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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Mesenteric Adipose-derived Stromal Cells From Crohn's Disease Patients Induce Protective Effects in Colonic Epithelial Cells and Mice With Colitis

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SUMMARY

Extracellular mediators from mesenteric adipose-derived stromal cells (ADSCs) promote colonocyte proliferation and the resolution of inflammation in a disease-dependent manner. ADSC-derived mediators such as lactoferrin may provide therapeutic potential to promote remission from intestinal inflammation in Crohn's disease patients.

Mesenteric adipose tissue hyperplasia is a hallmark of Crohn's disease (CD). Recently, we showed that mesenteric adipose-derived stromal cells (ADSCs) from CD, ulcerative colitis, and control patients synthesize and release adipokines in a disease-dependent manner. Here we examined the expression profiles of CD and control patient-derived mesenteric ADSCs and studied the effects of their extracellular mediators on colonocyte signaling in vitro and experimental colitis in vivo. ADSCs were isolated from mesenteric fat of control and CD patients. Microarray profiling and network analysis were performed in ADSCs and human colonocytes treated with conditioned media from cultured ADSCs. Mice with acute colitis received daily injections of conditioned media from patient-derived ADSCs, vehicle, or apolactoferrin. Proliferative responses were evaluated in conditioned media–treated colonocytes and mouse colonic epithelium. Total protein was isolated from cultured colonocytes after treatment with apolactoferrin for Western blot analysis of phosphorylated intracellular signaling kinases. Microarray profiling revealed differential mRNA expression in CD patient-derived ADSCs compared with controls, including lactoferrin. Administration of CD patient-derived medium or apolactoferrin increased colonocyte proliferation compared with controls. Conditioned media from CD patient-derived ADSCs or apolactoferrin attenuated colitis severity in mice and enhanced colonocyte proliferation in vivo. ADSCs from control and CD patients show disease-dependent inflammatory responses and alter colonic epithelial cell signaling in vitro and in vivo. Furthermore, we demonstrate lactoferrin production by adipose tissue, specifically mesenteric ADSCs. We suggest that mesenteric ADSC-derived lactoferrin may mediate protective effects and participate in the pathophysiology of CD by promoting colonocyte proliferation and the resolution of inflammation.
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Crohn's disease (CD) and ulcerative colitis, collectively known as inflammatory bowel disease (IBD), are chronic relapsing inflammatory disorders of the gastrointestinal tract. A specific feature of CD is white adipose tissue hyperplasia that likely develops from mesenteric depots residing in close proximity to the abdominal wall. Mesenteric adipose tissue expansion and wrapping of the involved intestinal segment occur during the onset of inflammation in CD.[1] This phenomenon, also known as "creeping fat", directly correlates with the extent of inflammation, ulceration, and wall thickness of the inflamed small or large intestine.[2] Thus, surgeons have long recognized mesenteric fat wrapping as a way of delineating areas of active disease during surgery.[3] However, the role of creeping fat in the development and progression of colitis and CD remains largely unknown.

In recent years, adipose tissue has become widely recognized as an active endocrine organ, playing key roles in the regulation of inflammation and immunity.[4–7] The cellular composition of adipose tissue is complex, including preadipocytes, mature adipocytes, and macrophages, derived from a dynamic precursor cell population referred to as adipose-derived stromal cells (ADSCs),[8] which

Abbreviations used in this paper: ADSC, adipose-derived stromal cell; CD, Crohn's disease; DSS, dextran sodium sulfate; i.c., intracolonic; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; IL, interleukin; PCR, polymerase chain reaction; RT, reverse-transcriptase; TNBS, trinitrobenzenesulfonic acid; VEGF, vascular endothelial growth factor.

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contribute to the metabolic and immunologic functions of fat by synthesizing and secreting hormones, cytokines, and chemokines.[9] The expansion of adipose tissue during obesity promotes the development of a chronic low-grade inflammation and is a significant risk factor for the development of many chronic inflammatory diseases.[10,11] Obesity has a negative impact on the course of CD, associated with increased severity of inflammation[12] and an earlier progression to surgery.[13] Previously, our laboratory demonstrated that mice with trinitrobenzenesulfonic acid (TNBS)–induced colitis display mesenteric fat wrapping of the intestine as well as major inflammatory changes in mesenteric ADSCs including increased expression levels of proinflammatory cytokines and neuropeptide receptors[14,15] as well as inflammatory infiltrate, neutrophil and leukocyte migration, and diapedesis.[14] Furthermore, we recently showed that CD patient-derived mesenteric ADSCs and whole mesenteric fat tissue exhibit differential patterns of cytokine release compared with control patients.[16]

Here we hypothesized that mesenteric ADSCs from CD and control patients exhibit differential gene expression profiles and induce disease-dependent responses in intestinal epithelial cells, thus participating in the pathophysiology of CD. To address this hypothesis, we injected conditioned media from cultured ADSCs in mice with experimental colitis in vivo. In addition, we performed microarray analysis of CD and control patient-derived mesenteric ADSCs and assessed the effects of their extracellular mediators on intestinal epithelial cell signaling and gene expression in vitro. Our profiling revealed several differentially regulated molecules, including lactoferrin, which has not been previously identified as a product of adipose tissue. Our results indicate that extracellular mediators released from CD patient-derived mesenteric ADSCs can attenuate the severity of experimental colitis and promote proliferative responses in intestinal epithelial cells, thus supporting a protective role for mesenteric adipose tissue during intestinal inflammation.

Materials and Methods

Patients

Mesenteric adipose tissue was resected from male and female patients with severe, active CD presenting for surgery (n = 46) and non-IBD control patients (n = 35) presenting for adenocarcinoma, gynecologic, vascular surgery, or other gastrointestinal complications. Subjects taking thiazolidinediones or steroids were excluded, and patients were fasted for at least 10 hours before surgery. Human studies protocols have been approved by the UCLA Institutional Review Board for Human Research (protocol #11-001527-AM-00003). All participants gave informed consent before taking part. Subjects with malignancies were not excluded because they may constitute an important subpopulation that could yield significant information for our intergroup comparisons. Tissues from Cedars-Sinai Medical Center were obtained after informed consent in accordance with procedures established by the Cedars-Sinai Institutional Review Board (protocol #3358 and #23705).

Isolation and Culture of Human Adipose-derived Stromal Cells

Mesenteric ADSCs were isolated from adipose tissue samples as previously described.[16–18] Briefly, 2–5 g adipose tissue was minced finely into small pieces by using sterile surgical scissors in 50 mL conical tubes containing 1 mg/mL collagenase (Worthington Biochemical Corporation, Lakewood, NJ) solution in ×1 phosphate-buffered saline (3 mL/g tissue). Minced tissue was vortexed and placed in a shaking water bath (100 rpm, 37°C) for 40 minutes before passing through a sterile 100-μm nylon mesh filter (Thermo Fisher Scientific, Waltham, MA). Homogenates were centrifuged at 1000 rpm for 10 minutes, and the resulting pellet was resuspended in 10 mL erythrocyte lysis buffer (Gibco, Thermo Fisher Scientific) before further centrifugation. The pellet was then resuspended in 10 mL Dulbecco's modified Eagle medium (ATCC, Manassas, VA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 0.1 mmol/L penicillin, and 0.06 mmol/L streptomycin (Gibco, Thermo Fisher Scientific) and plated on 100-mm culture dishes. Isolated cells were cultured as previously described[16–18] and passaged at least 3 times to ensure removal of macrophages, and conditioned media (1 mL; 24-hour incubation), RNA and protein were collected from confluent cells for analysis. The purity of these cultures was determined to be >90% in studies that compared single cell derived colonies with those of similarly treated skin and lung fibroblasts for their ability to accumulate lipid.[19]

NCM460 Cells

The human colonic epithelial cell line NCM460 (INCELL, San Antonio, TX) was incubated at 37°C with 5% CO_2 in M3:D culture medium (INCELL) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 10 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Thermo Fisher Scientific). ADSC-derived conditioned media (1 mL) from 6 CD and 6 control patients were added to confluent human colonic NCM460 cells and incubated for 24 hours. RNA was then isolated by using the miRNeasy mini kit (QIAGEN, Redwood City, CA) and subjected to mRNA expression analysis by microarray. In separate experiments, NCM460 cells were treated with human apolactoferrin (50 μg/mL; MyBioSource, Inc, San Diego, CA). Total protein was isolated from cells after treatment with conditioned medium or apolactoferrin by using RIPA buffer and subjected to multiplex protein analysis by using a custom 15-plex phosphoprotein panel or Western blot.

Microarray Analysis

The Human LncRNA Microarray V4.0 (Arraystar, Inc, Rockville, MD) was used to examine differential expression of 60,903 distinct transcripts including 20,730 protein-coding mRNAs in total RNA isolated from CD or control patient-derived ADSCs (6 patients/group, selected among those with the highest RNA quality). NCM460 cells were profiled with the GeneChip Human Gene ST Array (Affymetrix, Santa Clara, CA) after treatment with conditioned media from the same CD or control patient-derived ADSCs used for microarray profiling (n = 6 patients/group). The

mRNA expression data from both arrays were analyzed by using QIAGEN's Ingenuity Pathway Analysis (IPA; QIAGEN, www.qiagen.com/ingenuity).

Animals

Male C57BL/6J mice 8–10 weeks old were purchased from the Jackson Laboratory (Sacramento, CA). Mice were housed 4 per cage, maintained on a 12:12 hour light-dark cycle, and given access to food and water ad libitum. To induce colitis, dextran sodium sulfate (DSS; 3.5% or 4% w/v; Affymetrix) was dissolved in drinking water and supplied for 5 or 7 days. Mice received an intracolonic (i.c.) injection (0.1 mL) daily of vehicle (culture medium) or conditioned medium from control or CD patient-derived ADSCs (n = 3 patients/group, selected at random from those profiled above; n = 4 mice/patient) under isoflurane anesthesia. Separate groups of mice received daily injections of 10 mg/mL human apolactoferrin or saline (0.1 mL, i.c.). Mice were weighed and monitored daily and killed on day 5 or 7 by CO_2 asphyxiation and cervical dislocation. Severity of colitis was assessed by weight loss and determination of clinical and histologic damage scores as previously described.[20,21] Briefly, clinical scores were calculated on a scale of 0–3 for degree of weight loss, bleeding, stool consistency, and presence of rectal prolapse on day 5 or 7. Sections of colon (4 μm) fixed in 10% formalin, paraffin-embedded, and stained with H&E were used for histologic scoring by using an AxioImager .Z1 microscope equipped with AxioVision software version 4.6 (Zeiss, Jena, Germany). Scoring was performed by 2 independent investigators blinded to the treatment conditions, and resulting scores were averaged. Scores were assigned on the basis of the degree of crypt damage (0–4), polymorphonuclear neutrophil infiltrate (0–3), erosion (0–3), edema (0–3), and epithelial regeneration (0–3).[21] Adjacent sections were immunostained with a rabbit Ki-67 monoclonal antibody (dilution 1:200; Cell Signaling Technology, Danvers, MA). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes. Heat-induced antigen retrieval was performed in 0.01 mol/L citrate buffer, pH = 6 by using a decloaking chamber (Biocare Medical, Pacheco, CA) at 95°C for 25 minutes. The slides were then incubated with the primary antibody for 1 hour at room temperature. Signal was detected by using the rabbit horseradish peroxidase EnVision kit (Dako, Agilent Technologies Inc, Santa Clara, CA) and visualization by the diaminobenzidine reaction. Sections were counterstained with hematoxylin, air-dried, and coverslipped. Digital images were taken by using AxioCam under a ×20 objective lens, and pixel-based quantification of Ki-67 immunoreactivity was performed by using the AutoMeasure module to avoid selection bias or interobserver variation. Images were acquired, tiled, and stitched by using the MosaiX module and data collected from user-defined regions reflecting the entire epithelial surface of each section. All animal protocols were approved by the Institutional Animal Care and Use Committee and the Office of Animal Research Oversight at UCLA.

Real-time Quantitative Polymerase Chain Reaction

Total RNA was extracted from human ADSCs, NCM460 cells, and mouse colonic tissue using standard Trizol-based protocols, and complementary DNA was generated by reverse-transcriptase (RT) reaction (Promega, Madison, WI). An Applied Biosystems 7500 Fast Real-time Polymerase Chain Reaction (PCR) System was used with Fast Universal PCR Master Mix and validated TaqMan Gene Expression Assays for the detection of interleukin (IL) 1β, CCL2, CXCL1, IL6, IL17A, CCL23, lactoferrin, and vascular endothelial growth factor (VEGF) A (Applied Biosystems, Foster City, CA). Resulting data were calculated by using the 2-[delta][delta]Ct method, and the level of mRNA expression was normalized to Rn18S (Applied Biosystems). Expression of Rn18S was consistent across the treatments and conditions studied.

xCELLigence Real-time Cell Analysis

To assess NCM460 cell proliferation we used a label-free real-time cell analysis platform (xCELLigence; ACEA Biosciences, Inc, San Diego, CA) as previously described.[22] Briefly, 100 μL complete culture medium was added to each well of an E-plate 96 at RT. The E-plate 96 was connected to the system, and background impedance was measured during a period of 24 seconds. Cells were resuspended in complete culture medium and adjusted to 5000 cells/mL, which had been previously determined as the optimal seeding concentration. Resuspended cells were added to the E-plate 96 medium-containing wells. After 30 minutes of incubation at RT the E-plate 96 was placed into the cell culture incubator. Approximately 24 hours after seeding, when the cells were in the log growth phase (Cell Index >0.5), 100 μL ADSC conditioned medium from control and CD patients was added. In a separate experiment, cells were treated with 25 μmol/L NBP2-29332, an Akt 1/2/3 inhibitor (Novus Biologicals, Littleton, CO) or 10 μmol/L CHIR 99021, a glycogen synthase kinase-3 inhibitor (R&D Systems, Inc, Minneapolis, MN). Vehicle controls received non-conditioned medium. Cell proliferation was monitored and recorded every 15 minutes for 72 hours via the incorporated sensor electrode array of the E-plate 96. Electrical impedance was measured with the xCELLigence system software as a dimensionless parameter termed Cell Index, which directly correlates to the proportion of the plate surface occupied by adherent cells.[23]

Multiplex Phosphoprotein Immunoassay

Human mesenteric ADSCs were isolated and plated as described above, and media were collected at the end of the 24-hour period. NCM460 cells were then exposed to ADSC-derived conditioned medium for 24 hours, and total protein was collected by using RIPA buffer (Boston Bioproducts, Boston, MA) with protease and phosphatase inhibitors (Thermo Fisher Scientific). For phosphoprotein detection we used a custom panel that included antibody-loaded magnetic beads against Akt Ser473, GSK3b Ser9, p70SK6

Thr44/Ser424, p65 Ser536, c-Jun Ser63, STAT3 Tyr705, MEK1 Ser47/221, ERK1/2 Thr202/204/Thr185/Tyr187, p38 MAPK Thr180/Tyr182, JNK Thr183/185, PDGFR Tyr754, IRS1 Ser636/639, and IGFR-1 Tyr1131 (Bio-Rad, Hercules, CA), and the final data were obtained and analyzed via the Bio-plex 3D Suspension array system (Bio-Rad). In addition to loading volume, results were normalized for total protein.

Western Blot

Western blot analysis was performed in protein lysates from NCM460 cells. Cells were washed with ice-cold phosphate-buffered saline and incubated with radiolabeled immunoprecipitation buffer containing protease inhibitors and sodium orthovanadate (Santa Cruz Biotechnology, Inc, Dallas, TX) for 5 minutes. Cell lysates underwent sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred to polyvinylidene difluoride membranes. Blocking solution contained phosphate-buffered saline, 5% nonfat dry milk, and 0.05% Tween-20, and membranes were probed with primary antibodies for total Akt and phosphorylated Akt and s6p70K (dilution 1:1000; Cell Signaling Inc). The membrane was then blotted with the IRDye 800CW donkey anti-rabbit immunoglobulin G secondary antibody 1:15,000 (LI-COR Biosciences, Lincoln, NE) in blocking buffer for 1 hour. Visualization and quantitation were performed by using the Odyssey CLx Imaging system (LI-COR Biosciences).

Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay for lactoferrin was performed by using the human lactoferrin ELISA kit (LS-F12851; LifeSpan BioSciences, Inc, Seattle, WA), according to the manufacturer's instructions.

Data Analysis

Statistical analyses were performed by using GraphPad Prism software (Version 5.0a; GraphPad Software, La Jolla, CA). Differences between groups were determined by Mann-Whitney test or one-way analysis of variance, or two-way analysis of variance with Bonferroni or Tukey post-test. A P value $<.05$ was considered to be statistically significant. Data shown represent the mean ± standard error of the mean. All authors had access to the study data and reviewed and approved the final manuscript.

Results

Conditioned Media From Crohn's Disease Patient-derived Adipose-derived Stromal Cells Induce Protective Responses in Mice With Acute Dextran Sulfate Sodium Colitis

We first determined whether extracellular mediators from CD and control patient-derived mesenteric ADSCs could induce differential responses in the inflamed intestine. To accomplish this, we administered conditioned media from either cultured CD (n = 3) or control patient-derived ADSCs (n = 3) or vehicle via daily i.c. injections in mice with acute DSS (3.5% w/v) colitis for 5 days (Figure 1*A*). The DSS concentration and time point of these experiments were chosen to allow us to determine whether control or CD patient-derived media can enhance or limit the onset or severity of colitis. Although the degree of weight loss observed was not different among groups of mice with DSS colitis receiving vehicle, control patient-derived or CD patient-derived ADSCs (Figure 1*B*; n = 12/group), mice with DSS colitis receiving injections of CD patient-derived ADSC media had reduced clinical scores compared with mice receiving control patient media (1.375 ± 0.3239 versus 2.875 ± 0.4407, $P < .05$; Figure 1*C*) or vehicle injections (1.375 ± 0.3239 versus 4.125 ± 0.4795, $P < .001$; Figure 1*C*). Furthermore, mice receiving injections of CD patient-derived media had reduced histologic scores, compared with vehicle (3.833 ± 0.6009 versus 6.313 ± 0.7130, $P < .05$; Figure 1*D*) or control patients (3.833 ± 0.6009 versus 6.313 ± 0.7673, $P < .05$; Figure 1*D*). These results suggest that the course of acute DSS colitis is attenuated in the presence of extracellular mediators released from CD patient-derived ADSCs, supporting a protective role for mesenteric adipose tissue in the pathophysiology of CD.

In addition to a decrease in the severity of DSS colitis, conditioned media from patient-derived ADSCs altered mRNA expression of proinflammatory cytokines in colonic tissues of mice with acute DSS colitis (Figure 2*A–D*). Total RNA from full-thickness segments of mouse distal colon was used to measure mRNA expression of proinflammatory cytokines. Media from CD patient-derived ADSCs decreased mRNA expression of IL1β compared with vehicle ($P < .01$) or control patients ($P < .05$; Figure 2*A*). Media from CD patient-derived ADSCs reduced CCL2 and IL6 mRNA expression compared with vehicle ($P < .05$), whereas CXCL1 mRNA expression was reduced as compared with control patients ($P < .05$; Figure 2*B–D*). We also sought to determine whether media from control or CD patient-derived ADSCs could alter epithelial cell proliferation in vivo by immunostaining for Ki-67, a nuclear marker of cell proliferation. Quantification via pixel-based densitometry revealed that Ki-67 staining was increased in the colonic epithelium of mouse colons treated with conditioned media from CD patient-derived ADSCs compared with control patients (Figure 2*E*; $6.055e^6$ ± $1.623e^6$ versus $1.576e^6$ ± $4.28e^5$; $P < .05$). Thus, mesenteric ADSCs release extracellular mediators that can alter proinflammatory cytokine signaling and colonic epithelial cell proliferation in a disease-dependent manner.

Adipose-derived Stromal Cells From Crohn's Disease and Control Patients Exhibit Differential mRNA Expression Patterns and Release Extracellular Mediators That Alter Responses in Intestinal Epithelial Cells

We next examined the expression of 20,730 mRNA targets using the Human LncRNA Expression Microarray 4.0 in total RNA isolated from control and CD patient ADSCs (n = 6/group). Profiling revealed differential expression of

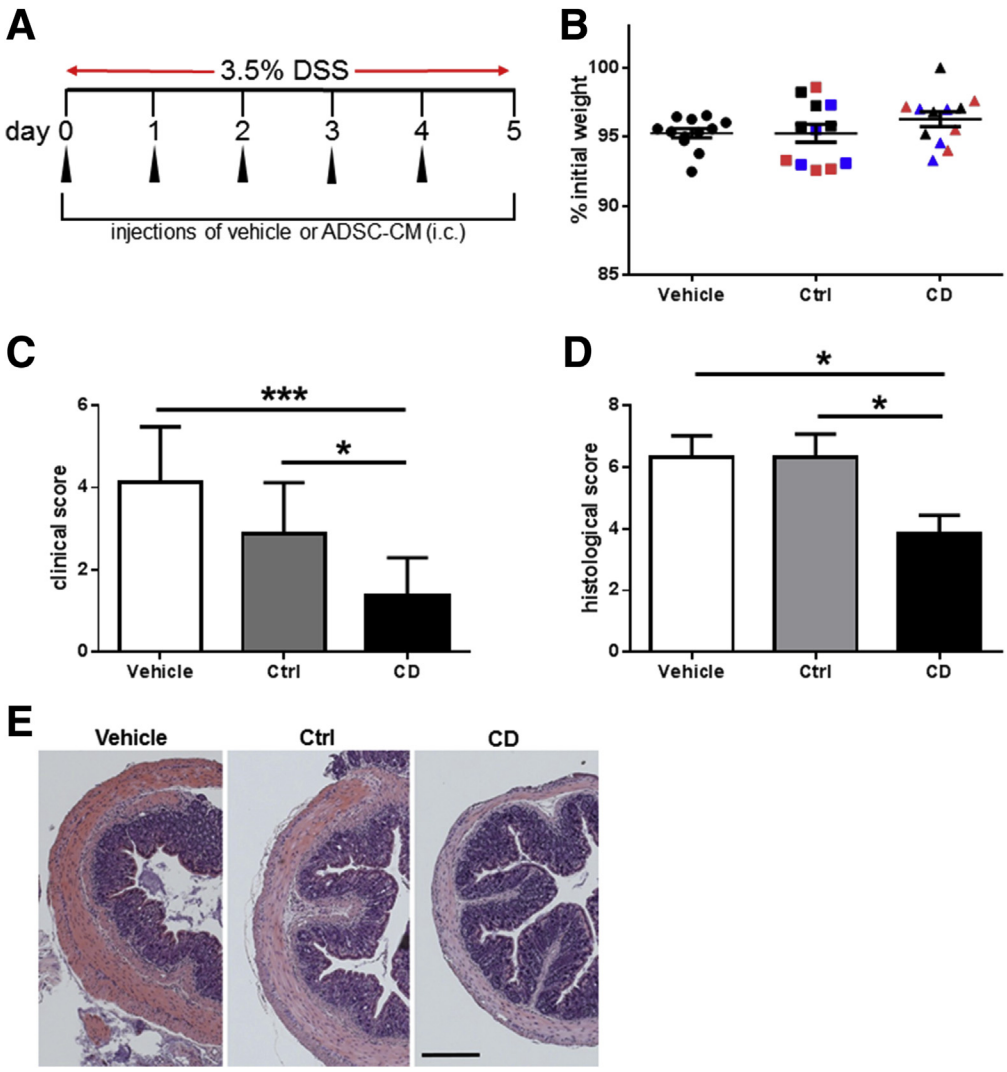

Figure 1. Mice treated with daily injections of conditioned media from CD patients displayed decreased severity of colitis as compared with mice treated with vehicle or conditioned media from control (Ctrl) patients (n = 12/group). (*A*) Mice with active DSS colitis (3.5% w/v) received i.c. injections of vehicle or patient-derived conditioned media daily for 5 days. Percentage of initial body weight did not differ between groups (*B*); individual patients within CD and Ctrl groups are reflected by color-coding of patients 1, 2, and 3 within groups. Clinical scores (*C*) and histologic scores (*D*) calculated on day 5 from DSS-treated mice. (*E*) Representative images from H&E-stained sections. **P* < .05, ***P* < .01, ****P* < .001.

992 mRNA transcripts (Supplementary Table 1) between groups (Figure 3*A*; *P* < .05, 2-fold change). Subsequent Ingenuity Pathway Analysis highlighted caspase-8 and p42/44 as central regulators of the top predicted networks differentially regulated in CD patient-derived ADSCs as compared with control patients, suggesting activation of cellular growth and proliferation pathways (Figure 3*B*).

To examine potential interactions between intestinal epithelial cells and mesenteric ADSCs that may contribute to the protective responses we observed in vivo, we also looked at the expression of 30,654 coding transcripts in RNA isolated from NCM460 cells incubated for 24 hours with conditioned media from cultured CD (n = 6) or control (n = 9) patient-derived ADSCs (n = 6/group) by using the GeneChip Human Gene 2.0 ST Array. Results from this whole-transcript array revealed 283 differentially expressed transcripts (Supplementary Table 2), and pathway analysis predicted alterations in injury and inflammation pathways, with *SERPINE1* identified as a central regulator of this network (Figure 4*A* and *B*). RT-PCR performed on RNA isolated from NCM460 cells after incubation with conditioned media from CD or control patient-derived ADSCs revealed increased expression of IL17A, CCL23, and VEGFA (*P* < .05; Figure 4*C*–*E*). Taken together, these findings suggest that the differential gene expression observed in ADSCs from human patients can induce specific disease-dependent responses in intestinal epithelial cells.

Extracellular Mediators Released From Adipose-derived Stromal Cells of Crohn's Disease Patients Promote Colonic Epithelial Cell Proliferation in Intestinal Epithelial Cells

To further investigate potential proliferative pathways as indicated by our microarray and network analysis of mesenteric ADSCs and conditioned media–treated intestinal epithelial cells, we applied CD and control patient ADSC conditioned media to NCM460 cells and monitored cell proliferation during a period of 120 hours in real-time by using the xCELLigence platform. We found that conditioned media from CD patient-derived ADSCs enhanced cell

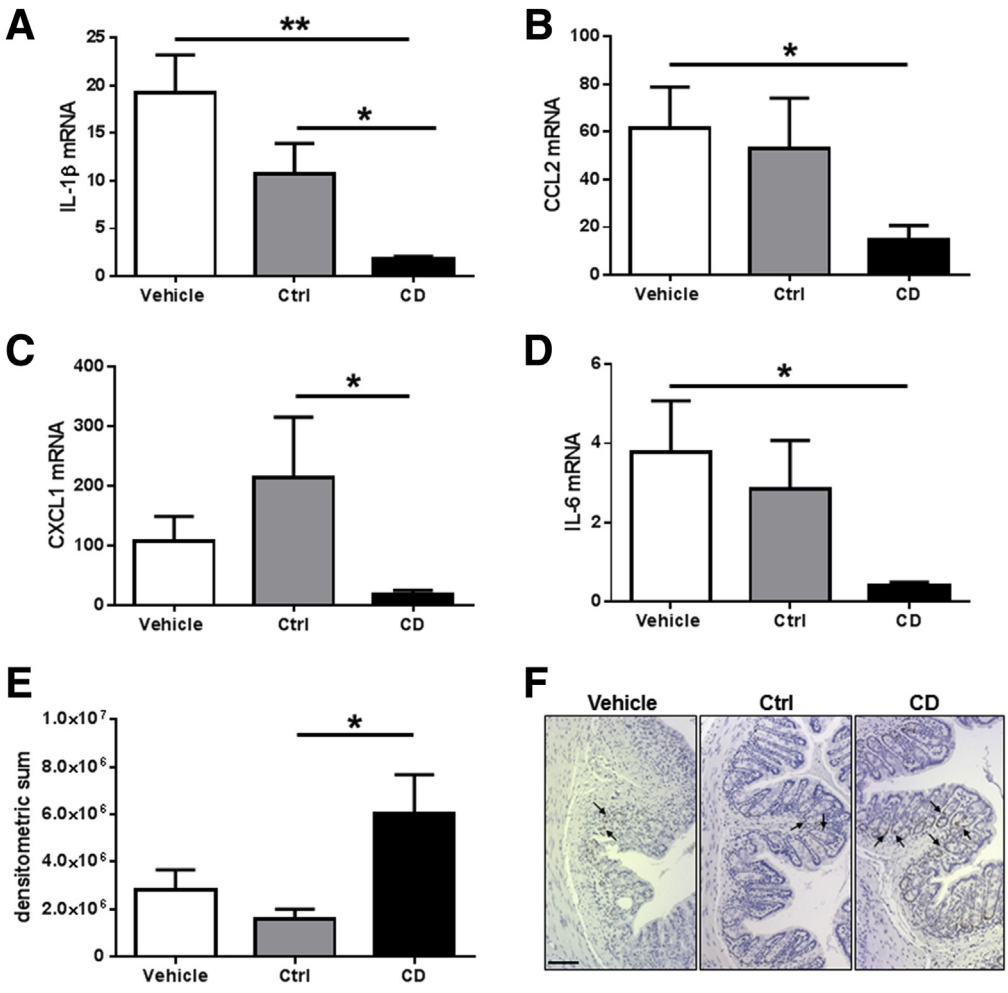

Figure 2. Proinflammatory cytokine mRNA expression is reduced in DSS-treated mice receiving daily injections of conditioned media from CD patients compared with mice receiving media from control patients (Ctrl) or vehicle-treated mice (Vehicle; n = 8/group). mRNA levels are reduced for IL1β (*A*), CCL2 (*B*), CXCL1 (*C*), and IL6 (*D*) from Vehicle or Ctrl. (*E*) Quantification of Ki-67 immunohistochemistry using pixel-based densitometry (n = 8/group). (*F*) Representative images from Ki-67 immunoreactive sections. Scale bar = 100 μm; *arrows* indicate immunoreactive intestinal epithelial cells. *$P < .05$, **$P < .01$.

doubling time ($P < .01$) and the Delta Cell Index compared with control patients ($P < .05$; Figure 5*A* and *B*). The conditioned medium used was from the same patients used for microarray profiling. These findings suggest that mesenteric adipose tissue–derived mediators may regulate proliferative responses in intestinal epithelial cells during intestinal inflammation.

In an effort to characterize the intracellular pathways underlying our observed effects, we analyzed protein extracts from NCM460 cells after treatment with CD or control patient-derived ADSC media (Figures 6, 7). Phosphokinase multiplex analysis using a 15-plex custom panel showed that CD patient ADSC-derived media induced activation of intracellular signaling pathways associated with cell survival and proliferation and, in particular, increased phosphorylation of Akt and of the p65 nuclear factor kappa B subunit (pro-proliferative; Figure 6*A* and *C*, $P < .05$) as well as GSK3β (pro-survival; Figure 6*B*, $P < .05$). It is known that Akt increases cellular proliferation via tuberin inactivation and the subsequent combination of activation of mTOR[24] and inhibitory phosphorylation and inactivation of GSK3β.[25] The p65 subunit of nuclear factor kappa B translocates into the nucleus and may induce either proinflammatory or pro-proliferative pathways. To further determine the involvement of pro-proliferative and pro-survival signaling pathways in vitro, we monitored NCM460 cell proliferation by using the xCELLigence platform in the presence of conditioned media from 6 patients and Akt (Figure 5*C*) or GSK3 inhibitors (Figure 5*D*). In colonocytes treated with conditioned media from control patient-derived ADSCs and the Akt inhibitor NBP2-29332, cell proliferation was inhibited to 66.92% ± 10.46% of control levels, whereas conditioned media from CD patient-derived ADSCs were inhibited to 43.89% ± 3.666% of control levels (Figure 5*C*; n = 6; $P < .05$). In cells that received the GSK3 inhibitor CHIR 99021, cell proliferation was inhibited to 68.84% ± 2.950% of control levels in the presence of conditioned media from control patient-derived ADSCs, whereas conditioned media from CD patient-derived ADSCs were inhibited to 45.04% ± 4.303% of control levels (Figure 5*D*; n = 6; $P < .01$). Collectively, our data suggest the potential of a CD-specific effect of ADSC-derived mediators on intestinal inflammatory and proliferative responses of intestinal epithelial cells during IBD that is both Akt and GSK3 dependent.

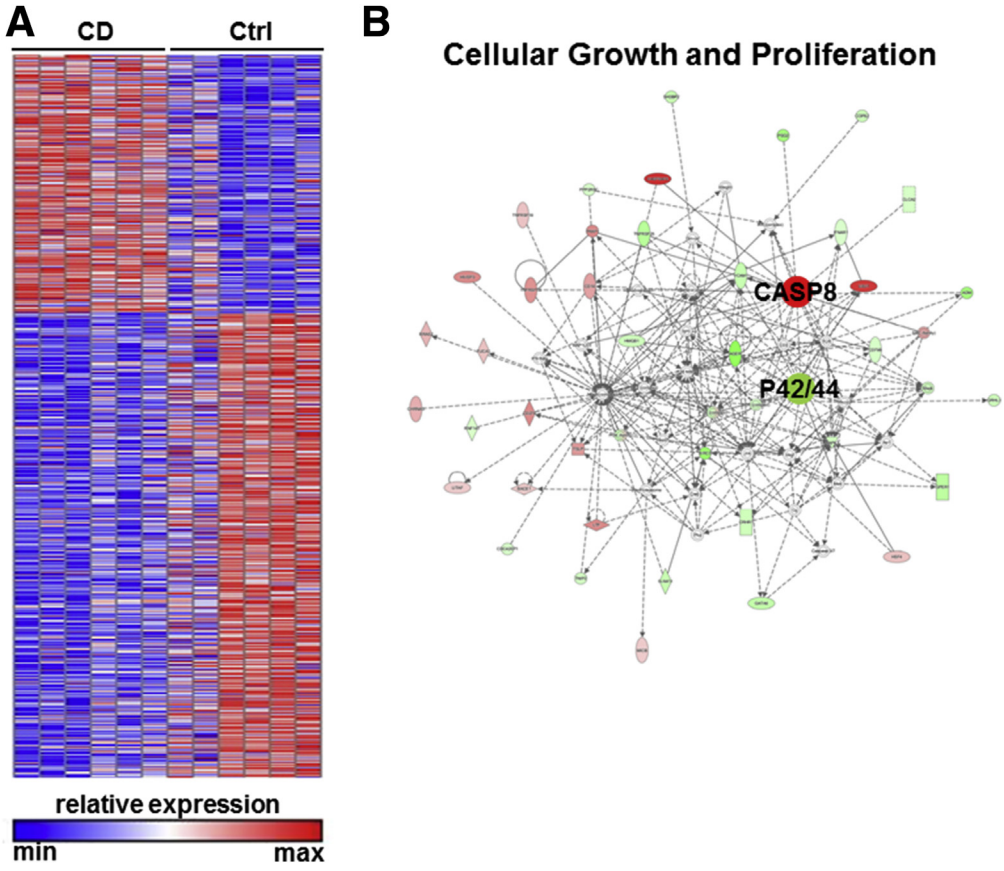

Figure 3. Mesenteric ADSCs isolated from human CD and control (Ctrl) patients exhibit differential mRNA expression patterns (n = 6/group). (*A*) Heat map represents relative expression levels of genes ranging from minimum expression (*blue*) to maximum expression (*red*). (*B*) Ingenuity Pathway Analysis of mRNA expression indicates convergence in pathways promoting cell growth and proliferation (*red and green* indicate increased and decreased mRNA expression, respectively).

Lactoferrin Is a Candidate Mediator of the Beneficial Effects Induced by Conditioned Media From Crohn's Disease Patient-derived Adipose-derived Stromal Cells During Dextran Sodium Sulfate Colitis

Deeper profiling of our mRNA expression data from patient-derived ADSCs revealed a subset of 65 differentially expressed mediators upregulated in CD patients compared with controls. Of these, 31 were moderately to highly upregulated (Figure 8*A*), and 34 were downregulated (Figure 8*B*), according to company guidelines (units >500). Lactoferrin was among the mediators with the largest fold-change and highest level of expression in CD ADSCs (Figure 8*A*) and was selected for further validation in a larger cohort of samples because of its pro-proliferative effects on human colonocytes.[26] Increased lactoferrin mRNA expression was verified via RT-PCR in ADSCs derived from 24 CD and 17 control patients (Figure 8*C*; 92.70 ± 18.41 versus 28.98 ± 5.681; $P < .05$). In addition, enzyme-linked immunosorbent assay of conditioned media revealed enhanced lactoferrin protein released from cultured CD patient-derived ADSCs compared with control patients (Figure 8*D*, 142.2 ± 5.653 versus 120.1 ± 3.664; $P < .01$). Together, these data indicate that lactoferrin production and release are increased in CD patient-derived ADSCs, suggesting lactoferrin as a potential mediator of intercellular communication between ADSCs and intestinal epithelial cells during CD.

Lactoferrin Induces Protective Effects in Mice With Dextran Sodium Sulfate–induced Colitis

We next investigated the effects of lactoferrin on the outcome of experimental colitis. DSS colitis (4% w/v) was administered to C57/BL6 mice (n = 8/group) for 7 days. A higher concentration of DSS and longer time point were chosen for these experiments because our data using conditioned media from ADSCs showed a protective effect during acute experimental colitis (Figure 1*A–D*). Daily injections of 100 μL human apolactoferrin or vehicle were administered intracolonically for 7 days (Figure 9*A*). On day 7, clinical scores were assessed, and mouse colons were harvested. Compared with mice receiving vehicle injections, mice receiving daily injections of lactoferrin had improved clinical scores (Figure 9*B*; 5.625 ± 0.565 versus 11.125 ± 0.743, n = 8) and colon length at day 7 (Figure 9*C*; 6.575 ± 0.1688 versus 5.613 ± 0.1445, n = 8). Body weight and total histologic scores were not significantly different between the vehicle-treated and lactoferrin-treated groups. In addition, we found epithelial cell proliferation was increased in the colons of lactoferrin-treated mice with colitis, compared with vehicle-treated controls (Figure 9*D* and *E*; $3.548e^{7}$ ± $1.547e^{6}$ versus $1.184e^{7}$ ± $2.915e^{6}$; $P < .01$). In an effort to

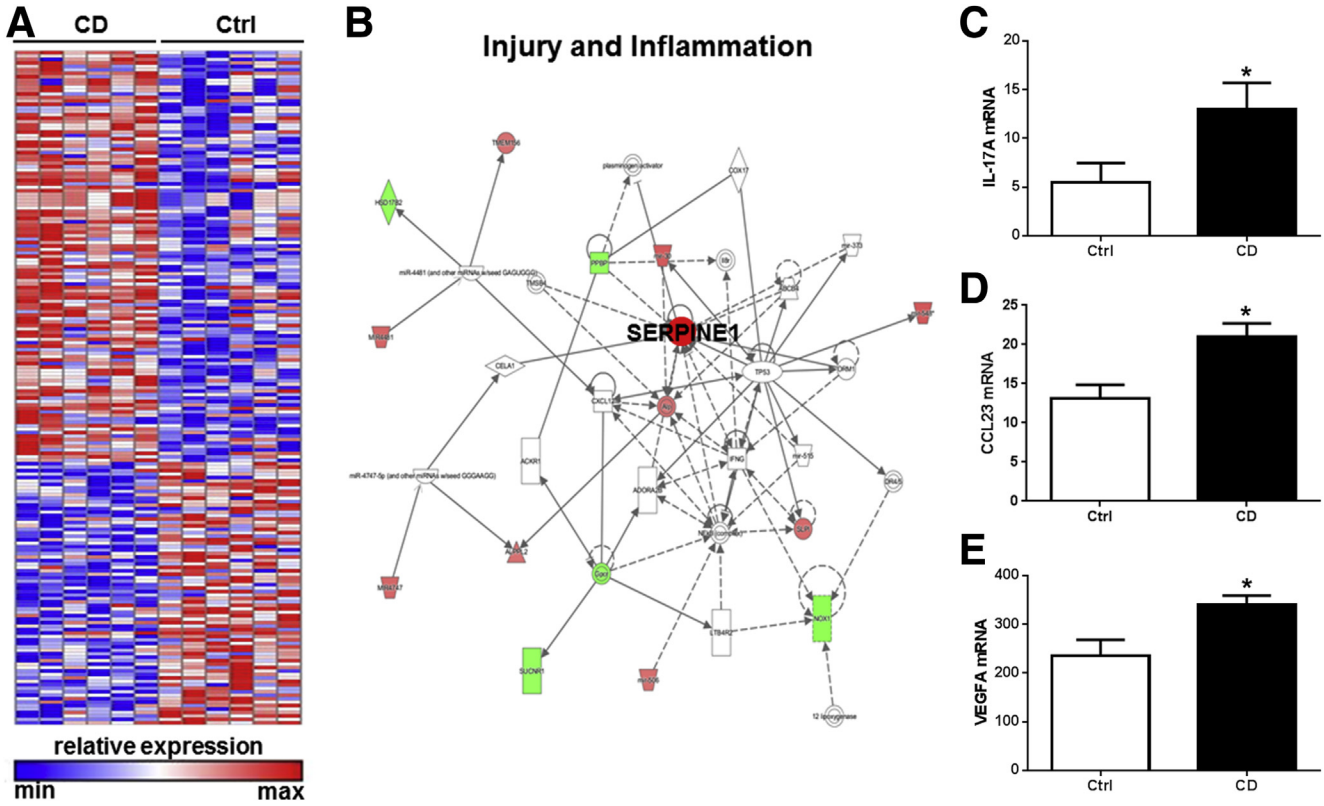

Figure 4. Conditioned media from human CD and control (Ctrl) and patient-derived mesenteric ADSCs induce differential responses in colonic NCM460 epithelial cells in vitro (n = 6/group). (*A*) Heat map represents relative expression levels of genes ranging from minimum expression (*blue*) to maximum expression (*red*). (B) Ingenuity Pathway Analysis of mRNA expression indicates convergence in injury and inflammation pathways (*red and green* indicate increased and decreased mRNA expression, respectively). Proinflammatory cytokine expression is elevated in colonic NCM460 epithelial cells treated with conditioned media from human CD patient-derived mesenteric ADSCs compared with control (Ctrl) patients (n = 6/group). mRNA levels are increased for IL17A (*C*), CCL23 (*D*), and VEGFA (*E*). *$P < .05$.

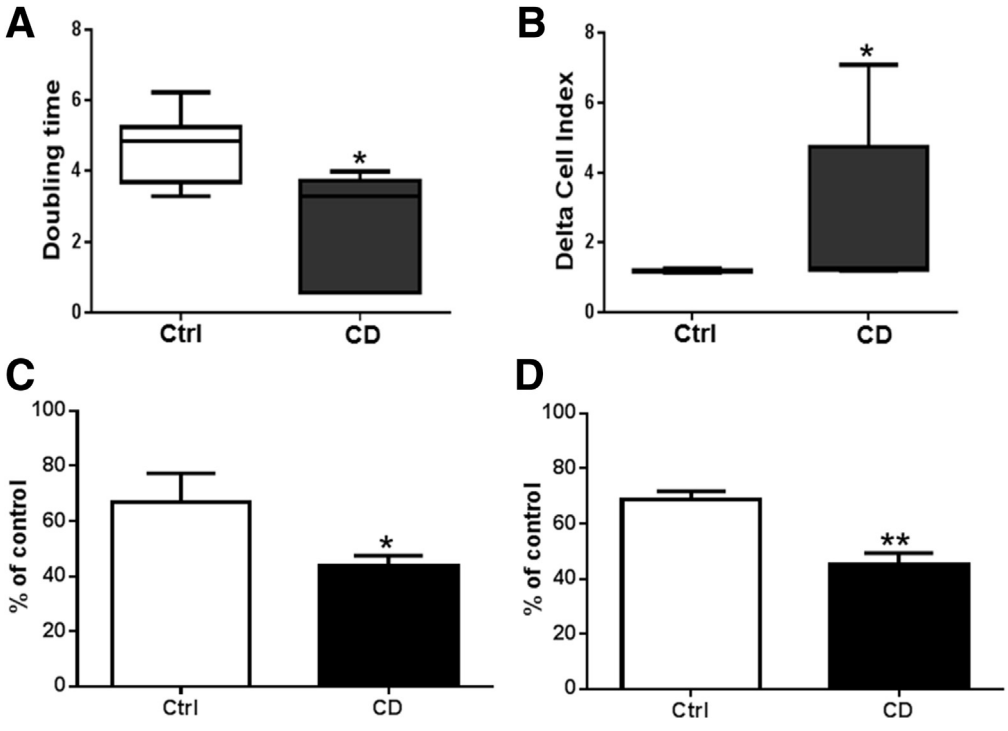

Figure 5. CD patient-derived conditioned media promotes NCM460 epithelial cell proliferation compared with control (Ctrl) patient media. Quantification of doubling time (*A*) and Delta Cell Index (*B*) is shown for n = 6 patients/group. NCM460 epithelial cell proliferation induced by CD patient-derived conditioned media was attenuated by pretreatment with AKT (*C*) and GSK-3 (*D*) inhibitors. The Delta Cell Index (Δ CI) is shown as percentage (%) of cells treated with inhibitors and media derived from control patients (n = 6, *$P < .05$, **$P < .01$).

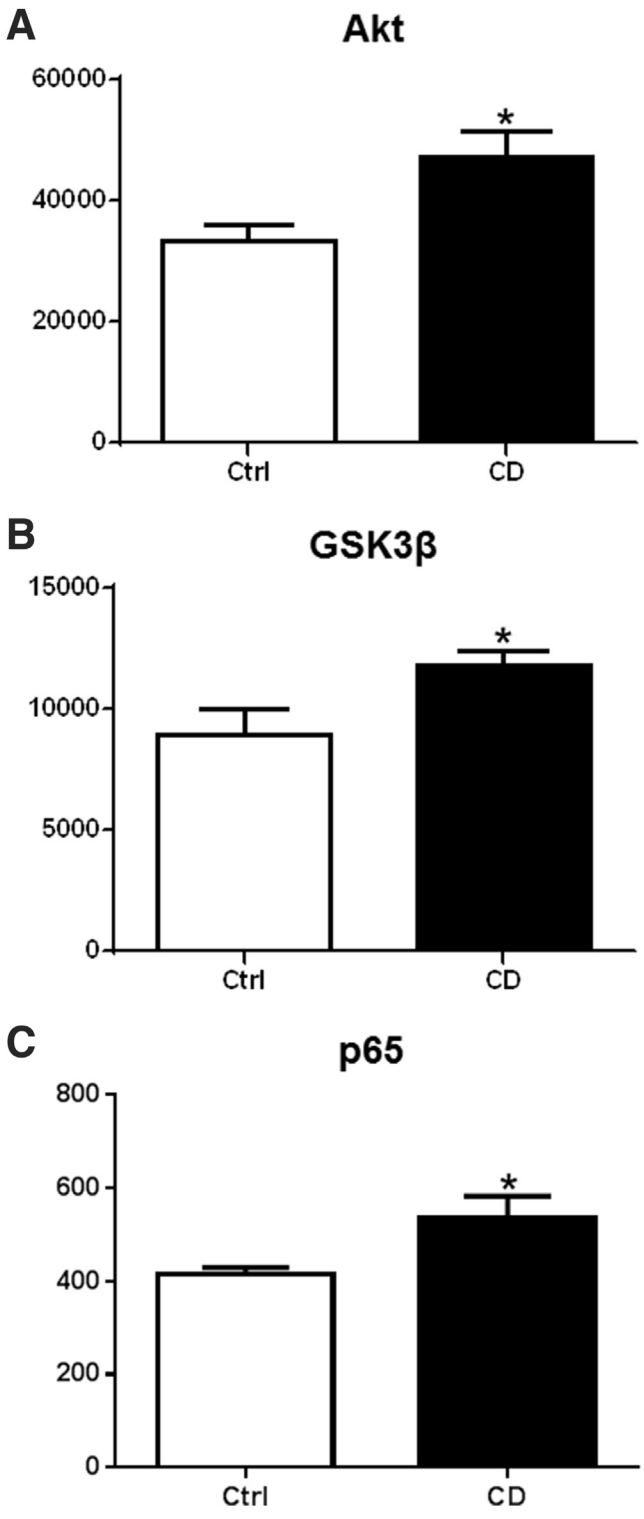

Figure 6. Protein extracts from NCM460 epithelial cells treated with conditioned media from CD patients promoted activation of proliferative and survival pathways. Phosphorylation levels for Akt, GSK3β, and the p65 subunit of nuclear factor kappa B were elevated after treatment with conditioned media from CD-derived ADSCs compared with control (Ctrl) patients. *$P < .05$.

determine which mechanisms may be responsible for these effects, we treated NCM460 cells with human apolactoferrin (50 μg/mL) and isolated total protein after 0, 5, 10, 20, 30, and 60 minutes of exposure for subsequent Western blot analysis of the phosphorylated intercellular signaling kinases Akt and p70S6K (Figure 9*F* and *G*; n = 4). Apolactoferrin treatment induced phosphorylation of Akt at 60 minutes of exposure (Figure 9*F* and *G*; $P < .05$) and s6p70 kinase at 30 and 60 minutes of exposure (Figure 9*F* and *G*; $P < .05$ and $P < .01$, respectively), consistent with a pro-proliferative action in the intestinal epithelium. Taken together, these data suggest that increased lactoferrin can mediate proliferative responses in colonocytes, and enhanced lactoferrin production from the mesenteric ADSCs of patients with CD may contribute to the protective effects observed after injection of conditioned media during DSS colitis.

Discussion

Altered adipose tissue physiology has become increasingly recognized for its involvement in pathologic conditions in several organs, including the intestine during IBD.[27,28] Despite the well-described creeping fat response around the inflamed intestine during CD, little is known about the functional interactions between mesenteric adipose tissue and the intestine. Here we show mesenteric ADSCs from CD and control patients exhibit CD-dependent gene expression profiles, and extracellular mediators from these cells induce differential responses in colonocytes. Mediators from CD patient-derived ADSCs also promote epithelial cell proliferation in vitro and in vivo. Furthermore, conditioned media from CD patient-derived ADSCs attenuate the severity of experimental colitis and reduce expression of proinflammatory cytokines in vivo. Collectively, these data suggest disease-dependent alterations in mesenteric adipose tissue ADSCs occur during CD in humans, and these cells release mediators that may protect intestinal epithelial cells during inflammation.

Previously, others have shown that mature adipocytes derived from CD and obese patients exhibit increased activation of inflammation-related pathways compared with control patients.[29] Here we have shown that the adipocyte precursor cells ADSCs exert anti-inflammatory actions on intestinal epithelial cells, demonstrating phenotypic differences between these 2 distinct adipose tissue cellular populations. Although the CD-induced changes in mRNA expression profiles of ADSCs and mature adipocytes seem to involve different physiological processes, collectively they support the concept that adipose tissue cellular populations are able to mount adaptive responses during CD that may affect the outcome of the disease, underscoring the complexity of the processes involved in CD pathophysiology. Furthermore, the present study offers evidence for a beneficial role for ADSCs during inflammation and raises the possibility of a novel target cell population for therapeutic interventions in CD. Future studies in this area will further define the interactions between ADSCs and

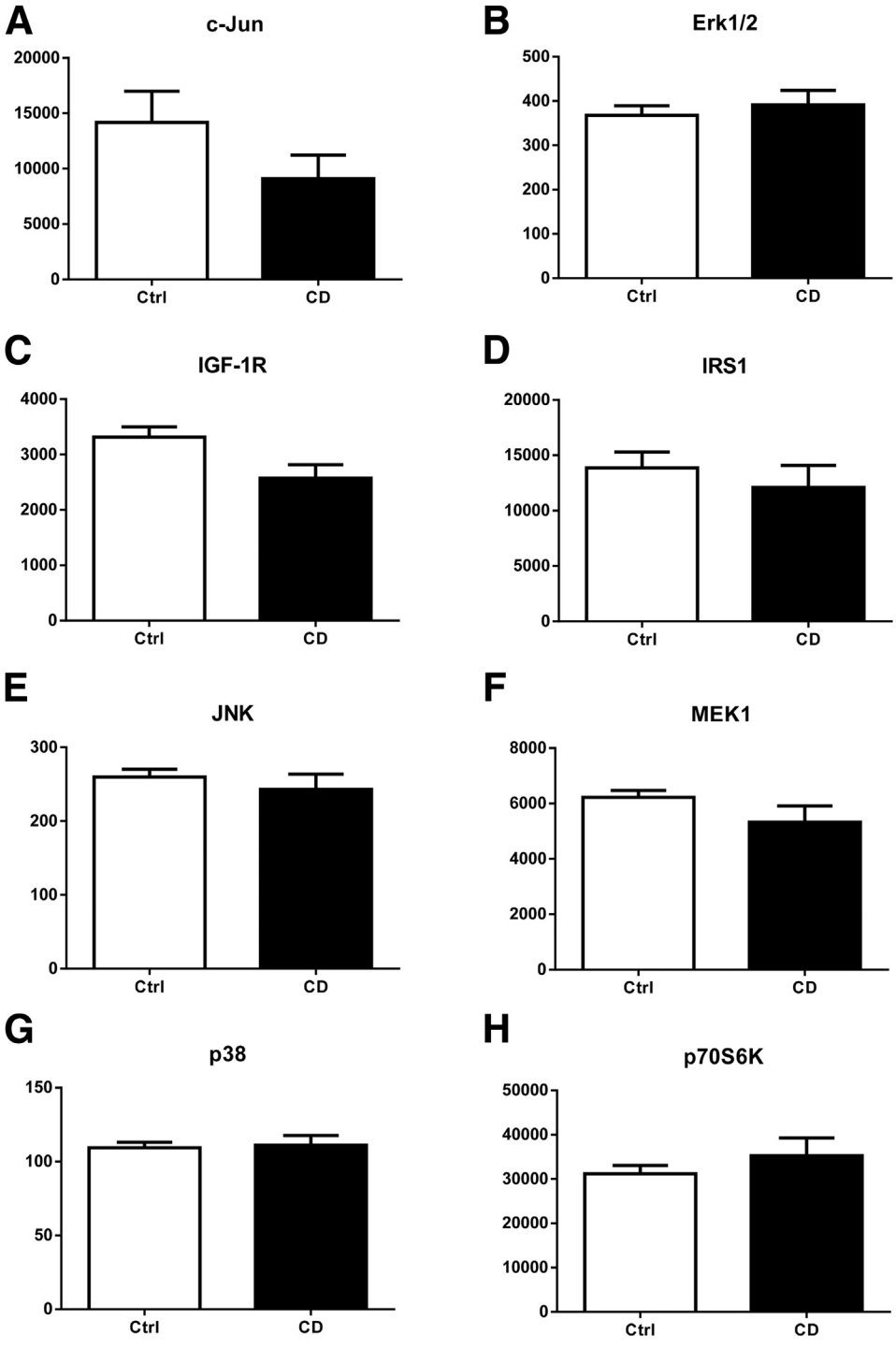

Figure 7. Phosphorylation levels of unaffected proteins from NCM460 epithelial cells treated with conditioned media from CD patients. Phosphorylation levels for p70S6K, c-Jun, STAT3, MEK1, ERK1/2, p38 MAPK, JNK, PDGFR, IRS1, and IGF-1R remained unaffected after treatment with conditioned media from CD-derived ADSCs compared with control (Ctrl) patients.

intestinal epithelial cells as well as additional cell types such as immune cells to fully characterize their impact on CD outcomes.

To assess the effects of ADSC-derived extracellular mediators on intestinal inflammation in vivo, we used the DSS model of intestinal inflammation. Although we acknowledge that our observed effects are limited to a single model of experimental colitis, this approach provided several important procedural and organizational advantages to our study. The DSS model results in a diffuse colonic inflammation that is restricted to the mucosal layer, thereby limiting potential risk for perforation with our protocol of daily i.c. injections in animals with active intestinal inflammation. Furthermore, we do not observe the creeping fat response in the DSS model of colitis as has been demonstrated in TNBS-induced colitis,[15] thereby

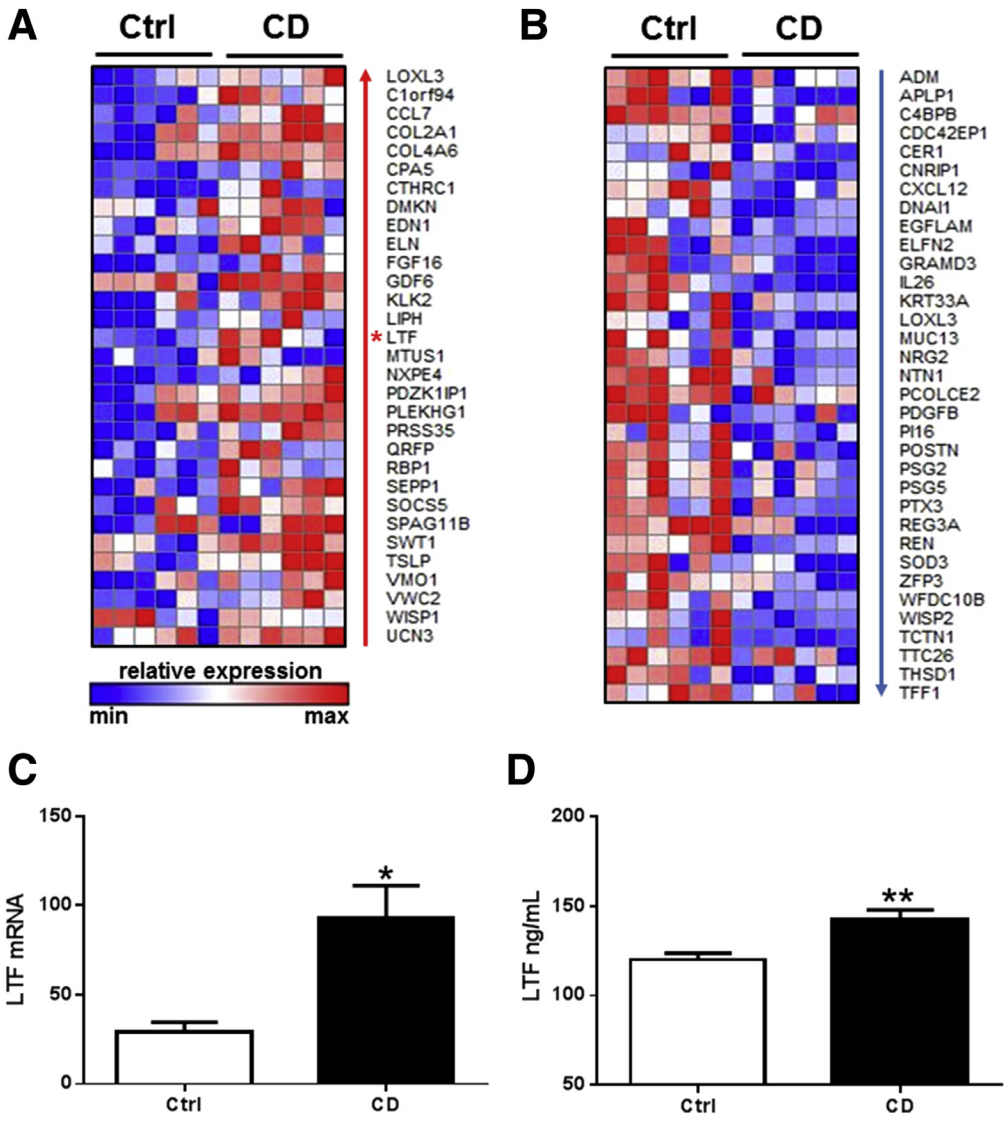

Figure 8. Heat maps from subprofiling of gene expression microarray data represent relative expression levels of genes coding for extracellular mediators upregulated in CD patient-derived ADSCs compared with Ctrl patients (*A*) and those downregulated in CD patient-derived ADSCs compared with Ctrl patients (*B*). mRNA (*C*) and protein (*D*) lactoferrin (LTF) levels are increased in human mesenteric ADSCs and conditioned media from CD patients, respectively (n = 6, $^{*}P < .05$, $^{**}P < .01$).

limiting potential effects of creeping fat–derived mediators from mouse adipose tissue at the serosal surface of the intestine. In addition, the initiation and course of intestinal inflammation are well-characterized in the DSS model, which allowed us to focus the timing of injections of our ADSC-derived conditioned media to the onset of acute colitis in vivo. Interestingly, these experiments included media from different CD patients yielding very consistent results.

Recently, patient-derived mucosal biopsy supernatants have been used by several groups to investigate the potential mechanisms of mucosal extracellular mediators in irritable bowel syndrome (IBS).[30–35] In these studies, interactions between mucosal mediators obtained from human biopsy supernatants were studied on rodent enteric neurons, leading to altered physiological responses that contribute to the manifestation of symptoms that characterize IBS. Here we adopted this approach to study the interaction between organs in the context of CD, the mesenteric adipose tissue depot and the intestine. In addition to providing evidence of CD-specific changes within mesenteric ADSCs, we have also shown that release of extracellular mediators from these cells can alter the course of experimental colitis in vivo, warranting further study of these interactions and identification of the specific mediators responsible for these effects, as well as their potential utility for therapeutic approaches in the treatment of IBD. In addition, we present evidence that adipokines released from mesenteric ADSCs can activate proproliferative responses in intestinal epithelial cells in vitro, supporting a potential functional role in wound healing responses in the inflamed intestine, which is consistent with our predicted network analyses from array data presented in Figure 4.

Cytokines represent key mediators of intestinal inflammation,[36] and therapeutic interventions targeted

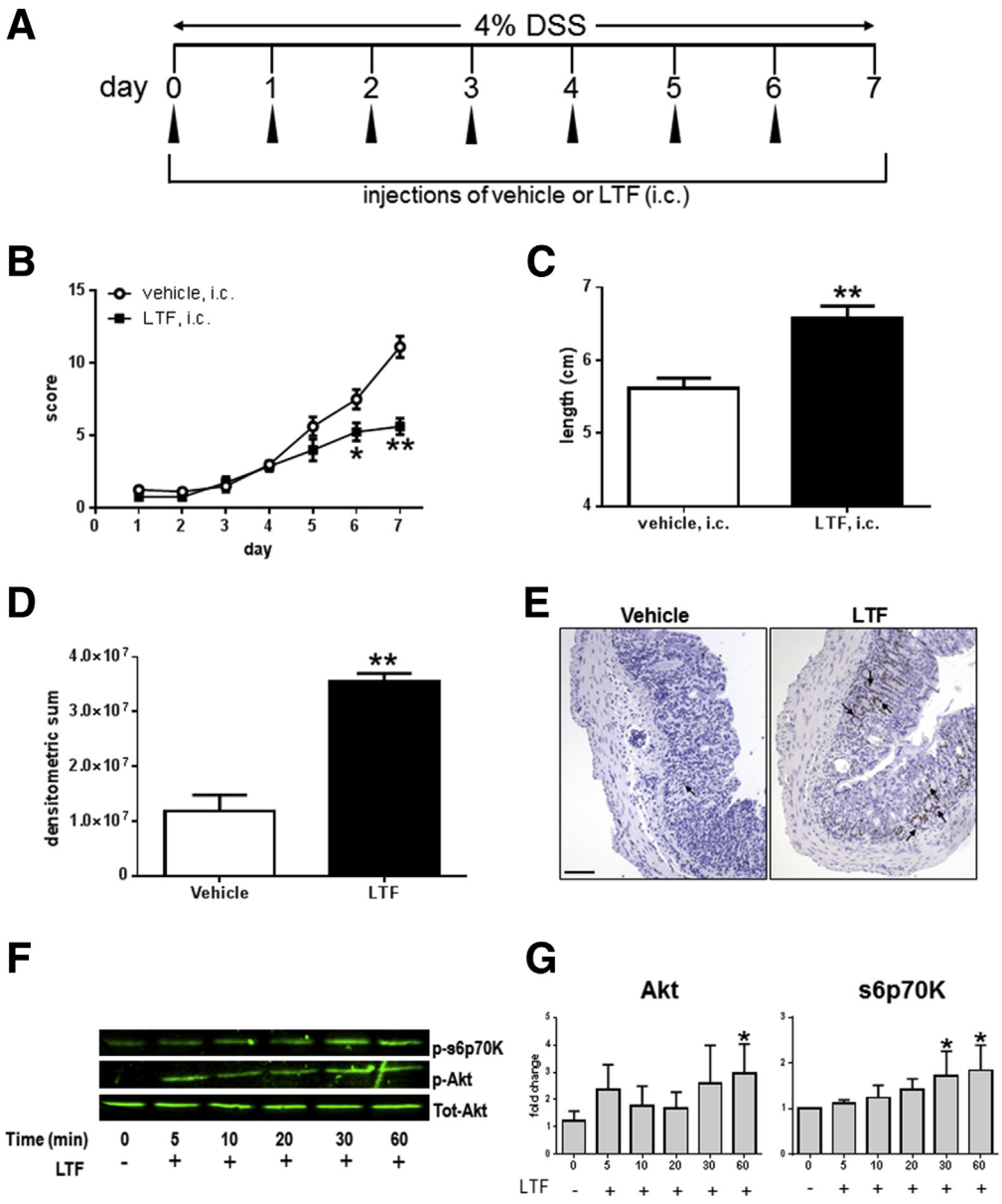

Figure 9. Mice treated with daily injections of lactoferrin (LTF) displayed decreased severity of colitis as compared with mice treated with vehicle or conditioned media from control (Ctrl) patients (n = 8/group). (*A*) Mice with active DSS colitis (4% w/v) received i.c. injections of vehicle or human apolactoferrin daily for 7 days. Clinical scores (*B*) were decreased and colon length was increased (*C*) in mice treated with LTF at days 6 and 7. (*D*) Quantification of Ki-67 immunohistochemistry using pixel-based densitometry (n = 3/group). (*E*) Representative images from Ki-67 immunoreactive sections. Scale bar = 100 μm; *arrows* indicate immunoreactive intestinal epithelial cells. (*F* and *G*) p70s6K and Akt phosphorylation in NCM460 cells, as assessed by Western blot (n = 3). *$P < .05$, **$P < .01$.

against them represent the current standard of IBD treatment.[36,37] Here we show reductions in proinflammatory cytokine expression in inflamed mouse colonic tissue in vivo. The concept that ADSCs from CD patients can promote anti-inflammatory actions in the inflamed intestine and reduce proinflammatory cytokine signaling suggests that disease-associated signals induce responses in mesenteric fat depots that, in turn, lead to the disease-dependent release of ADSC-derived mediators. Such protective effects from ADSCs are not surprising considering their progenitor-like properties and promising potential for tissue healing and regeneration in several pathologic conditions including myocardial infarction,[38,39] nerve damage,[40] and muscular dystrophy.[41] In our in vitro experiments, we observed that conditioned media from CD patient-derived ADSCs induced upregulation of several genes in colonic intestinal epithelial cells in vitro, including VEGFA, a central mediator of angiogenesis. CCL23 can also promote angiogenesis[42] and VEGF-dependent ERK phosphorylation.[43] Although angiogenesis is often associated with the promotion and maintenance of intestinal inflammation, proangiogenic factors also play a critical role in the initiation of wound healing responses.[44] Expression of IL17A was also upregulated in NCM460 cells in vitro after incubation with conditioned media from CD-derived ADSCs compared with control patients. IL17 levels are elevated in the mucosa of IBD patients compared with controls,[45] and our evidence suggests that adipokines released from mesenteric ADSCs during CD may be capable of mediating this response. Similar to VEGFA, IL17A has known roles in both the promotion of intestinal inflammation and wound healing responses,[46] and although its role in the

pathophysiology of intestinal inflammation is controversial, inhibition of IL17 was associated with a worsening of intestinal inflammation in DSS-treated mice[47,48] as well as patients in a clinical trial with moderate to severe CD,[49] supporting the concept that IL17A may exert a protective action in the intestine. Consistent with the potential for wound healing effects of ADSCs from CD patients is the observation of activation of known pro-proliferative pathways by these media on NCM460 colonic epithelial cells (Figure 6). However, additional studies are required to determine the precise roles of these factors during IBD, with particular attention paid to opposing proinflammatory and anti-inflammatory roles depending on the stage of inflammation.

The antimicrobial peptide lactoferrin is an iron-binding glycoprotein found in high concentrations in breast milk.[50] Orally administered lactoferrin leads to increased lactoferrin accumulation in the mesenteric adipose tissue depot, suggesting a direct action of lactoferrin on adipocytes.[51] Furthermore, lactoferrin suppresses adipocyte differentiation and mRNA expression of the adipogenic factors C/EBPα, PPARγ, aP2, and adiponectin in the MC3T3-G2/PA6 mouse ADSC cell line.[52] However, lactoferrin synthesis or release by human mesenteric ADSCs has not been previously shown. In the intestine, human and bovine lactoferrin treatment increases Caco-2 cell proliferation[53] via an ERK1/2-dependent mechanism[26] after binding to its receptor intelectin-1, highly expressed by both Caco-2 and small intestinal crypt cells.[54] Moreover, the porcine lactoferrin-derived peptide LFP-20 prevents lipopolysaccharide-induced damage in the colonic epithelium of mice via increased expression of tight junction proteins and corresponding reductions in intestinal permeability, intestinal epithelial cell apoptosis, and proinflammatory cytokine expression, as well as reduced infiltration of macrophages and leukocytes.[55] Clinically, lactoferrin has been identified as a target in the prevention of sepsis and necrotizing enterocolitis in neonates,[56,57] and fecal lactoferrin is an indicator of recurrent CD and inflammation in pediatric IBD.[58–60] Taken together with the recent observations that lactoferrin administration in mice with DSS colitis reduces intestinal inflammatory infiltrate and cytokine production[61] and can directly modulate T-cell phenotype,[62] our results suggest that enhanced lactoferrin expression and release from ADSCs during CD mediate, in part, the effects observed via administration of conditioned media from CD patient-derived ADSCs.

Overall, the current study demonstrates that mesenteric ADSCs exhibit differential mRNA expression profiles and promote colonocyte proliferation in a disease-dependent manner. We show evidence that these responses contribute, in part, toward protective effects observed during experimental colitis. Identification of ADSC-derived mediators such as lactoferrin offers more direct evidence for the participation of "creeping" fat in CD-associated responses within the intestine. In addition, we demonstrate lactoferrin production by adipose tissue (specifically, mesenteric ADSCs). Collectively, these data suggest that mesenteric adipose tissue–derived mediators, including lactoferrin, may participate in the pathophysiology of IBD.

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Study concept and design: JMH, AS, CP, IK; acquisition of data: JMH, AS, JJR, DS, IK; analysis and interpretation of data: JMH, AS, IK; drafting of the manuscript: JMH, IK; critical revision of the manuscript for important intellectual content: JMH, AS, CP, IK; statistical analysis: JMH, AS, IK; obtained funding: JMH, AS, JJR, CP, IK; administrative, technical, or material support: JMH, AS, JJR, DS, DQS, JRT, CP, IK; study supervision: CP, IK.

Conflicts of interest
The authors disclose no conflicts.

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