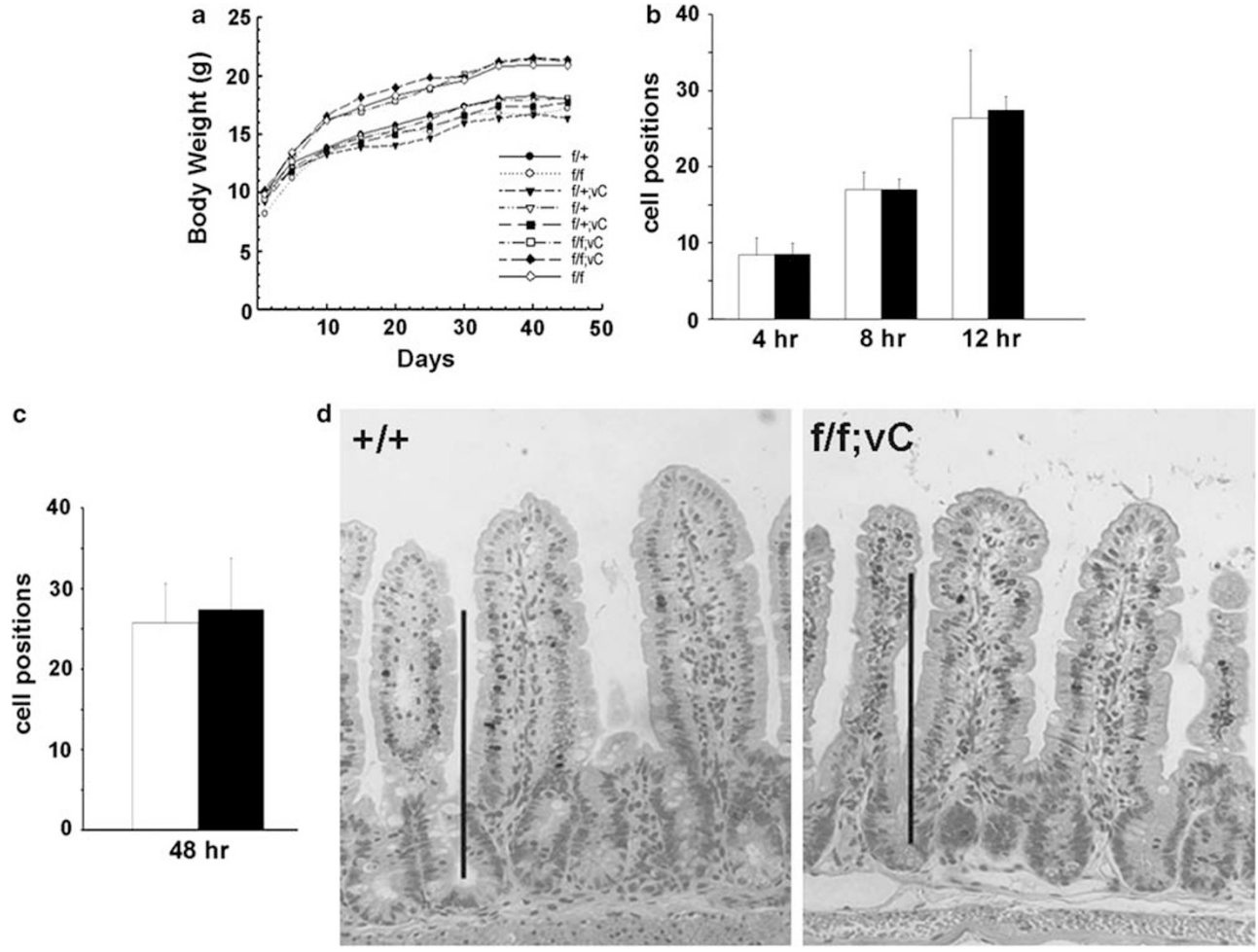


Figure 3 Mucosal architecture in CXCR4 conditional knockout mice. (**a**) Representative body-weight changes over time. CXCR4 mutant mice thrive and show no significant difference in body weight compared with control animals over time. Body weights diverge into two separate groups based on sex. Male littermates (top grouping) were slightly heavier than female littermates (bottom grouping). Two representative mice from over 20 homozygous, heterozygous, and control mice are shown. (**b**) Small intestinal epithelial cell proliferation. Floxed CXCR4-villinCre (CXCR4$^{f/f}$;vC) mutant (black bars) and wild-type control (white bar) animals were injected with bromodeoxyuridine (BrdU) in 1 ml/100 mg body weight and killed 4, 8, or 12 h later. The numbers of BrdU-positive cells were quantified from each of five intestinal crypts per field of view. Values represent 10 observations per mouse, 3–4 mice were quantified per time point in the small intestine. (**c**) Enterocyte migration in CXCR4$^{f/f}$;vC (black bar) and control (white bar) mice. Epithelial cell migration, defined as the number of cell positions moved from the site of BrdU incorporation in the crypt base to the highest BrdU-immunoreactive cell, was not significantly different between CXCR4$^{f/f}$;vC (black bar) and control (white bar) mice. Values are mean ± s.e.m. from 3 to 4 mice for each genotype. (**d**) Representative images from 3 to 4 separate BrdU-injected mice. The distance migrated from BrdU incorporation in the crypt along the villus axis is shown (solid black line). The number of cell positions migrated was measured at 10 separate locations in the small intestine. Sections were counter-stained with hematoxylin (× 10 magnification).

determined that length and width of the small and large bowel were not significantly altered, with villus height and crypt depth within the small intestine unchanged between $CXCR4^{+/+}$ and $CXCR4^{f/+}$;vC control mice and experimental $CXCR4^{f/f}$;vC animals (Supplementary Figure 2).

Previously, we have shown that CXCR4 regulates migration, barrier maturation, and restitution in model intestinal epithelium in culture,[15,16,18] suggesting a role for this signaling axis in the maintenance of barrier integrity. To investigate the *in vivo* potential for CXCR4 in regulating epithelial migration, mice were administered BrdU and its incorporation into intestinal crypts defined 4, 8, or 12 h later. As shown for the small intestine (Figure 3b), epithelial proliferation in wild-type mice was indistinguishable from conditional knockout mice after BrdU injection. Proliferation and emigration of BrdU-positive cells remained similar between mutant and control mice 8 and 12 h after injection. These data agree with our cell culture models that CXCL12 and CXCR4 do not significantly alter epithelial proliferation. Migration, defined as the number of cell positions from the crypt base 48 h after BrdU incorporation, showed that deletion of CXCR4 had little to no effect on movement of enterocytes up the crypt–villus axis (Figure 3c and d). Epithelial cell proliferation and migration was similarly equivalent in the colon of wild-type and CXCR4 mutant mice (data not shown). Consistent with these data, measurement of lumen-to-blood or blood-to-lumen permeability of sodium ferrocyanide or FITC-BSA, respectively, showed comparable barrier integrity and minimal difference between wild-type and mutant animals (data not shown). As epithelial proliferation and migration are tightly linked with enterocyte differentiation,[43] we quantified the number of goblet (Supplementary Figure 1C) and Paneth cells (data not shown) within the crypt–villus axis. In agreement with the minimal, if any, change in proliferation and migration, the numbers and ratios of terminally differentiated cells was not different in mutant CXCR4-null animals, compared with wild-type or heterozygous mice. Lastly, given the lack of CXCR4 expression by the epithelium, we postulated that enterocyte produced CXCL12 would increase the infiltration of intraepithelial lymphocytes or mononuclear cells into the mucosal compartment. However, we found little differences in the number of these cells in $CXCR4^{f/f}$;vC and control mice (Supplementary Figure 1). Combined, these data suggest that targeted deletion of epithelial CXCR4 has minimal roles in altering basal epithelial cell proliferation, differentiation, or migration.

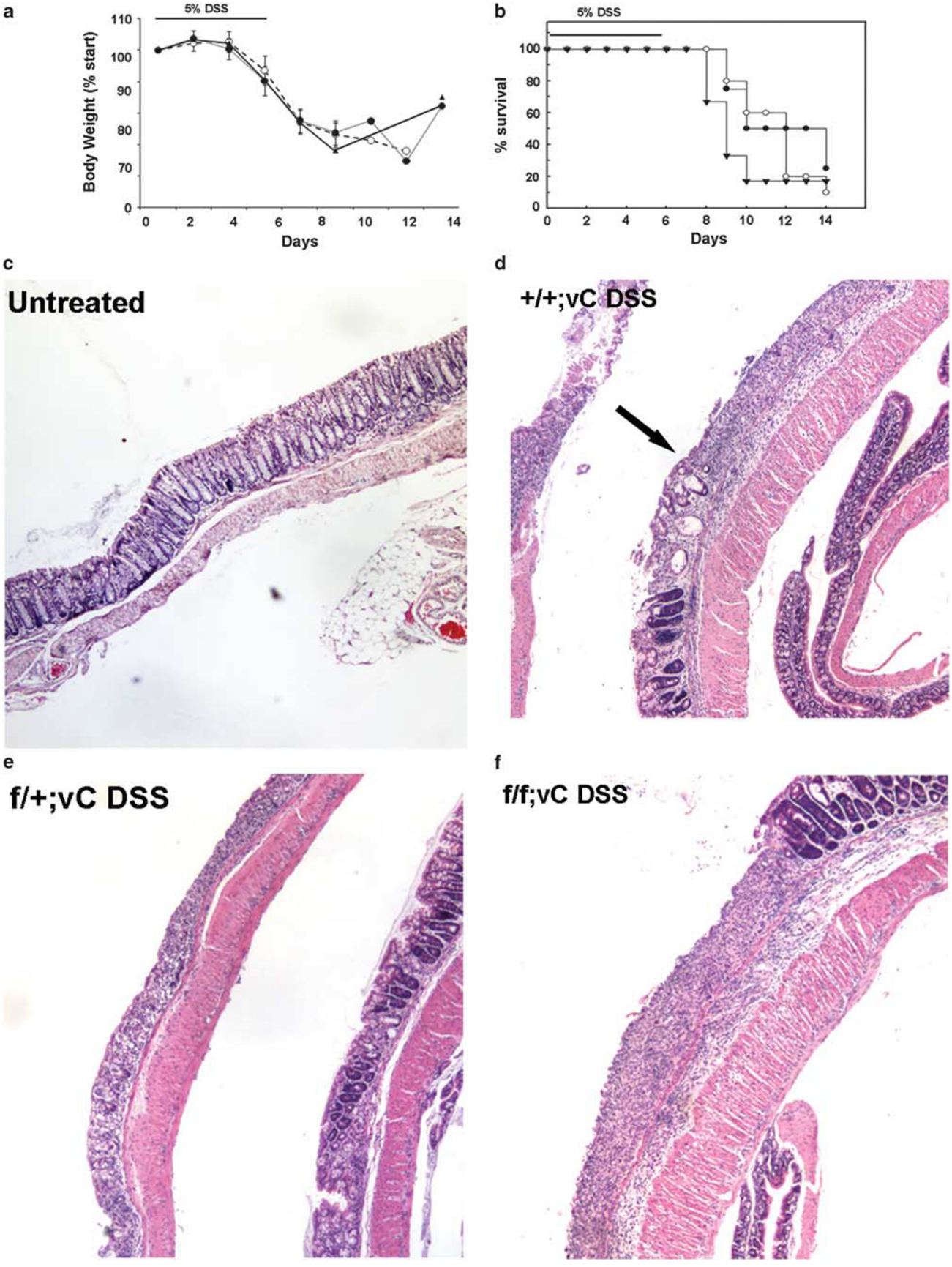
Re-Epithelialization in Conditional CXCR4-Deficient Mice

We next sought to test the hypothesis that ablation of CXCR4 from epithelial cells would modulate restitution in response to mucosal damage. For these studies, experimental floxed and control wild-type mice were administered the epithelial irritant DSS in their drinking water to disrupt and damage the colonic epithelium. Treatment with 5% (w/v) DSS for 7 days lead to a dramatic decrease in body weight, with experimental and control animals losing more than 30% of their starting body weight within 10 days from the start of the regimen (Figure 4a). There were no significant differences in weight loss (Figure 4a) or lethality (Figure 4b) as a result of DSS treatment among the genotypic groups. Necropsy of CXCR4-null and receptor-expressing mice at day 14 showed robust leukocyte infiltration with epithelial damage and ulceration compared with untreated animals (Figure 4c–f). Mice with the targeted deletion of CXCR4 in the cells of the intestinal epithelium were not statistically more susceptible to damage and inflammation in response to 5% DSS (Table 1), but were characterized by the marked absence of *de novo* crypt formation and re-epithelialization by the surrounding enterocytes (Figure 4d–f).

Given the severity of disease in mice treated with 5% DSS, we next sought to investigate the impact of epithelial CXCR4 knockout in a model of chronic, recurring colitis. After three repeated 5-day bouts of 3% DSS exposure (Figure 5a), animals were killed and the number of active ulcers enumerated. Active ulcers were defined as those that had a broken or discontinuous epithelium and active sites of inflammation. Consistent with the acute colitis, experimental and control mice showed comparable number of unhealed ulcers following three repeated DSS treatments. $CXCR4^{f/f}$;vC mice showed the same number of unhealed ulcers as the wild-type littermates, with markedly deeper ulcer beds in mice lacking epithelial CXCR4. Interestingly, $CXCR4^{f/+}$;vC animals showed significant improvement in ulcer healing, with a robust epithelium covering the wound (Figure 5b). These latter data suggest that signaling through epithelial CXCR4 is regulated, in part, through expression levels of the receptor. Taken together, these data support the model that the chemokine receptor CXCR4 regulates epithelial restitution *in vivo*.

Decreased Restitution and Dysregulated ERK1/2 Phosphorylation in CXCR4-Depleted Epithelium

Structural analyses indicate that CXCR4 exists primarily as homodimeric protein.[44,45] Further work implicates heterodimerization of chemokine receptors with differential receptor signaling.[46,47] We therefore next investigated potential mechanisms for the differential impact of CXCR4 on restitution. Mucosal injury repair *in vivo* is the sum effect of multiple cell types and mediators remodeling the injured tissue. Thus, we reasoned that variable re-epithelialization in CXCR4 mutant mice reflected differential activation or restitution signaling pathways. To investigate that possibility, RNAi approaches were first used to knockdown CXCR4 expression levels in model epithelia. Cells were separately transduced with equivalent levels of lentiviral particles expressing shCXCR4 or scramble sequences. Immunoblot analysis showed a significant 85% decrease in endogenous levels of the chemokine receptor CXCR4 compared with scramble sequence-expressing vectors or wild-type empty vector control cells (Figure 6a).


a
5% DSS
Body Weight (% start)
110
100
90
80
70
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0 2 4 6 8 10 12 14
Days
b
5% DSS
% survival
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60
40
20
0
0 2 4 6 8 10 12 14
Days
c
Untreated
d
+/+;vC DSS
e
f/+;vC DSS
f
f/f;vC DSS

We have previously shown that ERK1/2 signaling has a marked role in regulating CXCL12-induced intestinal epithelial migration[15] and colonic carcinoma anoikis.[48] Moreover, alterations in occupancy of dimeric CXCR1 receptors results in differential activation of the ERK1/2 signaling pathway.[46] Consistent with these reports, CXCR4-depleted cells stimulated with the cognate ligand CXCL12 were unable to induce ERK1/2 phosphorylation (Figure 6b). ERK1/2 was phosphorylated in cells transduced with the scrambled sequence. Stimulation with the cytokine EGF resulted in robust and equivalent ERK1/2 phosphorylation in CXCR4-competent and -deficient cell lines, underscoring that the signaling pathway was intact. CXCR4 knockdown cell lines were unable to migrate in response CXCL12 stimulation (Figure 6c). Baseline migration in CXCR4 knockdown and control cells was slightly, albeit not significantly, decreased, suggesting that depletion had little overall impact on constitutive migration *in vitro*. Taken together, these findings *in vitro* suggest that the absence of epithelial cells across the denuded surface of homozygous CXCR4 mutant mice reflects, in part, diminished migration during mucosal damage and inflammation.

Given our previous data indicating that ERK1/2 regulates inducible restitution *in vitro*, we next assessed the levels of active ERK1/2 in our experimental and control mice. Immunofluorescence detection showed active ERK1/2 immunoreactivity at the villus tip and to a lesser extent across the entire crypt–villus axis in wild-type mice. In contrast, phosphorylated ERK1/2 was decreased and notably absent at the villus tip of CXCR4$^{f/f}$;vC mice, with active protein localized to the dividing and differentiating crypt cells (Figure 6d). Heterozygous CXCR4 mutant mice displayed an intermediate phenotype with much of the crypt–villus axis demonstrating robust phosphorylated ERK1/2, whose activity was restricted to the villus apex and the very base of the intestinal crypts. Comparable changes in active ERK1/2 localization were observed in the colonic crypt–surface axis in heterozygous and homozygous CXCR4 floxed mice. Marked decrease in phosphorylated ERK1/2 (Figure 6d) may therefore account for decreased re-epithelialization in acute DSS-induced colitis, whereas increased ERK1/2 activity along the entire crypt–villus axis is correlated with increased restitution of DSS-treated heterozygous mice.

Table 1 Clinical damage scores for DSS-induced colitis

	Damage–activity index		
	+/+; vC	f/+; vC	f/f; vC
Acute (5% DSS)	12.2 ± 2.8	14.4 ± 1.3	14.5 ± 2.8
Chronic (3% DSS)	12.4 ± 2.0	12.2 ± 2.1	11.2 ± 2.7

Abbreviations: DSS, dextran sodium sulfate; vC, villin-Cre.

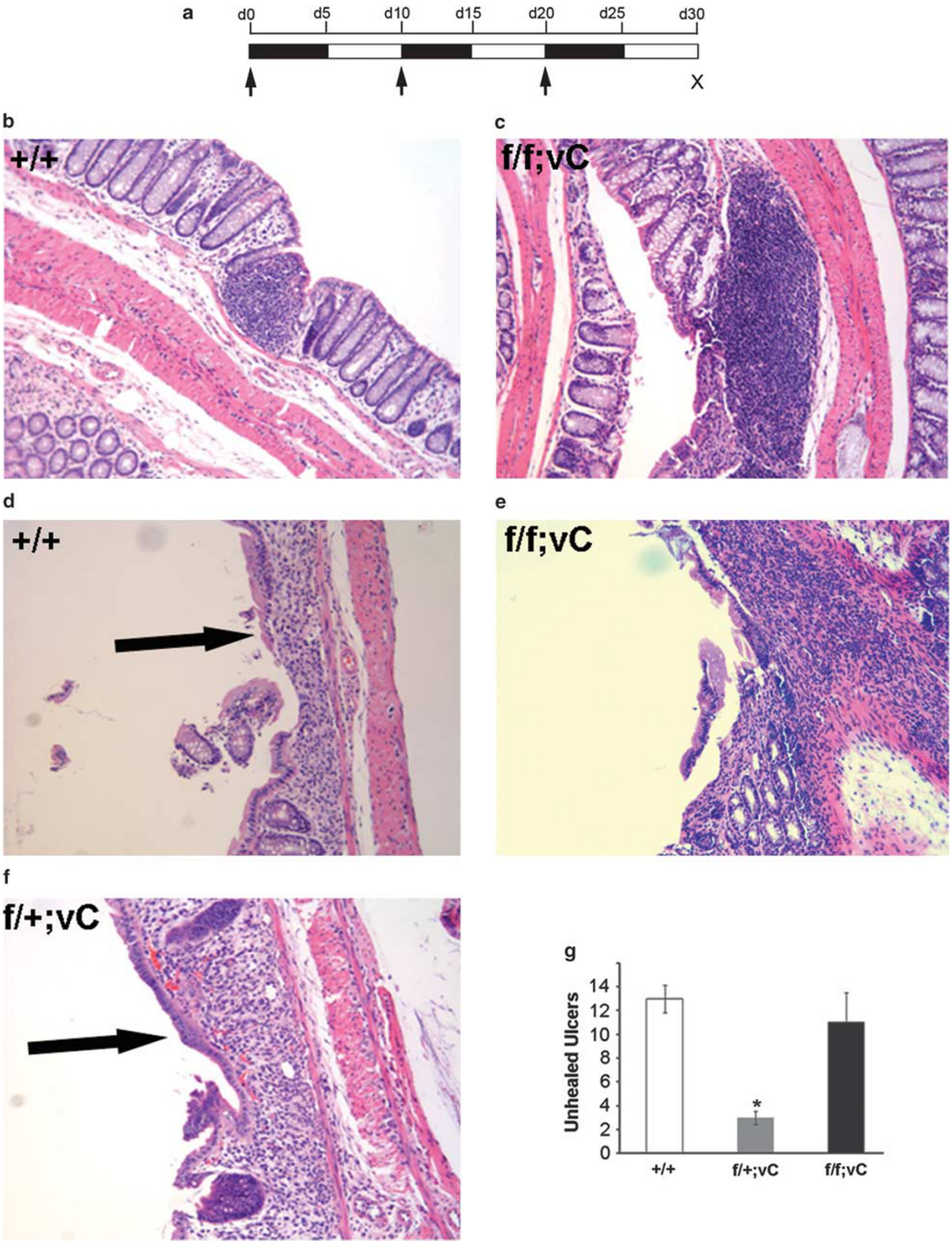
Values are mean ± s.e.m. Acute colitis, 7 days 5% DSS, $n = 8$–11. Chronic colitis, 5 days 3% DSS, 5-day recovery followed by two more 5-day treatments with 3% DSS, $n = 4$–6.

DISCUSSION

This study shows that loss of CXCR4 expression in the intestinal epithelium tightly regulates localization of active ERK1/2. We have previously shown that CXCL12, the ligand for CXCR4, is sufficient to increase restitutive migration of intestinal epithelial cells and barrier maintenance *in vitro*.[15,16,18] To determine roles for CXCR4 in injury repair *in vivo* conditional knockout mice were generated. In contrast to systemic CXCR4 knockout, conditional CXCR4 mice developed normally, establishing a new research tool with which to dissect roles for chemokines and chemokine receptors in the gastrointestinal mucosa. Using that model, we determined that depletion of CXCR4 from epithelial cells results in altered ability of epithelial cells to cover wounds in severe acute colitis, likely through decreased activation of ERK1/2. In contrast, heterozygous knockout of CXCR4 reversed the typical apical localization of active ERK1/2 and resulted in increased injury repair in acute and chronic colitis. Our data are the first to address the role of CXCR4 specifically in the intestinal epithelium *in vivo* and reveal critical roles for the chemokine receptor and ERK1/2 in mucosal injury repair.

Chemokines produced by numerous cell types regulate the directed trafficking of immunocytes into and out of the inflamed mucosa. We and others have previously determined that the intestinal epithelium of normal human bowel expresses an array of chemokine receptors in normal, healthy gut, suggesting that these cells are functional targets for chemokine action.[24,41] Additional studies suggest that alterations in chemokine or chemokine receptor expression levels are an important etiological step in the progression to cancer and inflammatory disorders.[29,49] We have significantly expanded upon these data and determined that in contrast to

Figure 4 Mucosal injury in acute dextran sodium sulfate (DSS) colitis. Mice were administered 5% (w/v) DSS for 7 days in drinking water. (**a**) Control CXCR4$^{f/+}$ mice (closed circle) lost weight starting 5 days after initial administration. Mice heterozygous for the floxed allele and expressing the Cre transgene (CXCR4$^{f/+}$;villin-Cre (vC); filled triangle) and homozygous CXCR4$^{f/f}$;vC (empty circle) showed similar initial decrease in body weight. Values are mean ± s.e.m. of 5–10 mice in each genotype. (**b**) Kaplan–Meier curve shows survival of control CXCR4$^{f/+}$ (closed circle) or heterozygous floxed vC (filled triangles) and homozygous CXCR4$^{f/f}$vC (empty circle) mice. Values are mean ± s.e.m. of 5–10 mice in each genotype. Untreated mice (**c**) show an intact crypt–villus axis and intact epithelium that is ulcerated, with pronounced edema (**d**) in wild-type mice expressing the Cre transgene (+/+;vC). Heterozygous (**e**) and homozygous (**f**) CXCR4 floxed vC mice show differential responses to challenge with DSS, with CXCR4$^{f/+}$;vC showing little damage and edema compared with the CXCR4$^{f/f}$;vC and wild-type mice. Re-epithelialization evident in wild-type mice (arrow) was absent in experimental CXCR4 mutant mice. Data in (**c**)–(**f**) are representative or 5–10 mice in each genotype. Representative healed and unhealed ulcers are shown (×40).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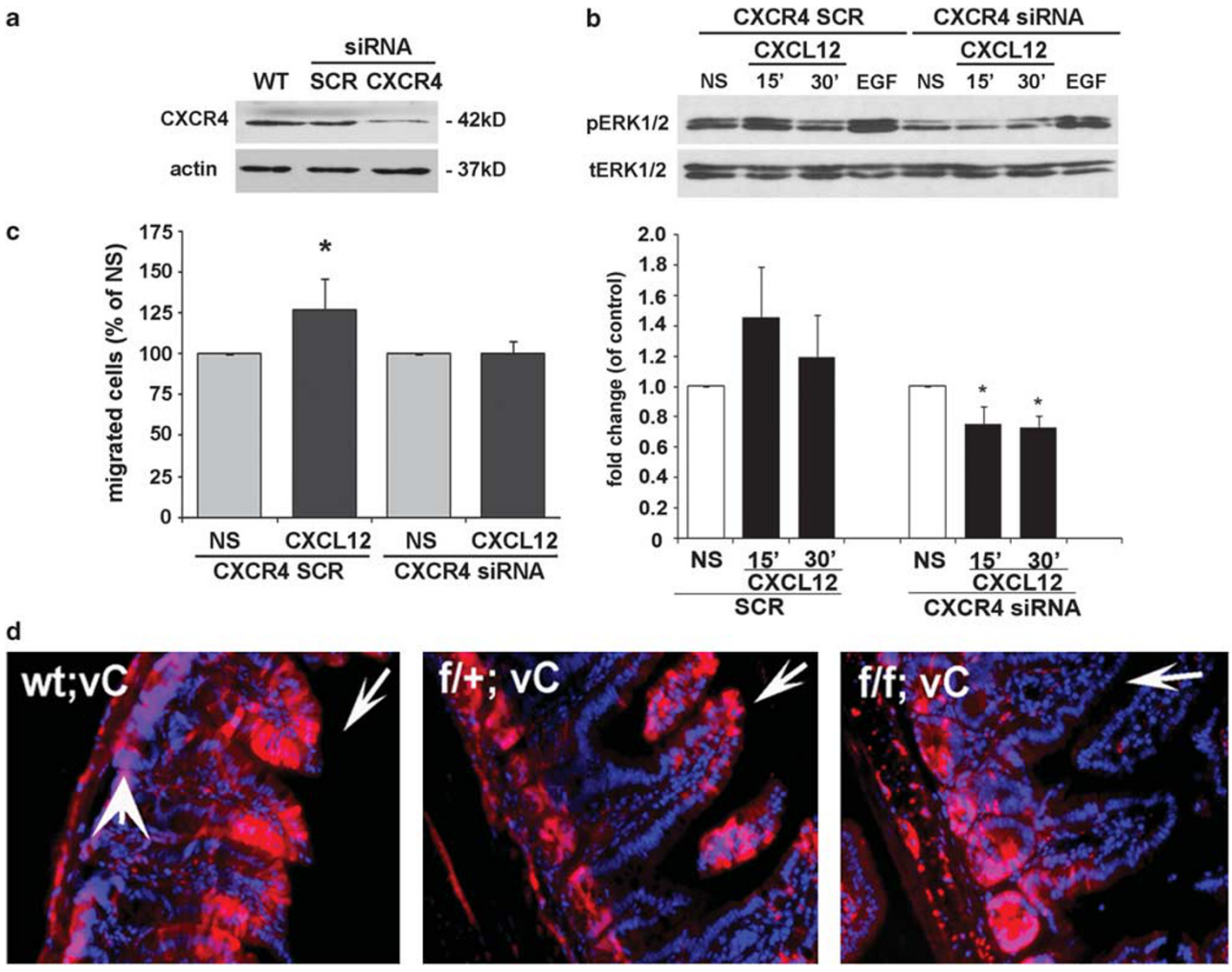

Figure 6 Targeted deletion of CXCR4 alters epithelial extracellular-regulated kinase-1/2 (ERK1/2) activity and localization. (**a**) IEC-6 cells transduced with lentiviral particles encoding CXCR4 small interfering (siRNA) probes showed a decrease in CXCR4 expression. Wild-type cells (WT) and cells transduced with control, scrambled sequences (SCR) showed comparable levels of CXCR4 protein. Actin levels were assessed as a loading control. (**b**) Decreased ERK1/2 activity in CXCR4-depleted cells. As shown by densitometric analysis (lower panel), depletion of CXCR4 blocked CXCL12 (20 ng/ml) induced levels of phosphorylated ERK1/2 (pERK1/2) compared with cells transduced with SCR sequence (upper panel). Levels of total ERK1/2 (tERK1/2) were not markedly different between CXCR4-depleted and SCR cells. Values are mean ± s.e.m. of five separate experiments. Asterisk indicates a statistically significant decrease ($P \leq 0.05$) from unstimulated controls. (**c**) CXCR4-depleted cells inhibited CXCL12-stimulated migration. IEC-6 cell monolayers were wounded with a sterile razor and treated with 20 ng/ml CXCL12. Control cells (NS) remained unstimulated. Migrating cells were counted 18 h later and presented as a percent of unstimulated cells. CXCL12 induced a robust and statistically significant increase in migration of control epithelial cells transduced with scrambled siRNA probes. Knockdown of CXCR inhibited CXCL12-induced migration. Values are mean ± s.d. of three separate experiments. Asterisk indicates statistically significant difference from unstimulated control. (**d**) Immunohistochemical analyses indicated that phosphorylated ERK1/2 (red) was demonstrably more abundant at the villus tip (arrows) in wild-type mice. Myenteric plexus regions were also immunoreactive for phosphorylated ERK1/2 (arrowhead). Phosphorylated ERK1/2 was localized to the villus tip (arrow) and the crypt base in heterozygous CXCR4 mutant mice expressing the Cre transgene (f/+;vC). Phosphorylated ERK1/2 was localized to the crypt base and absent from the villus tip (arrow) in mice homozygous for floxed CXCR4 and expressing the Cre transgene (f/f;vC). Tissue sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue) to visualize cell nuclei. Images were taken at × 100 magnification and are representative micrographs from four individual animals from each genotype. Data in panels a, b, and d are representative from 3 to 4 separate experiments.

Figure 5 Restitution in chronic dextran sodium sulfate (DSS)-induced colitis. (**a**) Three recurring bouts of acute colitis and healing were induced by administration of 3% (w/v) DSS for 5 days, followed by normal drinking water for 5 days. $CXCR4^{f/f}$;vC mice showed more consistent and larger lymphoid aggregates (**c**) relative to wild-type control animals (**b**). Images show incomplete re-epithelialization (arrows) over ulcer bed in wild-type (**d**) and homozygous $CXCR4^{f/f}$;vC mutant mice (**e**) compared with the robust epithelial covering in heterozygous $CXCR4^{f/+}$;vC mice (**f**). (**g**) Enumeration of healed and unhealed ulcers confirmed the improved healing in $CXCR4^{f/+}$;vC mice after repeated bouts of DSS exposure. Values are mean ± s.d. from 4 to 6 separate mice. Asterisk denotes statistical significance ($P \leq 0.05$). Hematoxylin- and eosin-stained tissues were from representative mice (× 100 magnification).

many chemokines, CXCL12 and CXCR4 levels are remarkably stable in the inflamed human colon. These data are in contrast to previous reports of CXCR4 and CXCL12 mRNA levels in IBD,[50,51] which may reflect the purity of colonic epithelial preparations, or differences in transcription and translation. Indeed, to investigate the latter possibility, we completed *in vitro* studies in which human T84 colonic epithelial cells or non-transformed rat IEC-6 epithelial cells were stimulated with TNF, an essential proinflammatory mediator of human inflammatory disorders. Stimulation of polarized T84 model epithelium resulted in little change in CXCR4 transcript or protein expression. The lack of CXCL12 regulation by TNF in IEC-6 or T84 cells agrees with the paucity of NF-κB or AP1 transcription sites within the *Cxcl12* promoter[52] and is supported by recent work showing that TNF is incapable of inducing CXCL12 transcript expression in non-transformed cells.[53] Thus, our data support the notion that CXCR4 and CXCL12 expression is sustained during gastrointestinal inflammatory disorders and support a previous report[54] suggesting that this axis provides a useful target for therapeutic intervention.

Epithelial restitution reflects an intrinsic enterocyte migration response to cover the denuded surface and limit the entry of harmful substances into the tissue. Constitutive epithelial migration is inducibly regulated in cell culture model systems by an array of cytokines, chemokines, growth factors, microbes, or extracellular matrix proteins.[55,56] Restitution *in vivo* is complex and reflects the input of additional cytokines and growth factors working in concert with extracellular matrix and the luminal microbiota. Consistent with this complexity, the mechanisms by which these molecules elicit their functions remain incompletely characterized and challenging to decipher. Tissue-specific deletion gene studies targeting the intestinal epithelium have proven increasingly useful relative to systemic knockout mice in unraveling roles for TGF-β receptor, epidermal growth factor receptor, cadherin, laminin, and Vav in integrity and repair of the gut mucosa.[57–61] Targeted deletion of CXCR4 results in a subtle phenotype with little outward signs of altered development or architectural changes in the gut. The mucosal architecture of tissue-specific depletion of CXCR4 agrees closely with a previous report interrupting TGF-β1 receptor signaling.[60,62] Taken together, these reports suggest that while disruption of CXCL12 and TGF-β1 minimally alters intrinsic basal epithelial migration, loss of these mediators negatively impacts the delicate balance of extracellular mediators regulating inducible migration needed to repair the epithelium. These reports further underscore the importance of constitutive and inducible restitution in the enteric mucosa and solidify that expression of numerous extracellular mediators coordinately regulate those processes *in vivo*.

We have previously shown that ERK1/2 signaling was a critical effector in chemokine-induced restitution, with little impact on the ability of epithelial cells to migrate.[15] Consistent with these data, we showed that loss of CXCR4 in the intestinal epithelium alters the localization of active phosphorylated ERK1/2 along the crypt–villus axis. These data indicate that G-protein-receptor-coupled receptors expressed along the crypt–villus axis tightly regulate basal ERK1/2 signaling *in vivo*. Consistent with these findings, mice with heterozygous CXCR4 depletion possessed increased wound healing concomitant with increased crypt and apical ERK1/2 activity in marked contrast to those mice in which CXCR4 was completely ablated from the cells of the epithelium. The altered re-epithelialization in mice lacking CXCR4 was correlated with ERK1/2 activity restricted to the intestinal crypt. Intestinal epithelial cell proliferation, migration, or barrier permeability was unchanged in CXCR4 conditional knockout mice, suggesting that reversing ERK1/2 phosphorylation to the crypt base does not significantly change proliferation or basal migration along the epithelial surface. We have previously determined that ERK1/2 activity has an important role in chemokine regulation of epithelial anoikis,[48] a specialized programmed cell death following cellular detachment for the subjacent matrix. Although not examined in our conditional knockout mice, it therefore remains possible that decreased ERK1/2 activation alters the ability of epithelial cells to adhere to or rapidly migrate across the remodeled matrix of the inflamed mucosa. Alternatively, multiple reports suggest a role for ERK1/2 in the regulation of tight junctions.[63,64] Thus, increased healing in heterozygous CXCR4 knockdown mice to DSS may reflect decreased ERK1/2 activity and in turn increased levels of tight junction proteins and increased barrier function of the intestine.

Given the complexity of *in vivo* restitution, we also asked if the targeted deletion of CXCR4 altered expression of chemokine ligands or chemokine receptors that might functionally compensate for the loss of that abundantly expressed receptor. As expected, a real-time analyses showed that CXCR4 mRNA levels were below detectable limits in the intestinal epithelium of conditional knockout mice, but present in the control wild-type and heterozygous littermates (Supplementary Table 1). Expression of the cognate ligand CXCL12 and a battery of inducible chemokine ligands and proinflammatory cytokines showed little change in mice lacking CXCR4 (Supplementary Table 2). These data suggest that increased CCR6 was not a result of increased inflammation in unstressed animals, as the transcription factor NF-κB and its proinflammatory target gene *TNF* was only slightly elevated, whereas other target genes including *CXCL1* and *TLR4* were not upregulated in mutant mice. Taken together, these data suggest that baseline, physiological inflammation was little affected following targeted deletion of CXCR4 from the cell of the intestinal epithelium. Furthermore, while the results of the real-time RT-PCR analysis show a trend to increased CXCL13, a B-cell chemoattractant, transcript levels of CCR6 were little changed between the wild-type and conditional knockout mice. Recent work indicates that CXCL12 may also bind and activate the newly characterized receptor CXCR7.[65] Although CXCR4 and

CXCR7 are thought to initiate functionally distinct signaling pathways, separate RT-PCR analyses indicate that CXCR7 mRNA levels were unaffected by the loss of CXCR4 (Supplementary Table 1). Other than CCR6, we noted a pronounced increase in transcript levels of the leukotriene B4 receptor-2, suggesting that additional G-protein-coupled receptors might have roles in epithelial functions. Taken together with our data from TNF-stimulated cell lines, these data suggest that post-transcriptional or post-translational modifications, or altered residence time of the surface receptor, have significant roles in regulating chemokine receptor expression and function. Our data also suggest that the loss of a single G-protein-coupled chemokine receptor stimulates the expression, or post-transcriptional, post-translational regulation of another, CCR6, in the *in vivo* intestine. The mechanisms whereby CXCR4 signaling regulates CCR6 or leukotriene receptor expression levels remain to be defined.

Surprisingly, we observed that heterozygous CXCR4 epithelial knockout mice were less edematous in acute colitis and had significant re-epithelialization in chronic DSS colitis. Alterations in ERK1/2 activity along the crypt–villus axis may best account for the improved healing in those animals, especially given the roles for that kinase in cellular proliferation, migration, and barrier maintenance. However, this begs the question as to how ERK1/2 activity is differentially regulated following alterations in CXCR4 expression. We speculate that ERK1/2 activity reflects increased protein levels of CCR6 (Supplementary Figure 2). Although CXCR4 has a well-characterized role in cell trafficking, CCR6, which is constitutively expressed by the epithelial cells of the colon and can be upregulated in response to inflammation,[41,66,67] also evokes cellular migration through receptor binding of its ligands CCL20 or human β-defensin 2.[17] We had previously shown in cell culture model systems that CCR6 activation by its chemokine ligand CCL20 or the human β-defensin HBD2 agonist are equally effective as CXCL12 in inducing restitution.[17] The upregulation of CCR6 suggests a potential compensatory mechanism by which the intestine can retain the ability to close ulcerations in the absence of the known wound healing signaling axis, CXCR4–CXCL12. Alternatively, recent findings in chemoattractant-driven migration of leukocytes suggest that agonist-biased signaling is regulated, in part, through structural changes in the G-protein-coupled receptor results in differential signaling and functional outputs.[46,68,69] Intriguingly, alterations in dimerization of the chemokine CXCL8 lead to changes in receptor utilization and the strength and duration of ERK1/2 signaling.[46] Studies in our laboratory indicate that the monomer–dimer equilibrium of CXCL12 has important roles in epithelial cell migration. Similarly, agonist-biased signaling may also reflect the dimerization status of the receptor.[69,70] Thus, it is possible that plasticity of re-epithelialization results from the deletion of one of the CXCR4 alleles and the concomitant formation of heterodimeric CXCR4:CXCR7 receptors.[47]

CONCLUSIONS

The importance of maintaining the epithelial barrier is clearly illustrated by the functional redundancy of chemokine receptor expression on epithelial cells. However, the functional importance of restitutive cell migration is not limited to chemokine receptor expression nor epithelial cells, as more recent reports have begun to link mucosal fibroblasts and T cells with important roles in injury repair.[3,37,71–74] The inflammatory status of the intestine is clearly linked to the wound repair activities of the organ. Although proinflammatory ligands may allow homing of immune cells to the intestine during the initial stages of inflammation, late stages may be characterized by low levels of those same ligands. Inflammatory ligands, such as inducible chemokine receptor ligands such as CCL20 or human β-defensins, similarly regulate cell migration and likely function to increase epithelial restitution. Roles for other inflammation-regulated mediators, such as members of the arachidonic acid metabolites known to bind and activate G-protein-coupled receptors in restitution and mucosal injury repair, remain to be fully-characterized. Previous reports link CXCL12 and CXCR4 with injury repair of the skin and heart.[75–77] Our studies using a conditional knockout mouse model have uncovered an important role for ERK1/2 signaling in intestinal restitution. Regulation of ERK1/2 activity may therefore provide a potential signaling pathway to improve injury repair in colitis.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

Author's contributions: NPZ participated in the design and implementation of all *in vivo* mouse studies as well as colony establishment and maintenance, histological evaluation and immunostaining, preparation of the manuscript, and data analysis. RAV performed studies involving *in vitro* lentiviral transfections and stimulations of cultured cells and analysis of human colon transcript expression. SLF contributed to the *in vivo* wounding assay, tissue and protein isolation, and performed animal husbandry and maintained the mouse colony. NHS performed histological evaluation of DSS-induced colitis. MBD contributed to the overall study design, implementation, data analysis, and all phases of drafting and editing the manuscript.

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