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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Substance P Mediates Proinflammatory Cytokine Release From Mesenteric Adipocytes in Inflammatory Bowel Disease Patients

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SUMMARY

Preadipocytes in inflammatory bowel disease (IBD) have acquired, differential disease-dependent characteristics that lead to changes in the release of inflammation-associated mediators after substance P (SP) treatment; interleukin 17 (IL-17) is the most consistently regulated mediator in isolated human mesenteric preadipocytes.

BACKGROUND & AIMS: Substance P (SP) neurokinin-1 receptors (NK-1Rs) are expressed in mesenteric preadipocytes, and SP binding activates proinflammatory signaling in these cells. We evaluated the expression levels of SP (Tac-1), NK-1R (Tacr-1), and NK-2R (Tacr-2) mRNA in preadipocytes isolated from patients with inflammatory bowel disease (IBD) and examined their responsiveness to SP compared with control human mesenteric preadipocytes. We investigated the effect of the neuropeptide SP on cytokine expression in preadipocytes of IBD versus control patients and evaluated the potential effects of these cells on IBD pathophysiology via SP-NK-R interactions.

METHODS: Mesenteric fat was collected from control, ulcerative colitis (UC) and Crohn's disease patients (n = 10–11 per group). Preadipocytes were isolated, expanded in culture, and exposed to substance P. Colon biopsy samples were obtained from control and IBD patients.

RESULTS: Tacr-1 and -2 mRNA were increased in IBD preadipocytes compared with controls, but Tac-1 mRNA was increased only in UC preadipocytes. SP differentially regulated the expression of inflammatory mediators in IBD preadipocytes compared with controls. Disease-dependent responses to SP were also observed between Crohn's disease and UC preadipocytes. Interleukin 17A (IL-17A) mRNA expression and release increased after SP treatment in both Crohn's disease and UC preadipocytes; IL-17RA mRNA increased in colon biopsies samples from IBD patients.

CONCLUSIONS: Preadipocyte SP-NK-1R interactions during IBD may participate in IBD pathophysiology. The ability of human preadipocytes to release IL-17A in response to SP together with increased IL-17A receptors in the IBD colon suggests that a fat-colonic mucosa inflammatory loop may be active during IBD. (*Cell Mol Gastroenterol Hepatol 2015;1:420–432; http://dx.doi.org/10.1016/j.jcmgh.2015.03.003*)

Keywords: Cytokines; Interleukin-17; Preadipocytes; Substance P.

Substance P (SP) is an endecapeptide[1] member of the tachykinin family of peptides and a product of the preprotachykinin-A (Tac1) gene.[2] SP signals via binding to three G-protein–coupled neurokinin receptors (NK-1R-2R-3R), with highest affinity for NK-1R.[2] SP is expressed in numerous tissues and organs, including the gastrointestinal tract.[2,3] SP is also expressed in cells of the immune system, and it functions both as a neurotransmitter and an immune modulator in many disease states, including several intestinal diseases with an inflammatory phenotype.[2,4]

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease, comprises complex diseases of unknown etiology. The pathophysiology of these diseases involves complex interactions between genetic, microbial, and immune factors.[5] Our group and others have shown that SP and NK-1R have a role in the pathophysiology of intestinal inflammation, including IBD.[6–8] NK-1R expression is increased in the intestinal mucosa of mice with

Abbreviations used in this paper: **b-FGF, basic fibroblast growth factor; BMI, body mass index; CSF-2, colony-stimulating factor 2; CXCL, chemokine (C–X–C motif) ligand; IBD, inflammatory bowel disease; IFNγ, interferon γ; IL, interleukin; IL-17RA, interleukin 17 receptor A; LTB, leukotriene B; MCP-1, monocyte chemotactic protein 1; MIP, macrophage inflammatory protein; NK-1R, neurokinin-1 receptor; PDGF, platelet-derived growth factor; RANTES, regulated on activation normal T-cell expressed and secreted; SP, substance P; Tac1, preprotachykinin-A; TNFα, tumor necrosis factor α; UC, ulcerative colitis; VEGFA, vascular endothelial growth factor A.**

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intestinal inflammation[9] as well as IBD patients.[7,10,11] Studies employing NK-1R knockout mice[12,13] and SP receptor antagonists[14–17] show that SP, via NK-1R, plays a dual role in the development of colitis. SP acts as a proinflammatory peptide in acute intestinal inflammation but also enhances proliferation and mucosal healing during chronic colitis[13,16,18,19] by activating distinct protective signaling pathways.[14,19,20] The mechanism involved in the proinflammatory NK-1R–associated responses includes interactions of SP with NK-1R on epithelial and inflammatory cells[10,21,22] and the release of cytokines[17,23,24] that modulate colitis and colitis-associated motility[3] primarily by activating pathways dependent on nuclear factor κB (NF-κB).[6]

A potential role for adipose tissue in IBD pathophysiology is suggested by clinical studies associating increased body mass index (BMI) with the development of active Crohn's disease and the requirement of patients for hospitalization.[25] Fat accumulation surrounding the inflamed intestine ("creeping fat") during Crohn's disease represents a hallmark of the disease.[26,27] Histologic examination of the mesenteric fat of patients with creeping fat demonstrated inflammatory changes[26] and alterations of adipokine levels in the circulation of IBD patients.[28] These data combined with the emergence of fat as an endocrine organ[29] suggest a role of intra-abdominal fat in IBD pathophysiology. Previously, we demonstrated the presence of NK-1R in human mesenteric preadipocytes[30] along with activation of inflammatory,[30] antiapoptotic,[31] and metabolic[32,33] pathways after SP treatment. We also reproduced the creeping fat phenotype in the intracolonic trinitrobenzylsulfonic acid (TNBS) mouse colitis model that was associated with increased proinflammatory cytokine expression in these depots.[30] However, modulation of expression of SP and NK-1R in adipose tissue during IBD has never been examined, and the responsiveness of IBD preadipocytes to SP has not been determined.

Here, we compared for the first time the effects of SP treatment on cytokine production in human mesenteric preadipocytes isolated from a substantial number of control, UC, and Crohn's disease patients. In these cells, we also compared the levels of expression of the Tac1 and NK-1R, NK-2R, and NK-3R genes. Initially, we demonstrate differential cytokine release from preadipocytes isolated from IBD patients compared with controls. We show that human mesenteric preadipocytes isolated from UC and Crohn's disease patients release express higher levels of NK-1R and NK-2R but not NK-3R. We also found that human mesenteric preadipocytes express Tac-1 mRNA, whose expression was elevated in UC but not Crohn's disease preadipocytes. Further, we present evidence that UC and Crohn's disease preadipocytes display differential responses after treatment with SP compared with cells from control patients. Our data also demonstrate IBD-disease dependent changes in SP-induced inflammatory stimulation of human preadipocytes, including increased interleukin 17A (IL-17A) transcription, while interleukin 17 receptor A (IL-17RA) mRNA expression is higher in colonic biopsy samples of both UC and Crohn's disease patients compared with controls.

Materials and Methods

Patients

Mesenteric fat tissues from male and female IBD (11 UC, 11 Crohn's disease) and non-IBD patients (adenocarcinoma surgery, other gastrointestinal complications, or vascular surgery, n = 10) were used. The group of control patients was either of Hispanic or (mainly) of Caucasian descent, mixed both males and females, and had an average BMI of 26.86. Their pathologies included four with adenocarcinoma, two with polyposis coli, one with Whipple disease, one with diverticulitis, one with idiopathic motility disorder, and one with tubular adenoma. The UC and Crohn's disease patients were also a mixed population of men and women with an average BMI of 27.23 and 24.12, respectively. The protocol was approved by the UCLA institutional review board for human research (11-001527-AM-00003).

All patients fasted for at least 10 hours before surgery and provided informed consent. Tissues from Cedar's Sinai were obtained after informed consent in accordance with procedures established by the Cedars-Sinai institutional review board (3358 and 23705). Tissues from Chicago were obtained in accordance with procedures established by the University of Chicago institutional review board (IRB 12960). Colon biopsy samples were collected from patients undergoing colonoscopy for colon cancer screening or IBD disease activity monitoring. The samples were obtained, immediately frozen, and used for RNA isolation.

Isolation and Cell Culture of Human Preadipocytes

We minced 2–5 g of mesenteric fat tissue from each patient into pieces. The samples were then placed in 50-mL tubes containing collagenase solution (1 mg/1 mL of phosphate-buffered saline, 3 mL solution/1 g tissue) and minced to a fine consistency. After vortexing, the tubes were placed in a 37°C shaking water bath (100 rpm) for 40 minutes. The solution was vortexed and filtered through a sterile 100-μm nylon mesh (Fisher Scientific, Hampton, NH). The homogenates were centrifuged at 1000 rpm for 10 minutes. The pellet was then resuspended in 10 mL of erythrocyte lysis buffer (cat. no. A1049201, GIBCO/Invitrogen, Grand Island, NY), placed in a 37°C shaking water bath for 5 minutes at 100 rpm, and then centrifuged at 1000 rpm for 10 minutes. The pellet was resuspended in 10 mL of plating medium (Dulbecco's modified Eagle medium, 0.1 mM penicillin, 0.06 mM streptomycin, 10% HI-fetal bovine serum, pH 7.4), vortexed, plated onto 100-mm dishes, and incubated at 37°C.

Culture of Human Preadipocytes

After 20 hours, the cells were washed three times with 10 mL of phosphate-buffered saline, and 1 mL of trypsin solution (Invitrogen, Carlsbad, CA) was added. The trypsin was inactivated with 5 mL of plating medium, and the cells were centrifuged at 1000 rpm for 10 minutes. After resuspension in plating medium, the cells were plated at 5×10^4 cells/cm^2 in plating medium and incubated at 37°C until

confluence. Previous studies have demonstrated that this isolation procedure yields >99% pure preadipocyte populations.[34] The cells were then subcultured three or four times to ensure the removal of macrophages. No ADAM8, F4/80, or macrophage inflammatory protein-1a mRNA (markers of macrophages) were detected by an Affymetrix array (Affymetrix, Santa Clara, CA) analysis of human mesenteric preadipocytes prepared using this protocol. Preadipocytes at passages three to four were then exposed to 10^{-7} M SP for 8 hours in 3 mL of human maintenance medium (Dulbecco's modified Eagle medium/Ham's F-12 medium with 23 mM HEPES, 25 mM $NaHCO_3$, 0.1 mM penicillin, 0.06 mM streptomycin, 10 mg/L transferin, 0.3 mM biotin, and 2 mM L-glutamine). The medium and the RNA of the cells were collected for analysis. Protein lysates were also collected from preadipocytes in plating medium.

Real-Time Polymerase Chain Reaction

The RNA was isolated from human mesenteric preadipocytes and colon biopsy samples using the TRIzol method. We reverse-transcribed 1 μg of RNA into cDNA as previously described elsewhere[33] and incubated with dual fluorogenic probes (Applied Biosystems, Foster City, CA).

The levels of the target mRNA were quantified using a fluorogenic 5′-nuclease polymerase chain reaction (PCR) assay using a 7500 Fast Real-Time PCR sequence detection system according to manufacturers instructions (Applied Biosystems). Cycle conditions were subject to change for higher efficiency as different targets required. The primers used were Hs00243225_m1 (Tac1), Hs00185530_m1 (Tacr1), Hs00169052_m1 (Tacr2), Hs00357277_m1 (Tacr3), Hs01064648_m1 (IL-17RA), Hs00994305_m1 (IL-17RC), and for normalization Hs03928990_g1 (Human Eukaryotic 18S rRNA) (all from Applied Biosystems).

Western Immunoblot Analysis

Proteins were collected from human mesenteric preadipocytes of control, UC, and Crohn's disease patients (n = 4 per group) in RIPA TRITON X100 (BP-116TX; Boston BioProducts, Ashland, MA) with protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). We loaded 30 μg of protein on a 10% polyacrylamide gel and electrophoresed it for 1.5 hours. The proteins were transferred on polyvinylidene fluoride membranes, and the membranes were blocked for 1 hour at room temperature in LI-COR blocking buffer (LI-COR Biosciences, Lincoln, NE). The membranes were blotted with a rabbit NK-1R primary antibody at a dilution of 1:100 overnight at room temperature (sc-15323; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary goat anti-rabbit antibody (1:15,000, cat. no. 926-32211; LI-COR Biosciences) was added for 1 hour at room temperature. Loading was normalized using a mouse β-actin primary antibody (1:1000, cat. no. sc-81178; Santa Cruz Biotechnology) and a goat anti-mouse secondary antibody (1:15,000, cat. no. 926-68170; LI-COR Biosciences). Bands were visualized and quantified using the Odyssey IR Imaging System (LI-COR Biosciences).

mRNA Multiplex Analysis

Total RNA was isolated as described earlier, and inflammation-related gene expression was analyzed using the 42-plex FlexScript LDA inflammatory panels 3 and 4 (Luminex, Austin, TX). We loaded 20 ng of total RNA in each well, and then we performed the treatments described in the company's manual (FlexScript LDA). The plate was run using Bio-Plex 3D suspension array system (Bio-Rad Laboratories, Hercules, CA). In addition to the total RNA concentration, the data were normalized to endogenous controls (GAPDH, B2M, β-actin) included within the gene panels.

Multiplex Cytokine and Phosphoprotein Immunoassays

Human mesenteric preadipocytes were isolated and plated as described previously, and media were collected at the end of the 8-hour exposure to SP. Cytokine concentrations in human preadipocyte media were determined using the Bio-Plex ProTM Human Cytokine 27-Plex, Group I, with magnetic beads (Bio-Rad Laboratories), and the final data were obtained and analyzed via the Bio-Plex 3D Suspension array system (Bio-Rad Laboratories). In addition to the loading volume, the results were normalized for cell plating number and total protein.

Immunohistochemistry

Paraffin-embedded whole-fat sections from UC and control patients (n = 4) were mounted on slides. The SP staining was detected using an anti-SP rabbit polyclonal antibody (AB1566; Millipore, Darmstadt, Germany) and the EnVision+ System HRP labeled Polymer Anti-Rabbit kit (DAKO, Carpinteria, CA). The staining was performed at the Translational Pathology Core, University of California at Los Angeles, following a standard procedure described in Millipore's manual for the primary antibody treatment (1:100 Ab dilution, pretreatment with citrate pH 6.0, antigen retrieval).

Determination of Endotoxin Levels

Aliquots from the SP preparations used in our treatments were diluted in cultured media as described earlier to match the concentration and conditions represented in our study. The Pierce LAL Chromogenic Endotoxin Quantification kit (Thermo Scientific, Rockford, IL) was used for the quantitative measurement of endotoxin levels using *Escherichia coli* 0111-B4 endotoxin as the standard. The endotoxin measurements in all treatment preparations were below detection levels (data not shown).

Statistical Analysis

The results were analyzed using the Prism professional statistics software program (GraphPad Software, San Diego, CA). Analyses of variances (ANOVA, one-way) as well as

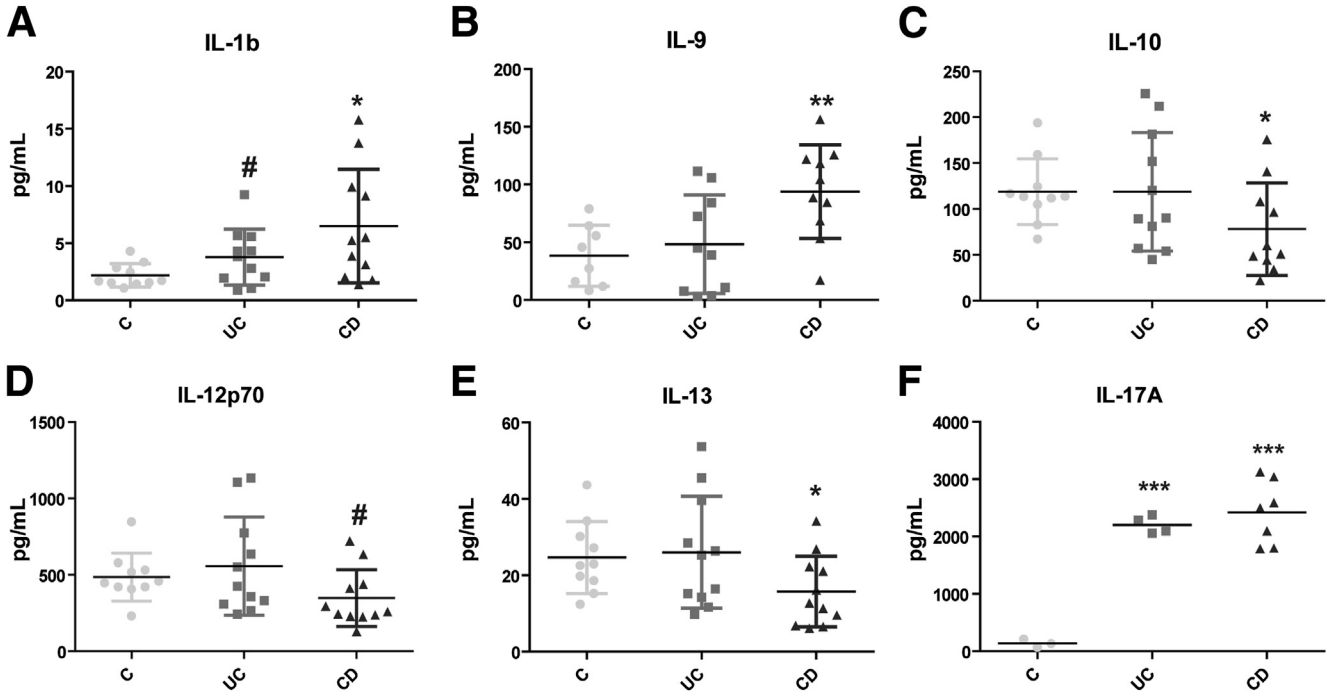

Figure 1. Preadipocytes isolated from Crohn's disease patients demonstrate different patterns of cytokine release compared with cells isolated from healthy controls (n = 4–11). Mesenteric preadipocytes were isolated from control ulcerative colitis (UC) and Crohn's disease (CD) patients and conditioned media were collected at the third passage. Multiplex analysis of a 27 human cytokine-containing panel revealed that preadipocytes isolated from CD patients secrete higher levels of (*A*) IL-1β, (*B*) IL-9, and (*F*) IL-17 while the levels of (*C*) IL-10, (*D*) IL-12, and (*E*) IL-13 were significantly lower compared with the controls (C). In preadipocytes from UC patients, strong trends toward an increase were observed for (*A*) IL-1β and significant increases in (*F*) IL-17 compared with controls. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{\#}P < .1$.

Mann-Whitney tests (for comparisons between two groups) were used for intergroup comparisons. $P < .05$ was considered statistically significant.

Results

Human Mesenteric Preadipocytes Isolated From Crohn's Disease Patients Demonstrate Distinct Mediator Release Compared With Controls

We have isolated preadipocytes from mesenteric fat depots of 10 control, 11 UC, and 11 Crohn's disease patients and expanded them in culture without prior freezing or external stimulation. At the end of the second passage, fresh medium was added to preadipocytes and collected after 8 hours for multiplex cytokine analysis. Analysis of the 27 cytokines showed changes in the release of mediators from human preadipocytes during IBD compared with the controls (Figure 1*A*–*F*). Preadipocytes isolated from Crohn's disease patients demonstrated statistically significant increases in the release of IL-1β, IL-9, and IL-17 (Figure 1*A*, *B*, and *F*) and significant decreases in the release of IL-10, IL-12 (trend in Crohn's disease), and IL-13 (Figure 1*C*–*E*) compared with the controls. Of the 27 mediators included in our panel, none exhibited differential secretion in preadipocytes from UC patients compared with the controls; only IL-1β and IL-17 were increased (strong trend for IL-1β, $P = .07$), and substantial differences were observed between preadipocytes isolated from UC and Crohn's disease patients (IL-9, IL-10, IL-12, IL-13, and b-FGF, data not shown), suggesting disease-dependent changes in these cells. Patients for whom the cytokine values fell outside the standard curve were excluded.

Human Mesenteric Preadipocytes From Ulcerative Colitis Patients Express Higher Levels of Tac1 Compared With Cells Isolated From Control and Crohn's Disease Patients

SP levels are modulated in the intestine during IBD.[35,36] However, there is no information about whether human preadipocytes express Tac-1 mRNA, the gene encoding for SP. To address this, we isolated RNA from preadipocytes from 11 control, 10 UC, and 11 Crohn's disease patients and examined the mRNA expression levels of Tac1 mRNA. We found that human mesenteric preadipocytes express Tac1 mRNA. We also found increased mRNA levels of Tac1 in preadipocytes isolated from UC patients (Figure 2*A*, $P < .05$, n = 9–11, extreme outliers were determined using the Grubbs test) compared with the control cells. There was no statistically significant difference (or trend) in Tac1 mRNA levels between preadipocytes from Crohn's disease and control patients. To verify the SP expression levels between control, UC, and Crohn's disease patients, we performed immunohistochemical analysis in whole-fat tissue isolated from these patients using an anti-SP antibody, and we observed increased expression of SP-positive cells in UC

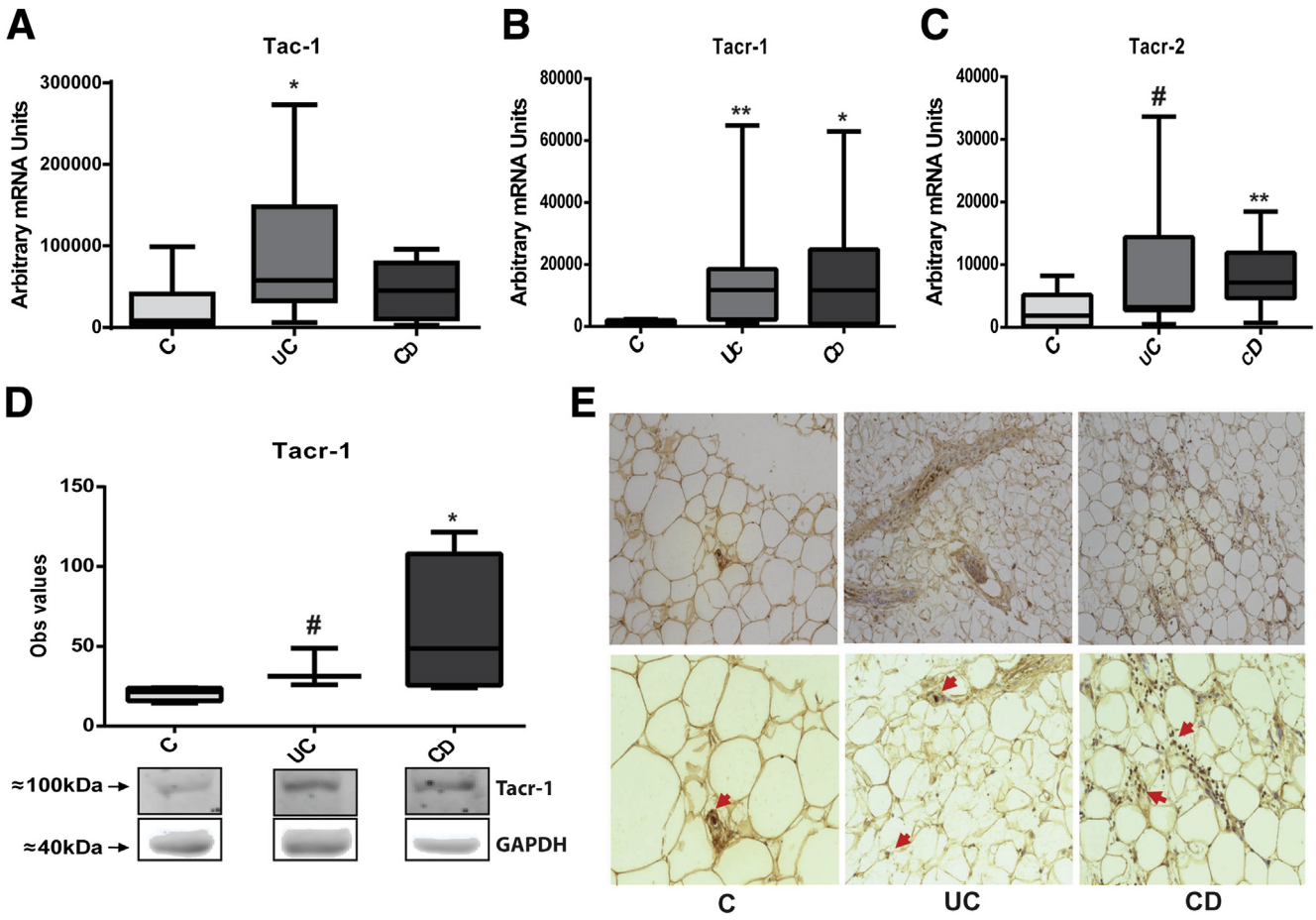

Figure 2. Regulation of SP and its receptors NK-1R and NK-2R during inflammatory bowel disease. Human mesenteric preadipocytes were isolated from control (C), ulcerative colitis (UC), and Crohn's disease (CD) patients and cultured until the third passage when the total RNA and protein were collected. (*A*) Real-time polymerase chain reaction analysis shows that Tac1 (SP mRNA precursor) mRNA levels increase in human mesenteric preadipocytes during UC but not during CD. Similar observations were made at the mRNA level for both (*B*) Tacr-1 and (*C*) Tacr-2 receptors (trend in UC). (*D*) Western blot analysis shows that NK-1R protein levels increase during UC (trend) and CD in human preadipocytes while (*E*) immunohistochemical staining with anti-SP antibody demonstrated an increased number of SP positive cells in whole mesenteric fat depots of UC and CD patients compared with controls. $^{*}P < .05$, $^{**}P < .01$, and $^{\#}P < .1$.

and Crohn's disease sections compared with the control patient sections (Figure 2*E*).

NK-1R and NK-2R mRNA Expression Is Increased in Human Mesenteric Preadipocytes From Inflammatory Bowel Disease Patients

We examined the expression levels of NK receptors in preadipocytes of control, UC, and Crohn's disease patients. Densitometric analysis of Western immunoblots demonstrated that Tacr-1 protein levels were increased in human mesenteric preadipocytes isolated from Crohn's disease patients, and there was a strong trend for increase in UC patient preadipocytes (Figure 2*D*, $P < .05$, n = 4). At the mRNA level, human preadipocytes isolated from UC and Crohn's disease patients expressed higher levels of the Tacr-1 receptor compared with the controls (Figure 2*B*, $P < .05$ and $P < .01$ for UC and Crohn's disease, respectively, n = 10–11, one extreme outlier was determined via Grubbs test). The levels of Tacr-2 were statistically significantly higher in the preadipocytes from Crohn's disease patients (Figure 2*C*, $P < .05$, n = 10–11) compared with the controls, although a trend for increased expression in the preadipocytes from UC patients was evident.

Substance P Induces Inflammation-Associated Cytokine mRNA Expression in Human Mesenteric Preadipocytes Isolated From Inflammatory Bowel Disease Patients

Previous studies from our group have demonstrated the ability of SP to induce inflammation-associated responses in preadipocytes and influence the metabolic responses of mice via the activation of intracellular signaling pathways in fat tissue.[30,32] Here we exposed human mesenteric preadipocytes from control, UC, and Crohn's disease patients to SP and examined their individual responses in the production of cytokines that may affect IBD pathophysiology. We observed that in preadipocytes from UC patients, SP increased the mRNA expression of IL-1β, IL-12B, regulated

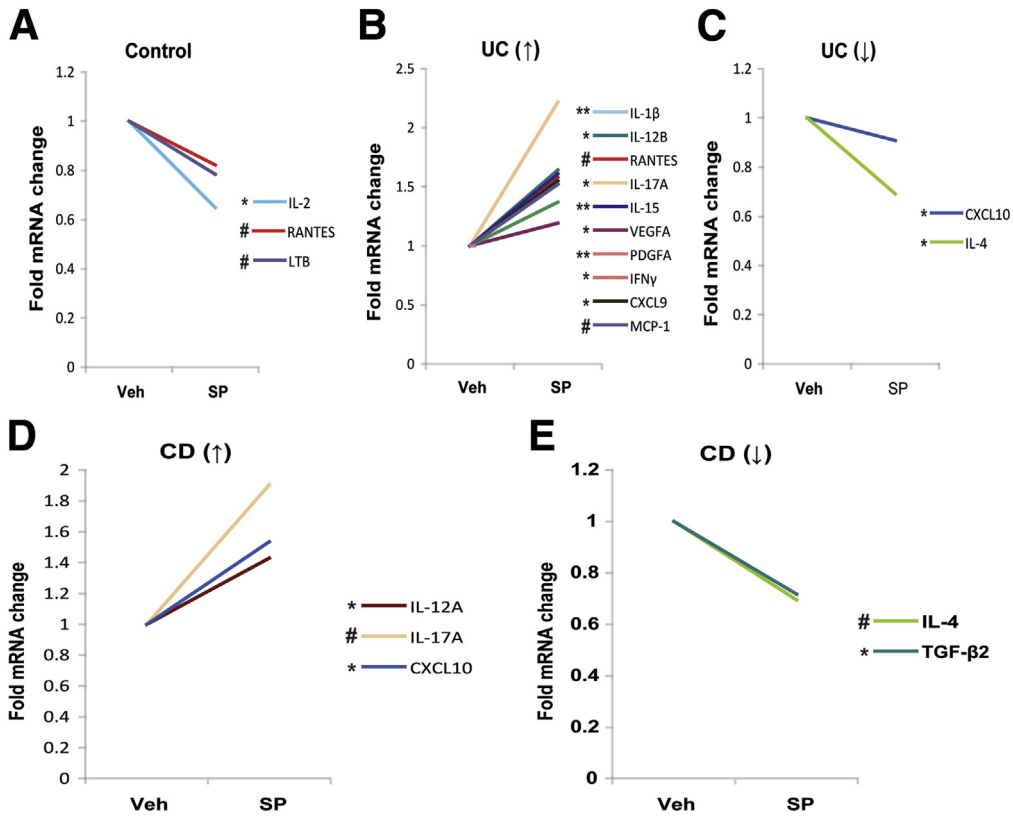

Figure 3. Substance P (SP) mediates cytokine mRNA expression from human mesenteric preadipocytes isolated from inflammatory bowel disease (IBD) patients. Human mesenteric preadipocytes were isolated from control and IBD patients and treated with SP for 8 hours. The total RNA was collected, and the cytokine mRNA levels were determined using real-time polymerase chain reaction. (*A*) SP decreased cytokine mRNA levels in mesenteric preadipocytes from control patients (C) and both (*B*) increased and (*C*) decreased cytokine expression in preadipocytes from ulcerative colitis (UC) patients. Preadipocytes isolated from Crohn's disease (CD) patients also demonstrated both (*D*) increased and (*E*) decreased cytokine mRNA expression after SP treatment. Despite the similarities in the responses to SP between the UC and Crohn's disease patient preadipocytes, they also demonstrated disease-dependent responses. Values are expressed as fold changes from untreated. $^{*}P < .05$, $^{**}P < .01$, and $^{\#}P < .1$.

on activation normal T-cell expressed and secreted (RANTES), IL-17A, IL-15, vascular endothelial growth factor A (VEGFA), platelet-derived growth factor subunit A (PDGFA), interferon γ (IFNγ), chemokine (C–X–C motif) ligand 9 (CXCL9), and monocyte chemotactic protein 1 (MCP-1) (Figure 3*B*) whereas the expression of CXCL10 and IL-4 was significantly decreased (Figure 3*C*, $P < .05$, n = 8). In preadipocytes isolated from Crohn's disease patients, SP treatment increased IL-12A, IL-17A, and CXCL10 mRNA levels (Figure 3*D*, n = 7) and decreased IL-4 and transforming growth factor β mRNA levels (Figure 3*E*, n = 7). In the control patient preadipocytes, IL-2, RANTES, and leukotriene B (LTB) mRNA expression (Figure 3*A*, n = 10) was reduced in response to SP treatment.

Substance P Induces Differential Inflammatory Bowel Disease–Dependent Cytokine mRNA Expression in Human Mesenteric Preadipocytes

We next investigated whether the higher NK receptor levels in IBD patient preadipocytes (Figure 2) reflect altered responsiveness to SP. Thus, we compared the fold difference changes in cytokine mRNA expression in isolated preadipocytes from control and IBD patients to examine whether their responsiveness to SP is significantly altered in IBD. We observed that several cytokines responded in a disease-dependent manner. The vast majority of responses demonstrated higher fold changes in cytokine expression in preadipocytes isolated from IBD patients compared with the controls. More specifically, we show five mediators that increase in response to SP only in preadipocytes isolated from UC patients (Figure 4*A*, $P < .05$ for IL-2, and IL-15; $P < .01$ for IL-17, VEGFA, and RANTES; and IL-17 also has a strong trend toward increased expression in Crohn's disease patients, $P < .1$, n = 7–8), three mediators that are increased only in preadipocytes from Crohn's disease patients (Figure 4*A*, $P < .05$ for LTB; $P < .01$ for IL-12A and CXCL10, and all mediators also show a trend for increase in UC, $P < .1$, n = 7–8), whereas for IL-12B, MCP-1, and CXCL9 we only observed a strong trend for increase in UC patient preadipocyte RNA ($P < .1$, n = 7–8). A mini–heat-map describing these changes in response to SP treatment

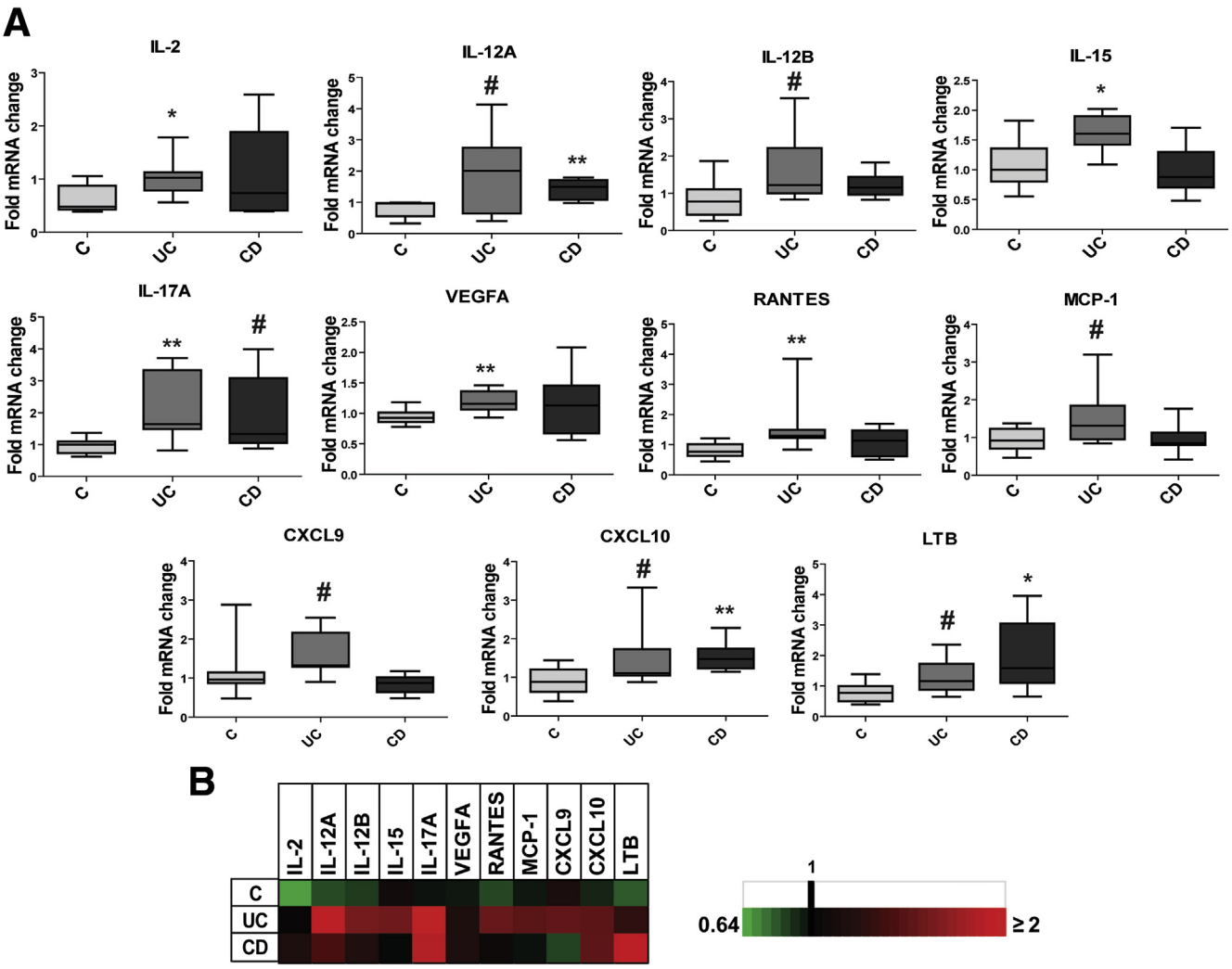

Figure 4. Human mesenteric preadipocytes isolated from inflammatory bowel disease (IBD) patients demonstrate increased responsiveness in cytokine mRNA expression compared with preadipocytes isolated from control patients. Mesenteric preadipocytes were isolated from control and IBD patients and exposed to substance P (SP) for 8 hours. Cytokine mRNA was measured using multiplex FlrxScript panels (42-plex), and the fold change in response to SP treatment was calculated for control (C), ulcerative colitis (UC), and Crohn's disease (CD) patient-derived preadipocytes. (A) SP treatment of UC and/or CD patient preadipocytes significantly increases the mRNA expression of IL-2, IL-12A, IL-12B, IL-15, IL-17A, VEGF, RANTES, MCP-1, CXCL9, CXCL10, and LTB compared with SP-treated preadipocytes from control patients. (*B*) Heat map depicting the changes in cytokine mRNA expression in SP-treated IBD and control patient preadipocytes. $^{*}P < .05$, $^{**}P < .01$, and $^{\#}P < .1$.

(including the strong trends toward change, $P < .1$) is also provided and includes the strong trends toward change (Figure 4*B*).

Substance P Induces Inflammation-Associated Cytokine Release in Human Mesenteric Preadipocytes Isolated From Inflammatory Bowel Disease Patients

At the protein level, in preadipocytes isolated from UC patients the SP treatment induced the release of IL-2, IL-17A, RANTES, PDGF-BB, macrophage inflammatory protein 1β (MIP-1β), and colony-stimulating factor 2 (CSF-2) (Figure 5*B*, n = 7) and inhibited the release of IL-6, IL-12p70, IL-13, VEGF, and eotaxin (Figure 5*C*, n = 7). Moreover, in Crohn's disease preadipocytes, SP induced the release of IL-1β, IL-2, IL-15, IL-17A, basic fibroblast growth factor (b-FGF), and MIP-1β (Figure 5*D*, n = 7) and inhibited the release of IL-7, IL-8, IL-10, IL-12p70, IL-13, and MIP-1α (Figure 5*E*, n = 7). Treatment of preadipocytes isolated from control patients with SP produced milder responses at both the mRNA and protein secreted levels. In preadipocytes isolated from control patients, IL-2, IL-17A, tumor necrosis factor α (TNFα), and IFNγ release (Figure 5*A*, n = 10) were reduced after SP treatment. These results indicate that mesenteric preadipocytes from Crohn's disease, UC, and control patients respond to SP by releasing different proinflammatory cytokines at the mRNA and protein level.

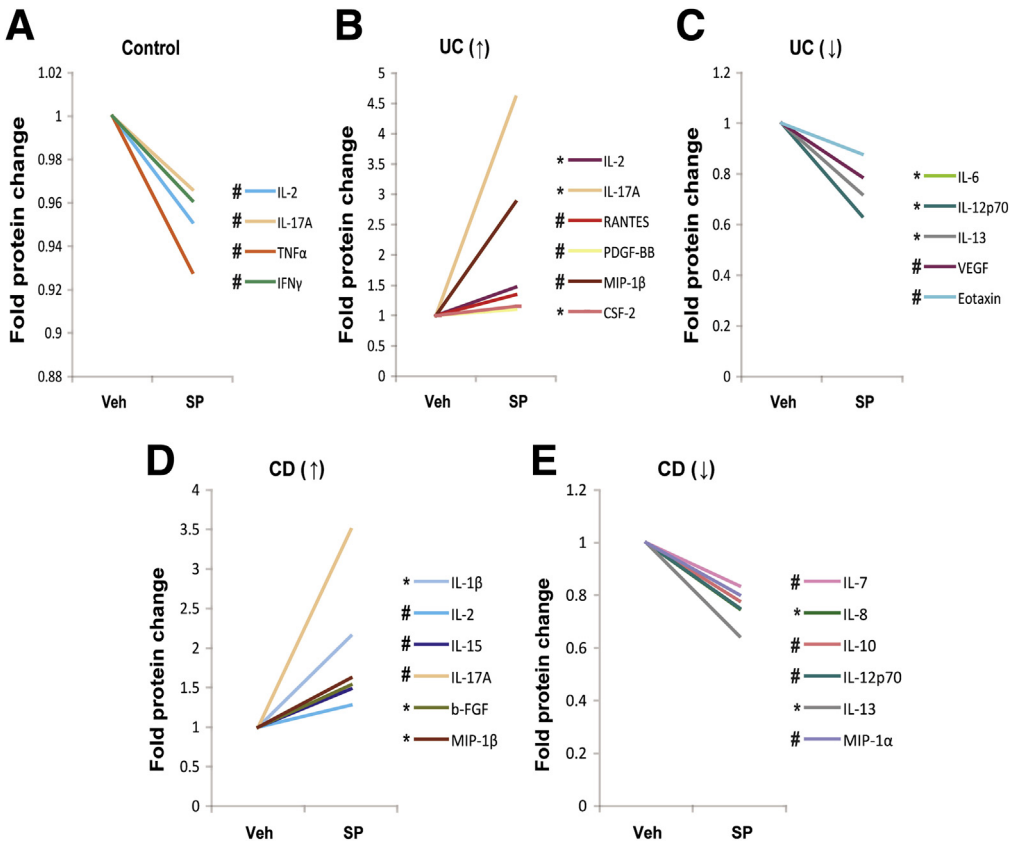

Figure 5. Human mesenteric preadipocytes from control and inflammatory bowel disease patients demonstrate different patterns of cytokine release in response to substance P (SP) treatment. Conditioned media were isolated from human mesenteric preadipocytes treated with SP. (*A*) In media from control patients, SP treatment decreased (strong trends) the release of cytokines from mesenteric preadipocytes. In media isolated from ulcerative colitis (UC) patients, SP both (*B*) promotes and (*C*) inhibits the release of cytokines from preadipocytes. In media isolated from Crohn's disease (CD) patients, SP also (*D*) induces the release of several inflammation-associated cytokines and (*E*) inhibits the release of a number of cytokines from mesenteric preadipocytes. The patterns of cytokine release between the conditioned media of UC and CD patients demonstrate similarities (IL-2, IL-17A, MIP-1β) but also differences, suggesting disease-specific contributions of preadipocytes in the course of these pathologic conditions. *$P < .05$, and #$P < .1$.

Substance P–Induced Cytokine Secretion Differs Significantly in Human Mesenteric Preadipocytes From Inflammatory Bowel Disease Patients Compared With Controls

We further analyzed the data included in Figure 5 to signify the potential disease-based differences in SP responsiveness at the protein level. Of the 27 cytokines tested in supernatants from SP-exposed UC and Crohn's disease preadipocytes, six cytokines showed statistically significantly increased secretion in both UC and Crohn's disease (Figure 6*A*, IL-1b, IL-2, IL-15, IL-17A, b-FGF, and MIP-1β, n = 7–11), and four showed an increased release or a trend toward increased release only in UC (Figure 6*A*, RANTES, PDGF-BB, TNF-α, and CSF-2, n = 7–11). Secretion of IL-13 and eotaxin in the supernatants of SP-exposed preadipocytes was either significantly decreased or had a trend toward significant decrease in both UC and Crohn's disease (Figure 6*A*, n = 7–11) compared with the controls. IL-12 was statistically significantly decreased in SP-treated UC preadipocytes (Figure 6*A*, n = 7–11), whereas IL-8 and IL-10 (Figure 6*A*, $P < .05$) were statistically significantly decreased in Crohn's disease preadipocytes after SP treatment, compared with the controls. A mini–heat-map depicting these changes in response to SP treatment (including the strong trends toward change, $P < .1$) is also provided (Figure 6*B*).

A modified Venn diagram (Figure 7) was created to summarize the mRNA and protein responses of preadipocytes from UC and Crohn's disease patients compared with the control patients to SP exposure and to highlight the similarities and differences between the two diseases. IL-17A was the sole mediator among 24 common molecules (between mRNA and protein panels used) that was increased after SP exposure of Crohn's disease and UC preadipocytes both in the mRNA and protein panels.

Preadipocytes Express Increased Interleukin-17A mRNA During Inflammatory Bowel Disease Whereas Interleukin-17 Receptor A mRNA Levels Are Increased in Colonic Biopsies of Inflammatory Bowel Disease Patients

Based on the proximity of mesenteric fat and the inflamed intestine during IBD, adipocyte-derived products

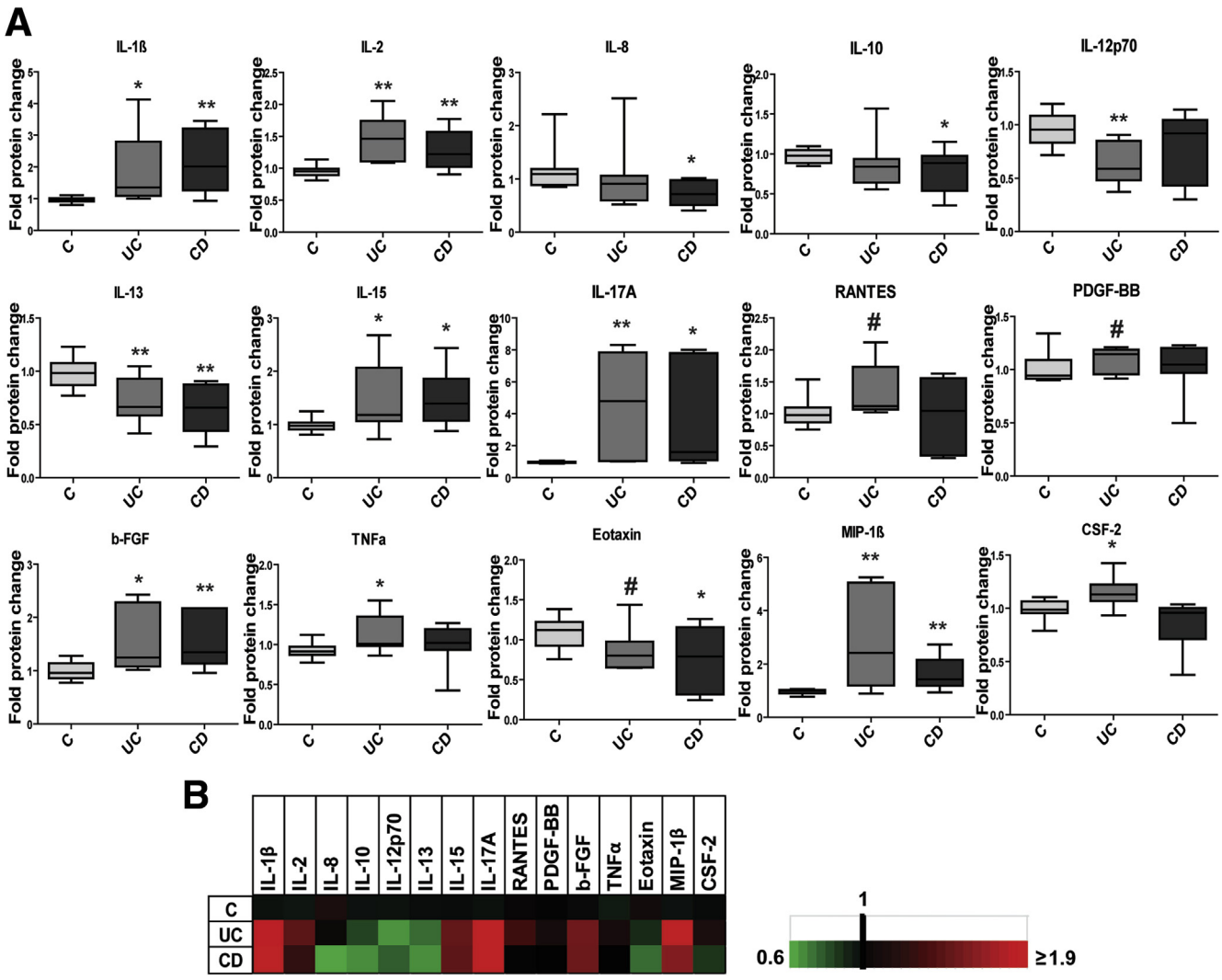

Figure 6. Human mesenteric preadipocytes isolated from inflammatory bowel disease patients demonstrate increased responsiveness in cytokine release compared with preadipocytes isolated from control patients. Mesenteric preadipocytes were isolated and cultured; the conditioned media were isolated after SP treatment, and the cytokines were measured using a multiplex magnetic assay kit (27-plex). (*A*) Substance P (SP) treatment induces a statistically significantly higher fold release of IL-1β, IL-2, IL-15, IL-17A, RANTES, PDGF-BB, b-FGF, TNFα (down-regulated in control), MIP-1β, and CSF-2 and significantly inhibits fold release of IL-8, IL-10, IL-12p70, IL-13, and eotaxin in preadipocytes isolated from ulcerative colitis (UC) and/or Crohn's disease patients compared with control (C) patients. (*B*) Heat map depicting the changes in cytokine release from SP-treated IBD and control patient preadipocytes. $^{*}P < .05$, $^{**}P < .01$, and $^{\#}P < .1$.

may reach the involved areas and affect the course of IBD. Moreover, our results showed that IL-17A is the only mediator modulated consistently after SP stimulation at both the mRNA and protein levels in both Crohn's disease and UC preadipocytes. As also shown in Figure 1*F*, mesenteric preadipocytes from IBD patients produce higher IL-17 protein levels even in the absence of SP stimulation. To investigate whether this change may potentially be important in the regulation of responses in the intestine during IBD, we examined the presence of IL-17 receptors in human colonocytes and compared their levels in colonic biopsy samples of IBD and control patients.

We first verified that IL-17RA (the high-affinity receptor for IL-17A) is expressed in significant amounts in NCM460 human colonic epithelial cells (data not shown). Furthermore, we examined the IL-17RA expression levels in colonic biopsy samples of control and IBD patients. We analyzed 19 non-IBD, 23 UC, and 30 Crohn's disease colonic biopsy samples, and we observed that IL-17RA is increased in biopsy samples from UC and Crohn's disease patients compared with the control samples (Figure 8, $P = .0693$ and $P < .05$, respectively). Expression levels of IL-17RA are shown separately for each patient, and the mean of the control samples was used as a cutoff value to signify high expression levels. Seven of 19 (36.84%) controls, 14 of 23 (60.86%) UC patients, and 19 of 30 (63.33%) Crohn's disease patients were above that cutoff value, signifying increased IL-17RA mRNA levels during IBD.

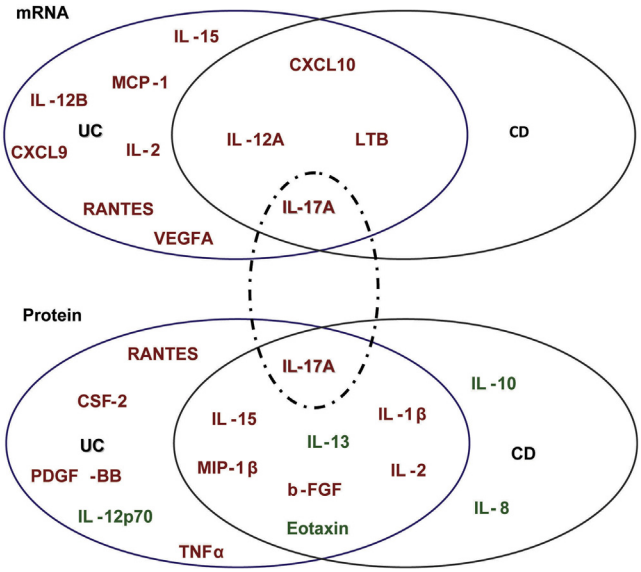

Figure 7. A Venn diagram summarizes the mRNA and protein responses of preadipocytes from ulcerative colitis (UC) and Crohn's disease (CD) patients compared with control patients to substance P (SP) exposure and highlights the similarities and differences between the two diseases. IL-17A is depicted as the mediator demonstrating the most consistent responses (both in Crohn's disease and UC and at both the mRNA and protein levels) to SP treatment in preadipocytes from inflammatory bowel disease compared with control patients.

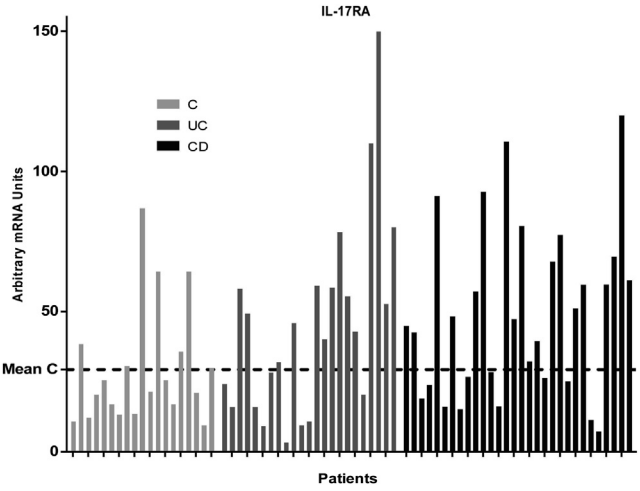

Figure 8. Increased interleukin 17 receptor (IL-17R) mRNA expression in human colonic biopsy samples during inflammatory bowel disease. Colonic biopsy samples were obtained from patients during colonoscopy and total RNA was isolated. Real-time polymerase chain reaction shows elevated IL-17RA mRNA levels in ulcerative colitis (UC) and Crohn's disease (CD) biopsy samples compared with controls (C).

Discussion

We found that mesenteric preadipocytes from control and IBD patients demonstrated differential mediator secretion patterns even after days in culture (Figure 1). Our results also indicate that SP exerts potent anti-inflammatory effects in preadipocytes from control patients compared with the mainly proinflammatory stimulation in cells from patients with IBD. This stark discrepancy indicates that components of the mesenteric fat depots have acquired disease-dependent characteristics, adding to the complexity of the factors that may contribute to the pathophysiology. Most importantly, our results (Figure 2) demonstrate that human mesenteric preadipocytes express Tac1 mRNA as well as both NK-1 and NK-2 receptors.

Our findings also show that preadipocytes isolated from IBD patients respond to SP in a considerably different manner from the controls, with profound differences in the responses between preadipocytes isolated from UC and Crohn's disease patients (Figures 3 and 5) possibly due to intrinsic characteristics these cells acquired during the course of IBD. Further analysis lead to the identification of an IBD-specific cytokine response pattern after exposure to SP (Figures 4 and 6). Overall, and despite the variability in patient backgrounds and potential treatments, we observed significant and quite consistent differences in the inherent ability of preadipocytes from patients from different disease groups to respond to SP. These responses can be proinflammatory and anti-inflammatory (at both the transcription and secretion levels), suggesting the potential involvement of mesenteric adipose tissue in disease manifestations and activity in the different phases of colitis.

We have shown that NK-1R is present in mouse mesenteric adipose tissue and that its expression is regulated during colitis.[30] However, this is the first evidence that expression of NK-1R is regulated differentially by IBD in preadipocytes, leading to specific inflammatory SP responses for UC or Crohn's disease. Previous observations suggested a possible role of creeping fat in the pathophysiology of Crohn's disease alone; however, ours is the first evidence that mesenteric adipose tissue may be involved in the pathophysiology of UC as well. This is an important observation because only Crohn's disease (and not UC) is associated with a creeping fat phenotype, suggesting different mechanisms for mesenteric fat activation in the two disease sates. Increased *Tacr-1* mRNA expression in IBD preadipocytes likely involves activation of nuclear factor κB (NF-κB) and binding of this transcription factor to sites at the promoter region of *Tacr-1*, shown to be important for its transcription.[37] SP alone can also increase transcription of *Tacr-1* in control mesenteric preadipocytes, as we previously showed elsewhere.[30]

The diverse differential responses described here in human mesenteric preadipocytes in response to SP during IBD are both proinflammatory and anti-inflammatory. Many of these molecules have been implicated in IBD pathophysiology, and their levels depend on disease activity and/or different cell populations involved in this group of diseases. For example, IL-1β polymorphisms are linked with IBD disease activity and phenotype, and IL-1β levels are elevated in serum and colonic biopsy samples of IBD and non-IBD colitis patients.[38] IL-12 and IL-15 are highly expressed in IBD, and

both cytokines represent potential therapeutic targets.[39,40] Antibodies against the p40 subunit of IL-12/23 are currently in clinical trials for IBD treatment.[41] IL-8 is increased in colonic intestinal epithelium in IBD and is a potent neutrophil attractant.[42] IL-2 polymorphisms seem to predispose to UC, and knockout animals for IL-2 or IL-10 are known to develop colitis.[43,44] IL-13 seems to have a protective role against colitis, and its levels are decreased in IBD colon biopsy samples of pediatric UC patients.[45,46] VEGF is also considered a susceptibility factor for IBD, linking angiogenesis with the development of colitis.[47] Biologic factors targeting TNFα are the most widely used and effective treatment for IBD currently.[48] Several of the cytokines demonstrating IBD-dependent responses to SP treatment in our study—eotaxin,[49] PDGF-BB, b-FGF,[50] CXCL9,[51] CXCL-10,[52] MIP-1β (CCL4),[53] RANTES (CCL5),[54] MCP-1 (CCL2),[30] CSF-2 (GM-CSF),[55,56] and LTB[57]—have been implicated in IBD pathophysiology. Collectively, the multitude of responsive mediators to SP in mesenteric preadipocytes from IBD patients highlights the potential magnitude of the involvement of mesenteric preadipocytes in IBD pathophysiology via regulation of inflammatory responses that may affect the involved intestine.

Mediators described here to be affected by SP treatment in human mesenteric preadipocytes from both UC and Crohn's disease patients have been implicated in the regulation of innate and adaptive immunity. In addition to their similarities, the combination of affected mediators by SP is mainly reminiscent of changes in macrophage responses in UC (IL-1β, IL-12, IL-15, CXCL10, GM-CSF, RANTES, MIP-1, IFNγ) and mostly resemble dendritic cell changes observed in Crohn's disease (IL-1β, IL-2, IL-12, IL-15, CXCL10, MIP-1) patient preadipocytes.[58,59] In both these cases, the described changes in response to SP can affect T-cell function (via IFNγ, IL-10, and IL4).[60,61]

Interestingly, a considerable number of SP-induced mediators in preadipocytes isolated from both UC and Crohn's disease patients (IL-1β, IL-12, IL-13, CCL2, CCL4) described here are downstream targets of IL-17A activation in macrophages, T helper cells, and intestinal epithelial cells.[62] Collectively, treatment of human preadipocytes isolated from UC and Crohn's disease patients with SP leads to the generation of responses that may be linked to inflammation, cellular development and proliferation, tissue development, connective tissue development and function, and hematologic tissue development and function, showing the potential involvement of SP and its signaling on preadipocytes on several aspects of IBD pathophysiology.

It is important to note that of all the inflammatory mediators that we were able to screen through in this study, IL-17A was the only one that exhibited consistent IBD-associated changes in mRNA expression and protein secretion levels in preadipocytes in response to SP treatment (schematically described in Figure 7) after SP stimulation compared with the controls. Previous studies indicated that IL-17 is regulated by SP in intestinal inflammatory T cells,[63] whereas an IL-23R haplotype, involved in the induction of IL-17A expression, is a risk factor for IBD.[5] Moreover, IL-17 levels are increased in colonic biopsy samples from UC and Crohn's disease patients.[64] Here, we present evidence that human mesenteric preadipocytes are a novel source of IL-17A, with higher expression during UC and Crohn's disease. In addition, human mesenteric preadipocytes from IBD patients have elevated NK-1R receptors and demonstrate increased expression and secretion of IL-17 in response to SP, and its receptor, IL-17RA, shows increased levels in colonic biopsy samples of IBD patients (Figure 8). Understandably, fat may not be the only source of IL-17 during colitis and may not reflect the cause behind the changes observed in the expression of IL-17RA in the gut during IBD. However, fat represents a novel reserve of IL-17 during the disease and may affect the progress of IBD by altering intestinal responses via interactions with IL-17RA.

This observation along with the identification of IL-17 as the most consistently regulated mediator in human mesenteric preadipocytes isolated from IBD patients in response to SP suggests a potential role for this neuropeptide in the regulation of inflammatory changes in the intestine during IBD (both UC and Crohn's disease) via modulation of IL-17 expression in the adjacent mesenteric preadipocytes. Furthermore, our observations introduce the preadipocytes as a novel cellular population with immune properties that are likely involved in the regulation of intestinal inflammation during IBD.

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The authors disclose no conflicts.

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