

CD40-Mediated Immune-Nonimmune Cell Interactions Induce Mucosal Fibroblast Chemokines Leading to T-Cell Transmigration

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Background & Aims: The CD40 pathway is a key mediator of inflammation and autoimmunity. We investigated cell adhesion molecule (CAM) up-regulation and chemokine production by CD40-positive human intestinal fibroblasts (HIF) and microvascular endothelial cells (HIMEC) induced by CD40 ligand (CD40L)-positive T cells and soluble CD40L and their effect on T-cell adhesion and transmigration. **Methods:** Expression of CD40, CD40L, and CAM was assessed by immunohistochemistry, confocal microscopy and flow cytometric analysis, and chemokine production using enzyme-linked immunosorbent assay. Calcein-labeled T cells were used to assay HIF adhesion and Transwell HIMEC transmigration. **Results:** Ligation of CD40-positive HIF and HIMEC by CD40L-positive T cells or soluble CD40L induced up-regulation of CAM expression as well as interleukin-8 and RANTES production. The specificity of these responses was shown by inhibition with a CD40L blocking antibody and by CD40 signaling-dependent p38 mitogen-activated protein kinase phosphorylation. On CD40 ligation, HIF increased their T-cell binding capacity and generated chemoattractants able to induce T-cell migration through HIMEC monolayers. **Conclusions:** Activation of the CD40/CD40L system in the gut mucosa may trigger a self-sustaining loop of immune-nonimmune cell interactions leading to an antigen-independent influx of T cells that contributes to chronic inflammation.

Both forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are characteristically chronic conditions that involve alternating periods of clinical exacerbation with remission.^{1,2} It is believed that IBD results from the interaction of genetic, environmental, microbial, and immune factors,³ but whether all of these factors come into play at once in the early stages of gut inflammation and continue to do so throughout the course of the disease or whether each plays a dominant but separate role at different stages of disease evolution is unknown. There is little doubt that IBD changes with time, as shown not only by its waver-

ing clinical manifestations but also its incremental therapeutic requirements.⁴ Even experimental IBD changes with time, switching from a typical Th1 pattern in early colitis to a Th2-like response in late disease.⁵

Progression from early to chronic gut inflammation is accompanied by multiple structural abnormalities in the affected bowel segments, where persistent infiltration by immune cells is associated with qualitative and quantitative changes of local nonimmune cells. Therefore, it is reasonable to assume that the cellular and molecular mechanisms underlying IBD vary during the course of the disease. In particular, it is possible that nonclassic immune cells (such as epithelial, endothelial, and mesenchymal cells) become increasingly more important in sustaining chronic inflammation. This notion, yet to be explored in IBD, has already gained wide acceptance in other chronic diseases.⁶ In rheumatoid arthritis, the concept of the vital contribution of T cell-independent, self-supporting pathways of synovial inflammation was put forward more than a decade ago.⁷ The interaction of synoviocytes with T and B cells perpetuates synovitis through complex paracrine and autocrine cytokine networks that lead to joint destruction.^{8,9} Another condition in which fibroblasts and other structural cells directly contribute to disease chronicity is airway inflammation, through the production of cytokines, chemokines, and growth factors.¹⁰ A chronic intestinal inflammatory condition in which nonimmune cells play a key pathogenic role is celiac disease.¹¹

Abbreviations used in this paper: CAM, cell adhesion molecule; CD40L, CD40 ligand; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; HIF, human intestinal fibroblasts; HIMEC, human intestinal microvascular endothelial cells; ICAM, intercellular cell adhesion molecule; IFN, interferon; IL, interleukin; LPT, lamina propria T cells; PBT, peripheral blood T cells; sCD40L, soluble recombinant CD40 ligand; VCAM, vascular cell adhesion molecule.

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Activated endothelial cells and fibroblasts are the prime source of tissue transglutaminases that selectively deaminate gliadin peptides, generating new epitopes recognized by mucosal T cells that proliferate, produce cytokines, and perpetuate inflammation.¹²

The complex cellular interactions brought to light in rheumatoid arthritis, chronic respiratory disease, celiac disease, and other chronic conditions likely occur in IBD, with a continuous interplay between immune and non-immune cells.¹³ Among the latter, mucosal endothelial cells and fibroblasts seem particularly important in fostering mucosal inflammation. Microvascular endothelial cells have long been recognized as gatekeepers of inflammation because they control both the quantity and quality of leukocytes transmigrating into the interstitium,¹⁴ and leukocyte-endothelial interactions have profound implications in gut inflammation.¹⁵ The fact that fibroblasts also play an active role in inflammation has finally been appreciated, and now fibroblasts are recognized as sentinel cells that not only modulate inflammation¹⁶ but actually regulate the switch from acute resolving to chronic persistent inflammation.¹⁷

Various molecular systems are in place that allow immune and nonimmune cells to exchange the signals that initiate or regulate inflammation. Among others, the CD40 receptor/CD40 ligand (CD40L; CD154) system has come under intense scrutiny because of the range and potency of its biological effects.^{18–20} Initially defined as a surface molecule present on activated CD4⁺ T cells inducing B-cell differentiation,²¹ CD40L was later shown to have a central regulatory role in cell-mediated immunity.¹⁸ After the discovery that essentially all types of immune and nonhematopoietic cells can express CD40 and that CD40L is expressed not only by activated CD4⁺ T cells, it became clear that the CD40/CD40L system plays a far broader role in immune regulation than previously recognized.¹⁹ In addition to T cells, B cells, and monocytic cells, CD40 is found on the surface of epithelial and endothelial cells as well as all types of mesenchymal cells, whereas CD40L is primarily expressed by activated CD4⁺ T cells and activated platelets.^{19,22} The distribution of CD40 and CD40L makes this molecular pair ideal to study the impact of activated T cells on nonimmune cells in disease states and, in fact, CD40 signaling is implicated in the pathogenesis of several chronic inflammatory conditions, such as atherosclerosis, graft-versus-host disease, and autoimmune disorders.^{20,23}

There is some evidence that the CD40/CD40L system is activated in IBD. The number of CD40- and CD40L-positive cells is increased in the intestine of patients with CD and UC,^{24–26} and CD40L-positive lamina propria T

cells (LPT) freshly isolated from IBD mucosa induce cytokine production by normal monocytes.²⁴ Various animal models of experimental IBD benefit from the administration of antibodies that disrupt CD40/CD40L interactions, including trinitrobenzene sulfonic acid-induced colitis, CD45RB^{high} cell transfer-induced colitis, tg ϵ 26-induced colitis, and the spontaneous colitis of C3H/HeJBir mice.^{27–30} However, none of these reports directly evaluated the molecular and biological effects of CD40 ligation on specific types of mucosal CD40-bearing cells or the mechanisms of these effects. Thus, we specifically investigated chemokine production and cell adhesion molecule (CAM) up-regulation by control and IBD CD40-positive human intestinal fibroblasts (HIF) and human intestinal microvascular endothelial cells (HIMEC) induced by CD40L-positive T cells and the impact of the CD40/CD40L system on T-cell adhesion and transmigration. The results show that on binding of CD40L-bearing T cells to CD40 on the surface of HIF and HIMEC, these cells up-regulate interleukin (IL)-8 (CXCL8) and RANTES (CCL5) production and intercellular cell adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 expression. In addition, HIF increase their ability to bind and retain T cells and produce bioactive chemoattractants that induce T-cell migration through HIMEC monolayers. Overall, these results suggest that activation of the CD40/CD40L system in the gut mucosa may induce a self-sustaining loop of immune-nonimmune interactions contributing to the persistence of IBD.

Materials and Methods

Isolation and Cultures of HIF and HIMEC

Surgical specimens used for isolation of HIF and HIMEC were all of colonic origin. Mucosal strips were obtained from histologically normal tissue of patients admitted for bowel resection due to malignant and nonmalignant conditions, including colon cancer, benign polyps, and diverticulosis, or tissue involved by CD or UC. All diagnoses were confirmed by clinical, radiologic, endoscopic, and histologic criteria. Isolation of HIF and HIMEC was performed as previously reported.^{31,32} Briefly, HIF were obtained as explants of surgically resected intestinal mucosa, grown to subconfluence in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS) and antibiotics, and then established as long-term cultures that were fed twice a week and split at confluence. Isolation of HIMEC consisted of enzymatic digestion of intestinal mucosal strips followed by gentle compression to extrude endothelial cell clumps, which adhered to fibronectin-coated plates and were subsequently cultured in MCDB131 medium supplemented with 20% FBS, antibiotics, heparin, and endothelial cell growth factor. Cultures of HIF and HIMEC were maintained at 37°C in 5% CO₂, fed twice a week, and split at

confluence. Both HIF and HIMEC were used between passage 3 and 10. The study was approved by the Institutional Review Board of University Hospitals of Cleveland.

Isolation, Culture, and Activation of T Cells

Depending on the experimental conditions, different T cells were used, including transformed T-cell lines (D1.1, Jurkat, and MOLT-4), freshly isolated peripheral blood T cells (PBT) and LPT, and IL-2-derived T-cell lines. CD40L-positive D1.1 T cells,²¹ CD40L-negative Jurkat T cells, and MOLT4 cells were obtained from American Type Culture Collection (Rockville, MD), cultured in RPMI 1640 with 10% FBS, and split and fed twice weekly.

Lamina propria mononuclear cells from the same mucosal specimens described in the preceding paragraph and peripheral blood mononuclear cells were isolated as previously described.³³ To obtain LPT and PBT, adherent cells were removed by plating 30×10^6 cells in RPMI 1640 (Biowhitaker, Walkersville, MD) with 5% FBS onto tissue culture dishes at 37°C and 5% CO₂ for 1 hour. Nonadherent cells were collected; incubated for 30 minutes at 4°C with magnetically labeled anti-CD19, CD14, and CD16 antibodies (Miltenyi Biotec Inc., Sunnyvale, CA) against B cells, monocytes, and neutrophils, respectively; and T cells negatively selected by magnetic activated cell sorting. Flow cytometric analysis showed that purified T cells were >97% CD3 positive.

To generate IL-2-derived T-cell lines, freshly isolated peripheral blood mononuclear cells were suspended at 10⁶/mL in RPMI 1640 with 10% FBS containing 1 ng/mL phorbol myristate acetate (Sigma Chemical Co., St. Louis, MO) and 0.02% phytohemagglutinin (Difco, Detroit, MI). After 48 hours, cells were rinsed twice and kept at 37°C in 5% CO₂ in RPMI 1640 supplemented with 10% FBS and 20 U/mL of human recombinant IL-2 (Chiron, Emeryville, CA). Cells were fed twice weekly and used 12–20 days later in the transmigration assay (see following text). At that time, the T-cell lines were composed of >96% CD3-, CD4-, CD8-, TCR $\alpha\beta$ -positive cells and their viability exceeded 95%.³⁴

To induce CD40L expression by PBT and LPT, cells were activated for variable periods (3–48 hours) with a combination of phytohemagglutinin (0.02%) and phorbol myristate acetate (1 ng/mL), cross-linked CD3 monoclonal antibody (10 μ g/mL) (Ortho Diagnostic Systems, Raritan, NJ), cross-linked CD3 with soluble CD28 monoclonal antibody (1 μ g/mL) (Ansell Corp., Bayport, MN), or polystyrene beads coated with murine monoclonal antibodies to human CD3 and CD28 (DYNAL, Lake Success, NY).³⁵

Evaluation of CD40 Protein Expression by Immunohistochemistry and CD40L by Fluorescent Confocal Microscopy

For identification of CD40-expressing cells in intestinal tissues, paraffin-embedded sections of histologically normal control and IBD-involved colonic mucosa were cut at 3- μ m thickness, deparaffinized and then hydrated, blocked for endogenous peroxidase using 3% H₂O₂/H₂O, and subse-

quently subjected to microwave epitope enhancement using a Dako Target retrieval solution (Dako, Carpinteria, CA), pH 10.00. Incubation with the primary CD40 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was performed at 1:200 dilution for 30 minutes at room temperature. Detection was achieved using a standard streptavidin-biotin system (Dako), and antigen localization was visualized with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.).

For identification of CD40L-expressing cells in intestinal tissues, paraffin-embedded sections of colonic mucosa were cleared and rehydrated in sequential incubations of Clear Rite 3 (2 \times 3 minutes), Flex 100 (1 minute then 2 minutes), Flex 95 (1 minute then 2 minutes) (all reagents from Richard Allen Scientific, Kalamazoo, MI), and finally tap water (1 minute). The tissue sections were preincubated with Hank's balanced salt solution (HBSS) containing 2% FBS for 30 minutes at 25°C. After removing the medium, the sections were incubated with a solution containing a CD40L-directed monoclonal antibody (10 μ g/mL) (BD Pharmingen, San Diego, CA) in HBSS containing 2% FBS for approximately 16 hours at 4°C. The sections were washed 3 times with HBSS and then incubated with a solution containing Alexa 568 conjugated anti-mouse immunoglobulin (Molecular Probes, Eugene, OR) at 1:1000 in HBSS containing 2% FBS. This secondary incubation was performed for 1 hour at 25°C. The sections were washed 3 times in HBSS and coverslips mounted to the slides in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc.). The slides were then sealed with nail polish and stored at -20°C until microscopic observations could be performed.

Confocal images were obtained using a Leica TCS-SP laser scanning confocal microscope (Leica, Heidelberg, Germany), which is equipped with 3 lasers and photodetectors that permit detection of distinct fluorochromes.

Evaluation of CD40 Messenger RNA Expression by Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from HIF and HIMEC monolayers using the guanidinium thiocyanate method described by Chomczynski and Sacchi,³⁶ quantified in a Beckman DU 640 spectrophotometer, and stored in 3 \times ethanol at -80°C. One-microgram aliquots of total RNA were reverse transcribed and CD40 messenger RNA (mRNA) amplified using the following primers: sense, 5'-ATGGTTCGTCTGCCTCTGCAG-3'; antisense, 5'-CGTACAGTGCCAGCCTTCTTC-3'.³⁷ The thermal cycle commenced with a hot start at 94°C for 5 minutes followed by 30 cycles each consisting of 94°C for 30 seconds, annealing for 30 seconds at 63°C, and extension at 72°C for 60 seconds and terminated after a final 10-minute period at 72°C. The products were run on a 1% TAE agarose gel with 0.25 μ g/mL ethidium bromide, and β -actin was used for loading control.

Flow Cytometric Analysis for Evaluation of CD40, CD40L, and CAM Expression

HIF and HIMEC were grown to subconfluence and then kept in culture medium with and without 500 U/mL interferon (IFN)- γ (R&D Systems, Minneapolis, MN). After 24, 48, or 72 hours, the confluent monolayers were thoroughly rinsed with HBSS and harvested after 2–5 minutes of treatment with 0.5% trypsin/ethylenediaminetetraacetic acid. In some experiments, the monolayers were washed thoroughly to remove IFN- γ and then cultured in fresh medium for an additional 24–72 hours before harvest. Harvested HIF or HIMEC were washed twice with cold phosphate-buffered saline containing 1.0% bovine serum albumin and 0.1% sodium azide and then suspended in 0.1 mL of wash buffer containing 1.0 μ g of purified mouse anti-human CD40 (BD Pharmingen) or 1.0 μ g of isotype control antibody (Dako). After a 30-minute incubation on ice, the cells were washed and resuspended in 0.1 mL wash buffer containing fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (Biosource, Camarillo, CA). Following an additional 30-minute incubation in the dark on ice, HIF and HIMEC were washed again, fixed with 0.5% paraformaldehyde, and analyzed by flow cytometry (Beckman Coulter, Miami, FL). Control, CD, and UC cells were used for studies of CD40 and CAM expression (see following text). Because no differences were noted between CD and UC HIF or HIMEC, these cells are referred to as IBD cells.

To assess CD40L expression, 1×10^6 T cells were washed and suspended in 0.1 mL wash buffer containing fluorescein isothiocyanate-conjugated mouse anti-human CD40L or isotype control antibody (BD Pharmingen), incubated in the dark on ice for 30 minutes, and then washed, fixed with 0.5% paraformaldehyde, and analyzed by flow cytometry.

To evaluate HIF and HIMEC ICAM-1 and VCAM-1 expression, cells were submitted to the same protocol used for CD40 assessment, including a 48-hour incubation with IFN- γ , followed by addition of irradiated (6000 rads) D1.1 cells, Jurkat cells, or 1 μ g/mL of soluble recombinant CD40L (sCD40L) (Immunex Corp., Seattle, WA). A 10:1 ratio of T cells to HIF or HIMEC was used, and in some experiments a blocking antibody to CD40L (M90; Immunex Corp.) was added to the cocultures at 4 μ g/mL. Cultures were incubated for 48 hours, washed to remove T cells, harvested, washed, and incubated with buffer containing 1.0 μ g of purified mouse anti-human ICAM-1 (provided by Pharmacia & Upjohn, Kalamazoo, MI) or 1.0 μ g of purified mouse anti-human VCAM-1 (BD Pharmingen) on ice. This was followed by incubation with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G and analysis by flow cytometry as previously described.

Induction of Chemokine Production by HIF and HIMEC

Control or IBD cells were seeded in 24-well cluster plates at 3×10^4 /well/mL of their respective medium and

grown to subconfluence when 500 U/mL IFN- γ (R&D Systems) or fresh medium was added. After 48 hours of incubation, the confluent monolayers were extensively washed and T cells at various T-cell:HIF/HIMEC ratios were added in fresh medium containing 2% FBS. In some wells, IL-1 β , tumor necrosis factor α , lipopolysaccharide (Sigma Chemical Co.), or sCD40L were added instead of the T cells as stimulatory controls. In other wells, a blocking antibody to CD40L (M90) was used to hinder the CD40/CD40L interaction. Stimulation was allowed to continue for an additional 72 hours, at which point T cells were removed by centrifugation and supernatants were harvested for storage at -20°C . Chemokine content in the supernatants was measured by enzyme-linked immunosorbent assay (ELISA) for IL-8 and RANTES (R&D Systems).

Western Blotting

Using the experimental conditions described for induction of chemokine production, confluent HIF and HIMEC monolayers were left alone or stimulated with sCD40L, D1.1, or Jurkat cells for 0, 5, 15, 30, and 60 minutes. In preliminary experiments using visual inspection and flow cytometric analysis, it was determined that after 4–5 washings with cold phosphate-buffered saline, virtually all D1.1 or Jurkat cells were removed from the monolayers and that a 15-minute coculture was optimal for phosphorylation of the protein of interest. Protein extraction was obtained using a lysing buffer containing 50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 10% glycerol, 1% Triton X-100, and 50 mmol/L protease plus 50 mmol/L phosphatase inhibitor cocktail (Sigma Chemical Co.). The concentration of proteins in each lysate was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Immunoblotting was performed as previously described.³⁸ Equivalent amounts of proteins (20 μ g) were fractionated on a 10% Tris-glycine gel and electrotransferred to a nitrocellulose membrane (Novex, San Diego, CA). Nonspecific binding was blocked by incubation with 5% milk in 0.1% Tween 20/Tris-buffered saline (Fisher Scientific, Hanover Park, IL), followed by overnight incubation at 4°C with the primary anti-phospho-p38 mitogen-activated protein kinase polyclonal antibody (Cell Signaling, Beverly, MA). Membranes were washed 6 times with 0.1% Tween 20/Tris-buffered saline, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), washed again, and incubated with the chemiluminescent substrate (Super Signal; Pierce, Rockford, IL) for 5 minutes, after which they were exposed to film (Amersham, Arlington Heights, IL).

Adhesion Assay

The adhesion assay was performed as previously described with some modifications.³⁹ Briefly, 5×10^4 HIF were plated onto 24-well cluster plates (Costar, Corning, NY); after 24–48 hours, the resulting monolayer was fed with fresh medium alone or containing 500 U/mL IFN- γ (R&D Systems). After 48 hours, irradiated D1.1 or Jurkat cells were added at a 10:1 ratio (T cells:HIF) or 1 μ g/mL of sCD40L was

added. In some experiments, the blocking antibody to CD40L (M90) was added to the cocultures. Monolayers were incubated overnight, rinsed extensively to remove all T cells or the SCD40L, and then overlaid with 1 mL of medium containing 10^6 calcein-labeled (Molecular Probes) MOLT-4 cells. After 1 hour of HIF-MOLT-4 coculture at 37°C , the wells were gently rinsed 4 times with calcium- and magnesium-containing phosphate-buffered saline to remove all nonadherent T cells. Fluorescent adherent T cells were quantified by an imaging system (Image Pro Plus; Media Cybernetics, Silver Spring, MD) connected to an Optronics Color digital camera (Olympus) on an inverted fluorescence microscope. All experiments were performed in duplicate wells and results expressed as adherent cells/ mm^2 .

T-Cell Transmigration Assay

A modification of the T-cell transmigration system described by Roth et al. was used.⁴⁰ The method is based on a cluster Transwell plate containing polycarbonate porous filter inserts (3402; Costar) separating the upper and lower chambers. In preliminary experiments using membranes with different pore sizes (3–12 μm), it was determined that a 3- μm pore allowed the most discriminatory, gradient-dependent transmigration. A HIMEC monolayer was established on the upper chamber filter by seeding 75×10^3 HIMEC in MCDB131 medium containing 20% FBS.³² Monolayers were grown for 7–10 days until complete confluence was reached and verified by microscopic evaluation of the histochemically stained (Diff-Quick Stain Set; Dade Diagnostics, Aguada, Puerto Rico) monolayer. Pretreatment of the monolayer with 0.1% sodium azide drastically reduced the number of T cells recovered in the lower chamber, indicating the need for live and metabolically active HIMEC for transmigration to occur.

On the day of the assay, the HIMEC monolayer was thoroughly rinsed with MCDB131 medium to remove all serum. T cells were generated as IL-2–derived peripheral blood T-cell lines,³⁴ suspended at $2 \times 10^6/\text{mL}$ in phosphate-buffered saline with 5% FBS, and labeled at 37°C with 4 $\mu\text{mol/L}$ calcein (Molecular Probes). After 20 minutes, T cells were rinsed twice, resuspended in transmigration medium consisting of 50% RPMI 1640 and 50% MCDB131 medium containing 0.5% bovine serum albumin at $2 \times 10^6/\text{mL}$, and added to the upper chamber at 1×10^6 cells/0.5 mL/insert. The insert was placed in a well of the cluster plate containing 1.5 mL of transmigration medium (50% RPMI 1640 and 50% MCDB131 with 0.2% bovine serum albumin), HIF-conditioned medium, or optimal concentration of recombinant IP-10 or RANTES in transmigration medium.^{41,42} HIF-conditioned medium was generated using the same protocol for HIF chemokine production (see previous text) except that HIF stimulation was performed in transmigration medium. In preliminary experiments using various incubation times (0.5–6 hours), a 4-hour period was optimal for an efficient T-cell transmigration; all subsequent experiments were performed with this time interval.

Plates were incubated at 37°C in 5% CO_2 for 4 hours, after which the inserts were removed and the bottom surface of the filter was gently scraped to recover cells that had not completely migrated into the lower chamber. The cell suspension in the lower chamber was allowed to settle, and fluorescent cells were quantified using the same imaging system described for the adhesion assay. To assess dose dependency, supernatants were serially diluted or concentrated using a 3000 molecular weight cutoff Centricon filter (Millipore Corp., Bedford, MA). To control for chemokinesis, in some experiments T cells in the upper chamber were suspended in the same medium of the lower chamber (recombinant chemokines or HIF-conditioned medium).

Analysis of Data

Statistical analyses were performed using Student *t* test, Kruskal–Wallis test, and Dunn's and Bonferroni's multiple comparison tests. Results are expressed as mean \pm SEM, and significance was inferred at $P < 0.05$.

Results

Expression of CD40 and CD40L in Normal and IBD-Involved Colonic Mucosa

Immunohistochemistry for CD40 of histologically normal colonic tissue from control subjects showed staining of some mononuclear cells in the lamina propria and weak staining of microvascular endothelial cells in the submucosa (Figure 1A). Muscularis mucosae and mesenchymal cells were negative for CD40. In actively inflamed CD tissue, there was strong and diffuse CD40 staining of mononuclear, endothelial, and mesenchymal cells in the mucosa and submucosa (Figure 1B), and the same cells were also stained in actively inflamed UC tissue (Figure 1C). Confocal microscopy of control normal mucosa detected only occasional CD40L-positive mononuclear cells in the lamina propria (Figure 1D), but these were abundantly distributed in CD and UC tissue samples (Figure 1E and F).

Induction and Modulation of CD40 Expression by HIF and HIMEC

To determine whether T cells could interact with nonimmune cells through the CD40-dependent pathway, we initially investigated the expression and modulation of CD40 by HIF and HIMEC.

As determined by reverse-transcription polymerase chain reaction, unstimulated cultures of both HIF and HIMEC contained low levels of CD40 mRNA that substantially increased on treatment with IFN- γ (Figure 2). These results were confirmed by flow cytometric analysis showing that unstimulated HIF cultures displayed low levels of CD40 surface expression that were progressively

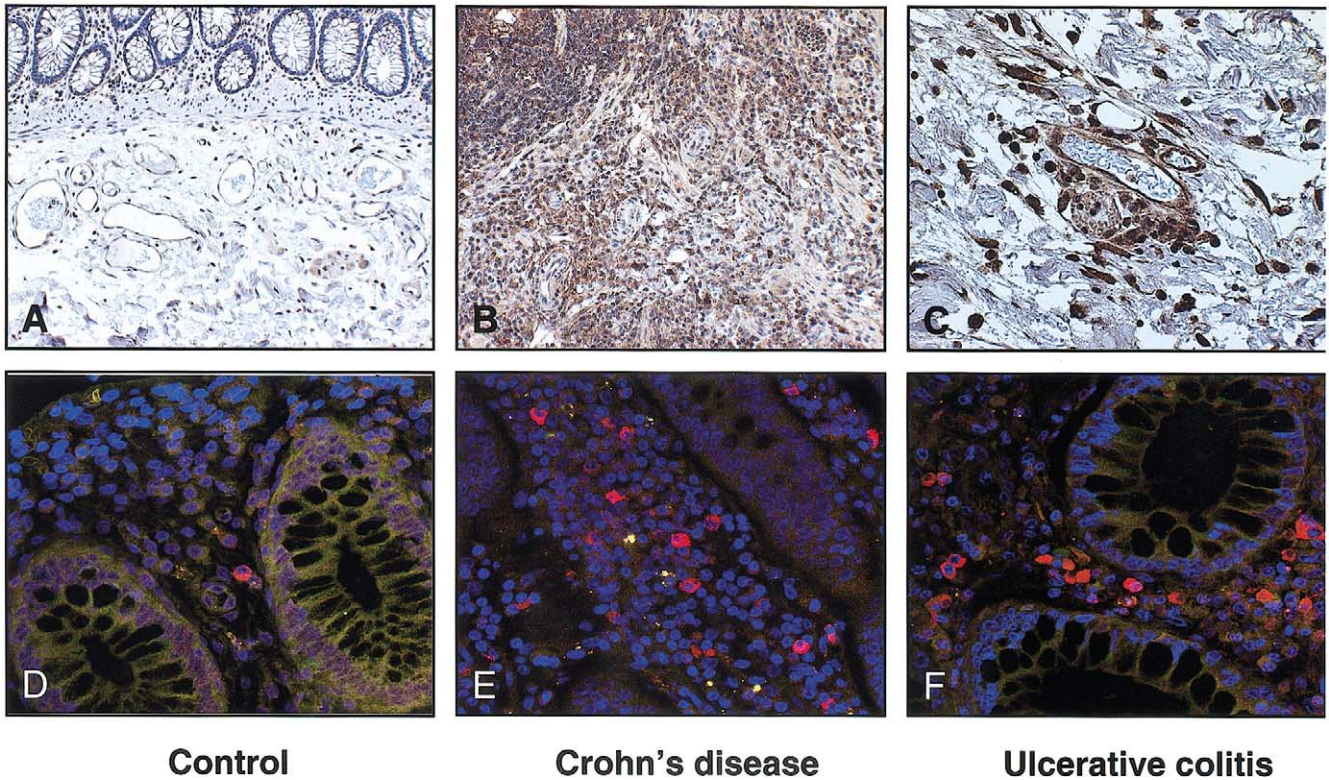


Figure 1. Expression of CD40 and CD40L in normal and IBD-involved colonic tissue. (A–C) CD40 immunohistochemical staining of colonic mucosa and submucosa from (A) controls, (B) patients with CD, and (C) patients with UC. (Original magnification: A and B, 100 \times ; C, 200 \times .) (D–F) Confocal micrographs of colonic mucosa from (D) controls, (E) patients with CD, and (F) patients with UC, where the red color in the tissue sections indicates CD40L and the blue color (4',6-diamidino-2-phenylindole) indicates nuclei. A–C are representative of 5 control, 7 CD, and 4 UC samples, and D–F are representative of 3 control, 3 CD, and 3 UC samples.

up-regulated on exposure to IFN- γ for up to 72 hours, when maximal expression was reached (Figure 3). Exposure of HIF to other proinflammatory stimuli, such as IL-1 β , tumor necrosis factor α , or lipopolysaccharide, did not significantly increase CD40 expression (not shown). When tumor necrosis factor α and IFN- γ were

added concomitantly, this only resulted in a marginal increase of CD40 expression over that induced by IFN- γ alone, whereas a combination of IFN- γ with lipopolysaccharide or IL-1 β did not lead to any further enhancement (not shown). Once maximal expression was achieved at 72 hours, HIF continued to express high levels of CD40 for at least an additional 72 hours even after removal of IFN- γ . Similarly to HIF, HIMEC also expressed low spontaneous levels of CD40 that were up-regulated by IFN- γ and maintained for at least 72 hours on removal of IFN- γ (Figure 3). The expression level and response to cytokine modulation of CD40 was comparable in control and IBD HIF and HIMEC.

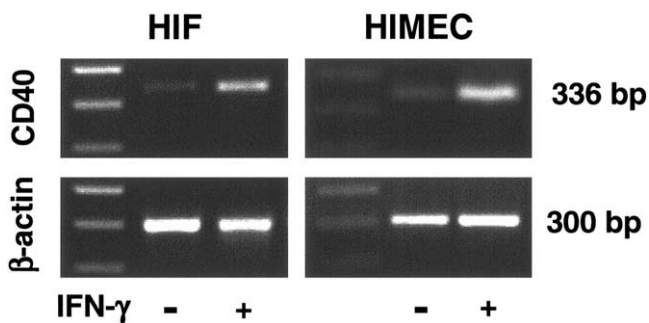


Figure 2. Expression and modulation of CD40 mRNA by HIF and HIMEC. Unstimulated HIF and HIMEC express low levels of CD40 mRNA, which is markedly up-regulated on exposure to IFN- γ . HIF and HIMEC monolayers were cultured in the absence (–) and presence (+) of IFN- γ for 24–72 hours, and total RNA was extracted and submitted to reverse-transcription polymerase chain reaction amplification using CD40 and β -actin-specific primers. This figure is representative of 4 separate experiments with control cell lines.

Induction and Modulation of CD40L Expression by T Cells

Having shown the expression of CD40 by non-immune cells, we next assessed the expression of CD40L by T cells to be used for induction of chemokine and CAM by HIF and HIMEC. Flow cytometric analysis confirmed that D1.1 cells spontaneously express high levels of CD40L, whereas this was absent in Jurkat cells (Figure 4).²¹ Freshly isolated unstimulated PBT did not

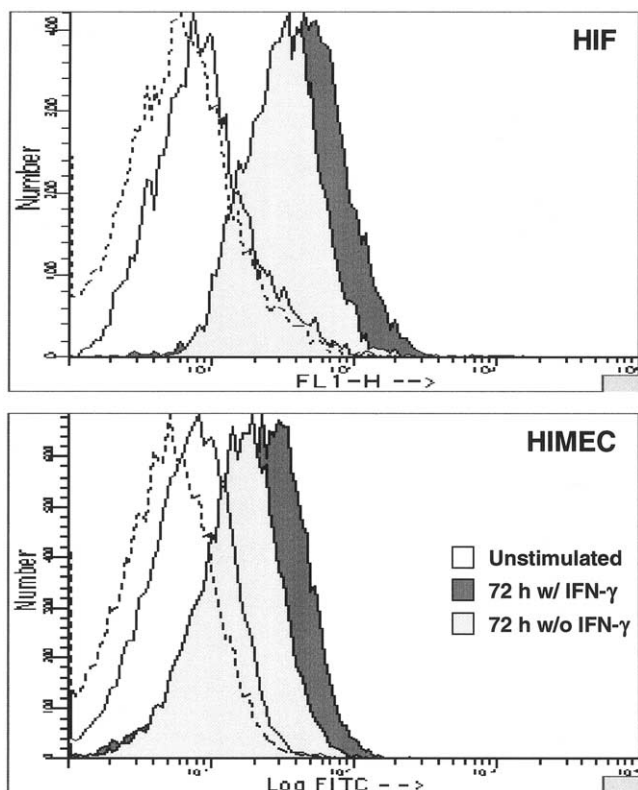


Figure 3. Expression and modulation of surface CD40 by HIF and HIMEC. Flow cytometric analysis shows that unstimulated HIF and HIMEC display low levels of CD40 surface expression that is markedly up-regulated by exposure to IFN- γ and remains elevated after removal of IFN- γ . HIF and HIMEC monolayers were cultured in the absence and presence of IFN- γ for 72 hours. Identical cultures were started 3 days earlier; at 72 hours, the monolayers were washed to remove IFN- γ and cultured for an additional 72 hours. All flow cytometric analyses for CD40 were performed at the same time for the 72- and 144-hour cultures. The *dashed line* represents the isotype control. This figure is representative of 6 separate experiments with 4 control and 2 IBD cell lines.

express detectable levels of CD40L (Figure 4). Stimulation by a combination of phytohemagglutinin and phorbol myristate acetate or cross-linked anti-CD3 antibody, with and without soluble CD28 antibody, induced only low and transient levels of CD40L (not shown). To maximize the level and extend the duration of CD40L expression by PBT, anti-CD3/CD28-coated beads were used for stimulation.³⁵ This resulted in high levels of CD40L as early as 6 hours that lasted for at least 48 hours (Figure 4). Similarly, activation of LPT by anti-CD3/CD28-coated beads resulted in substantial CD40L up-regulation of comparable magnitude as that of PBT (Figure 4).

Induction and Modulation of CAM Expression by HIF and HIMEC

To determine the effect of CD40-dependent activation on HIF and HIMEC expression of CAM, cell

monolayers were cultured in the presence of sCD40L or D1.1 cells. Flow cytometry of suspended cells showed that unstimulated or IFN- γ -treated HIF expressed ICAM-1, which was further up-regulated when sCD40L was added to IFN- γ -pretreated HIF (Figure 5). HIMEC also spontaneously expressed ICAM-1, which was up-regulated by IFN- γ and further enhanced by the addition of sCD40L (Figure 5). When the same experimental conditions were used to study the expression of VCAM-1 by HIF and HIMEC, results comparable to those seen for ICAM-1 were observed (Figure 5).

To verify whether the modulatory effect of sCD40L on CAM expression could be reproduced by cell-bound CD40L, parallel experiments were performed by exposing the same HIF and HIMEC cultures to D1.1 cells in the presence or absence of CD40L blocking antibody. Addition of D1.1 cells to IFN- γ -pretreated HIF caused a substantial up-regulation of both ICAM-1 and VCAM-1 expression. The dependency of this effect on the CD40 pathway was confirmed by a nearly complete blockade of D1.1 cell-induced up-regulation when the M90 antibody was added (Figure 6). The response pattern of HIMEC to CAM modulation by D1.1 cells was comparable to that observed with HIF, with a marked increase in VCAM-1 levels that was also CD40 dependent (Figure 6). No differences between control and IBD HIF or HIMEC were observed in regard to the induction and modulation of ICAM-1 and VCAM-1.

CD40L Dose-Dependent Chemokine Production by HIF and HIMEC

Because one of the aims of this investigation was to determine whether HIF and HIMEC can produce IL-8 and RANTES when activated through the CD40/CD40L pathway, experiments were performed to establish optimal stimulatory conditions by sCD40L and D1.1 cells. D1.1 cells produce a small amount of IL-8 and RANTES before or after exposure to IFN- γ (averaging 100 and 40 pg/mL, respectively). Unstimulated and IFN- γ -treated HIF produced low levels of IL-8 and small amounts of RANTES (Figure 7). A dose-dependent increase in IL-8 and RANTES production was observed in IFN- γ -pretreated HIF exposed to increasing concentrations of sCD40L (Figure 7). A dose-dependent increase in chemokine production was also observed in IFN- γ -pretreated HIMEC that, however, produced substantially higher levels of IL-8 and RANTES than HIF (Figure 7). Of note, spontaneous IL-8 production was much higher in HIMEC than in HIF, and CD40 ligation resulted in a relatively more pronounced increase in RANTES than IL-8 production. When increasing numbers of D1.1 cells were used instead of sCD40L, a dose-dependent increase

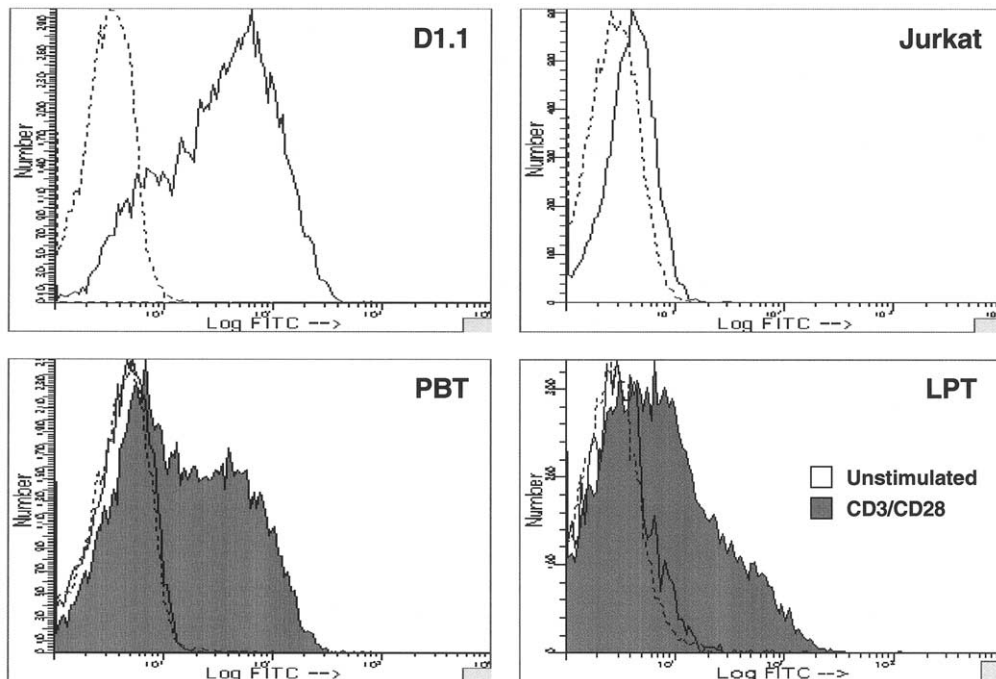


Figure 4. Expression and modulation of surface CD40L by D1.1 and Jurkat cells, PBT, and LPT. Flow cytometric analysis shows that D1.1 cells spontaneously display high levels of CD40L surface expression that is absent in Jurkat cells. Unstimulated PBT and LPT do not express CD40L, which is induced after costimulation of the CD3 and CD28 receptors. Flow cytometric analysis for CD40L was performed at 0 hours and 3–48 hours after co-culture with anti-CD3/CD28-coated beads. The *dashed line* represents the isotype control. This figure is representative of 3 separate experiments with control cells.

in chemokine production was also observed in both HIF and HIMEC (Figure 8). Compared with cell-free CD40L, cell-bound CD40L was far more effective in inducing production of IL-8 and RANTES from HIF but equally effective in inducing the same chemokines from HIMEC (Figures 7 and 8).

CD40-Dependent Chemokine Production by HIF and HIMEC

To show that D1.1 cell-mediated chemokine production by HIF and HIMEC was truly CD40 dependent, a series of experiments was performed aimed at blocking CD40-CD40L interaction. In addition, Jurkat cells were

Figure 5. Soluble CD40L-induced enhancement of CAM expression by HIF and HIMEC. Flow cytometric analysis shows that unstimulated control HIF and HIMEC display moderate to high levels of ICAM-1 and VCAM-1 surface expression that are up-regulated by IFN- γ and further enhanced by addition of sCD40L to IFN- γ (except for HIMEC ICAM-1). HIF and HIMEC monolayers were cultured in the absence and presence of IFN- γ or sCD40L plus IFN- γ for 48 hours. The *dashed line* represents the isotype control. This figure is representative of 6 separate experiments with 4 control and 2 IBD cell lines.

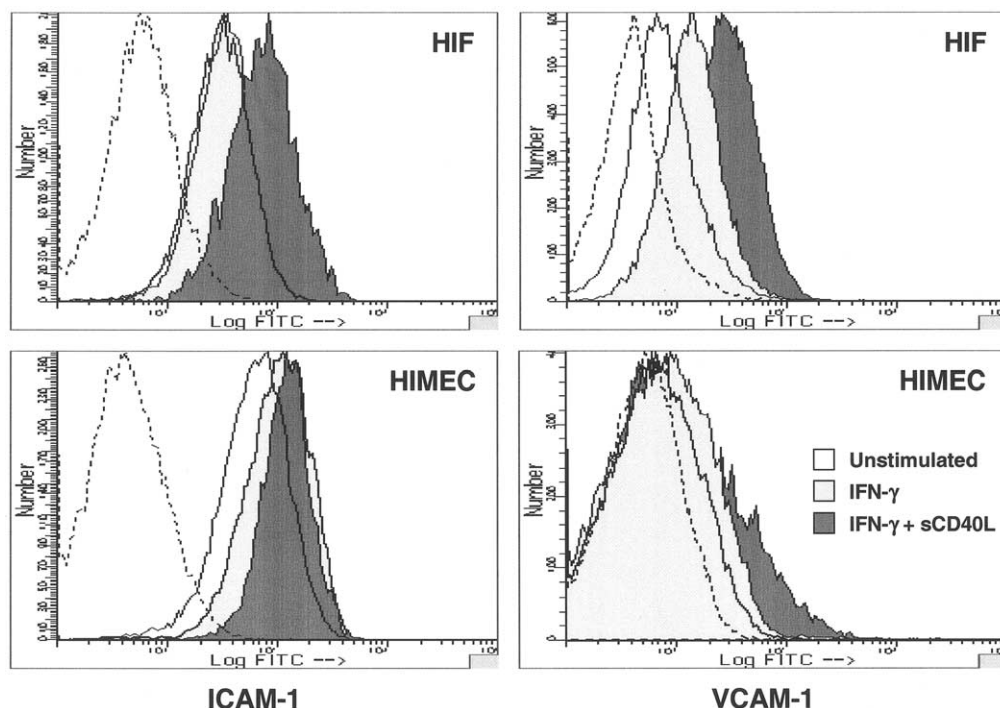
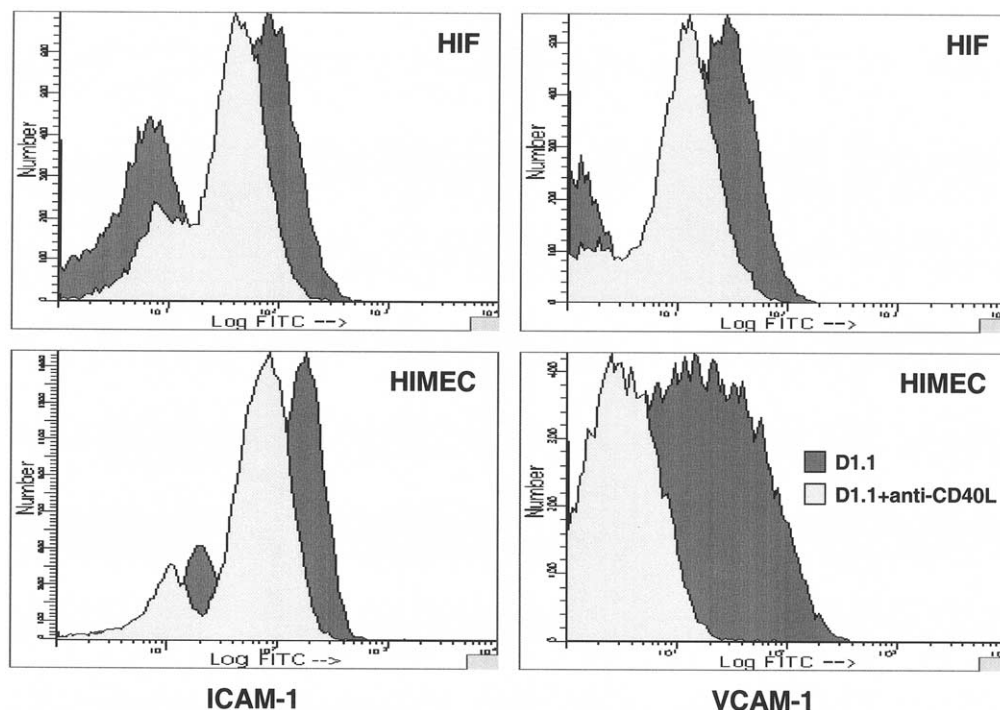


Figure 6. Inhibition of cell-bound CD40L-mediated enhancement of HIF and HIMEC CAM expression by CD40L blockade. Flow cytometric analysis shows that a CD40L blocking antibody substantially reduces the high levels of HIF and HIMEC surface expression induced by coculture with CD40-positive D1.1 cells. IFN- γ -stimulated HIF and HIMEC monolayers were cultured in the presence of D1.1 cells with and without a CD40L blocking antibody for 48 hours. The curves showing the isotype control and the ICAM-1 and VCAM-1 levels under unstimulated and IFN- γ -stimulated conditions are the same as for Figure 4 and were omitted for ease of interpretation. The smaller peaks represent ICAM-1 and VCAM-1 expression by residual D1.1 cells. This figure is representative of 3 separate experiments with control cell lines.



used instead of D1.1 cells to determine the possible effect of CD40L-negative T cells on chemokine production. IL-1 β was used as a CD40-independent positive control. Control HIF exposed to D1.1 cells alone significantly ($P < 0.05$) increased IL-8 production compared with unstimulated or IFN- γ -treated monolayers (Figure 9). IL-8 production was strikingly greater when HIF were pretreated with IFN- γ and then cocultured with D1.1 cells, not only compared with unstimulated HIF ($P < 0.001$) but also D1.1 cell-stimulated HIF ($P < 0.001$) (Figure 9). Blockade of CD40L on D1.1 cells with the M90 neutralizing antibody markedly reduced IL-8 production to the same level obtained with D1.1 cells alone (Figure 9). Jurkat cells induced a lower but still significant ($P < 0.001$) increase in IL-8 production by IFN- γ -pretreated HIF, but this effect was not inhibited by the CD40L antibody (not shown). As expected, IL-1 β was a potent inducer of IL-8 by HIF. When RANTES was measured in the supernatants of the same cultures, the production profile was noticeably different from that of IL-8. Although maximal production was seen when D1.1 cells were added to IFN- γ -pretreated HIF ($P < 0.001$ compared with unstimulated and D1.1 cell-stimulated HIF), blockade of CD40L completely abrogated RANTES production, in contrast to the partial inhibition seen for IL-8 (Figure 9). Moreover, Jurkat cells failed to up-regulate RANTES production, and IL-1 β only induced low levels of RANTES from HIF.

When control HIMEC chemokine production was evaluated, spontaneous IL-8 production was observed

that was moderately inhibited by IFN- γ . D1.1 cells alone enhanced IL-8 production, although not significantly; when they were added after pretreatment with IFN- γ , HIMEC secretion of IL-8 further and significantly ($P < 0.01$) increased compared with unstimulated HIF (Figure 10). In contrast to the partial inhibitory effect seen with HIF, up-regulation of IL-8 production by D1.1 cells was completely abrogated by blockade of CD40L. Also in contrast to HIF, Jurkat cells failed to up-regulate IL-8 production by HIMEC. On the other hand, RANTES production by HIMEC exhibited a profile similar to that of HIF, including a complete blockade by anti-CD40L and a weak stimulatory effect by IL-1 β (Figure 10). Induction of comparable levels of IL-8 and RANTES production by HIF and HIMEC was also seen when CD40L-positive PBT or LPT were used instead of D1.1 cells (not shown).

The same experiments were performed using HIF and HIMEC derived from IBD. When D1.1 cells were used to induce chemokine production from IBD monolayers, the results obtained essentially reproduced those seen with control HIF and HIMEC, including the effect of CD40L blockade and Jurkat cells (Figure 11). Stimulation of IBD monolayers with IFN- γ alone had little or no effect on chemokine production, whereas IL-1 β induced high levels of IL-8 and low-moderate levels of RANTES from IBD HIF and HIMEC, respectively, comparably to what was found using control cells (not shown).

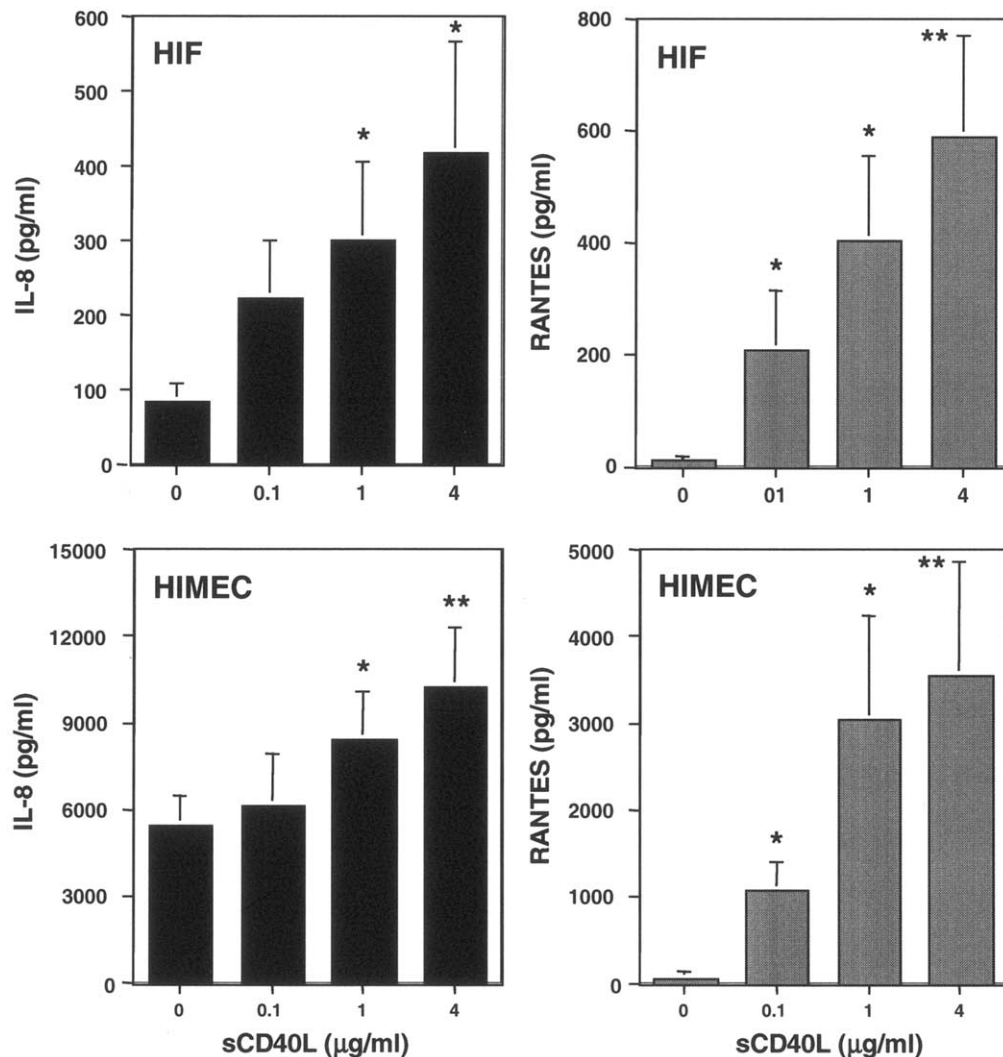


Figure 7. Dose-dependent induction of IL-8 and RANTES production by soluble CD40L in control HIF and HIMEC. IFN- γ -pretreated HIF and HIMEC monolayers were exposed to increasing concentrations of sCD40L for 72 hours, after which supernatants were harvested and chemokine content measured by ELISA. The data represent the mean \pm SEM of 3 separate experiments. * P < 0.05 and ** P < 0.01 compared with spontaneous production.

CD40-Dependent Phosphorylation of p38 Mitogen-Activated Protein Kinase in HIF and HIMEC

Ligation of CD40 triggers a complex signaling cascade involving numerous transduction molecules, among which p38 plays a central role.⁴³ To show that this pathway is set in motion in HIF and HIMEC by CD40 ligation, we measured the levels of phosphorylated p38 in monolayers cocultured with D1.1 cells, sCD40L, and Jurkat cells. Whereas both cell types alone exhibited minimal or no phosphorylation of p38, they markedly up-regulated the level of phospho-p38 when cocultured with D1.1 cells or exposed to sCD40L but not when cocultured with Jurkat cells (Figure 12). Control and IBD HIMEC and HIF exhibited similar degrees of p38 phosphorylation.

Enhancement of T-Cell Adhesion to CD40-Activated HIF

To investigate the functional relevance of CD40-mediated up-regulation of CAM on HIF, we examined whether the increase in ICAM-1 and VCAM-1 expression resulted in a corresponding increased capacity of HIF to bind T cells.³⁹ Under the same experimental conditions used for chemokine induction, IFN- γ stimulation resulted in a significantly (P < 0.05) increased adhesion of MOLT-4 cells above the level observed with unstimulated HIF (Figure 13). The addition of D1.1 cells or sCD40L to IFN- γ -pretreated HIF further and significantly (P < 0.001) enhanced adhesion above the degree obtained with IFN- γ alone. The specificity of this phenomenon was shown by antibody blockade of CD40L, which significantly (P < 0.01) inhibited D1.1

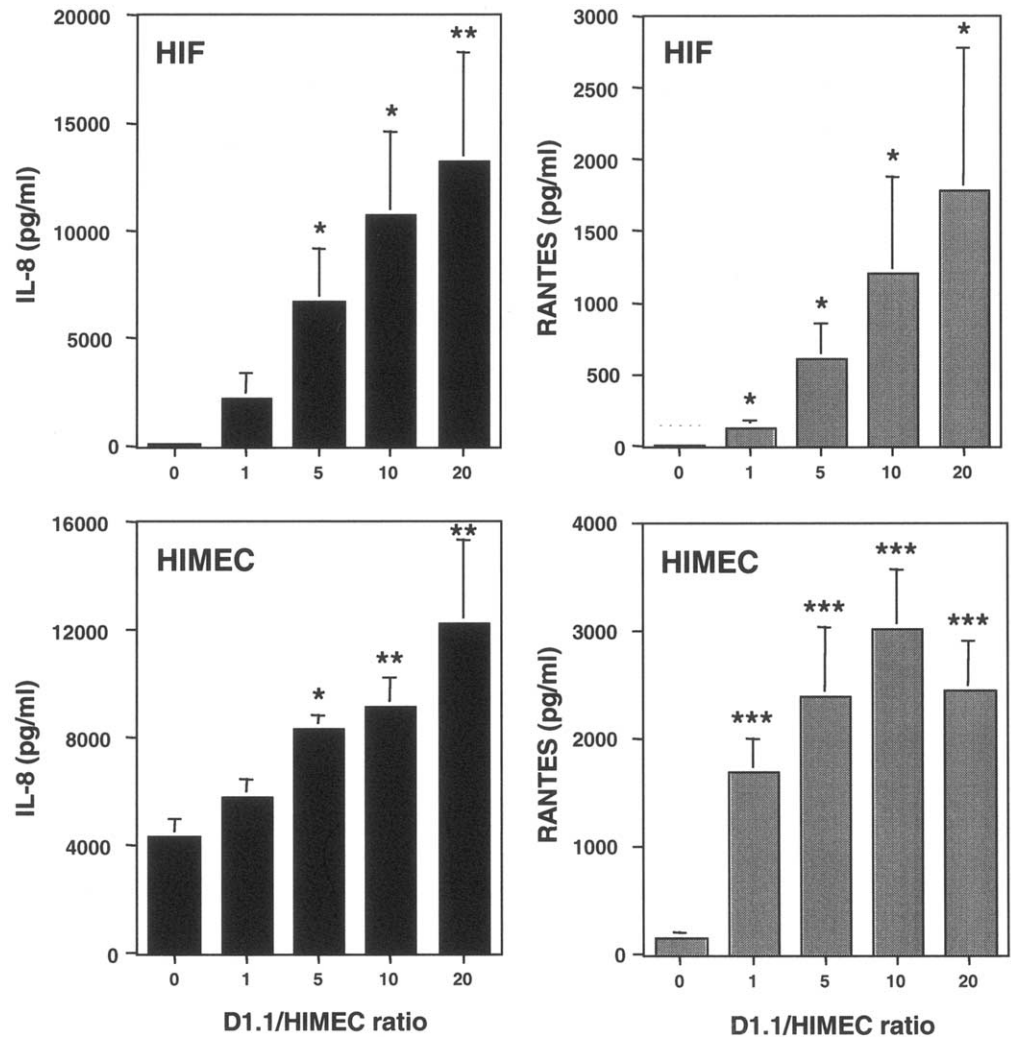


Figure 8. Dose-dependent induction of IL-8 and RANTES production by cell-bound CD40L in control HIF and HIMEC. IFN- γ -pretreated HIF and HIMEC monolayers were exposed to increasing ratios of D1.1 cells/HIF or D1.1 cells/HIMEC for 72 hours, after which supernatants were harvested and chemokine content measured by ELISA. The data represent the mean \pm SEM of 3 separate experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with spontaneous production.

cell-induced MOLT-4 adhesion, lowering it to the level induced by IFN- γ alone. In addition, stimulation with CD40L-negative Jurkat cells failed to up-regulate MOLT-4 cell binding above the level induced by IFN- γ . As control for CD40-independent modulation of HIF binding capacity, enhancement of MOLT-4 cell adhesion by IL-1 β approximated the degree achieved by CD40 stimulation with D1.1 cells or sCD40L. The same results were obtained regardless of whether control or IBD HIF were used.

Induction of T-Cell HIMEC Transmigration by Chemoattractants Produced by CD40-Activated HIF

The demonstration that CD40-activated HIF can lead to the production of immunoreactive chemokines (IL-8 and RANTES) prompted us to investigate whether biologically active chemoattractants were actually produced. This was evaluated in a Transwell system that measures transmigration of T cells through a control

HIMEC monolayer. Compared with culture medium alone, supernatants from unstimulated HIF induced twice as many T cells to transmigrate ($P < 0.01$) (Figure 14). When IFN- γ -pretreated HIF were cocultured with CD40L-positive D1.1 cells, the resulting supernatants induced a >3-fold increase in transmigration compared with medium alone and 2-fold that of unstimulated supernatants ($P < 0.001$ for both). This effect was dose dependent, as shown by the serial dilutions and concentration of D1.1 cell-stimulated HIF supernatants (0.5 \times , 1 \times , and 10 \times) that resulted in a corresponding decrease and increase in T-cell transmigration (6.6%, 9.2%, and 22.8%, respectively). Chemokinesis was excluded as a cause of T-cell migration due to the lack of transmigration when medium alone or HIF supernatants were placed in both the upper and lower chamber (not shown). The effect of D1.1 cell-conditioned supernatants was essentially abolished when the M90 CD40L blocking antibody was added to the D1.1 cell-HIF cocultures

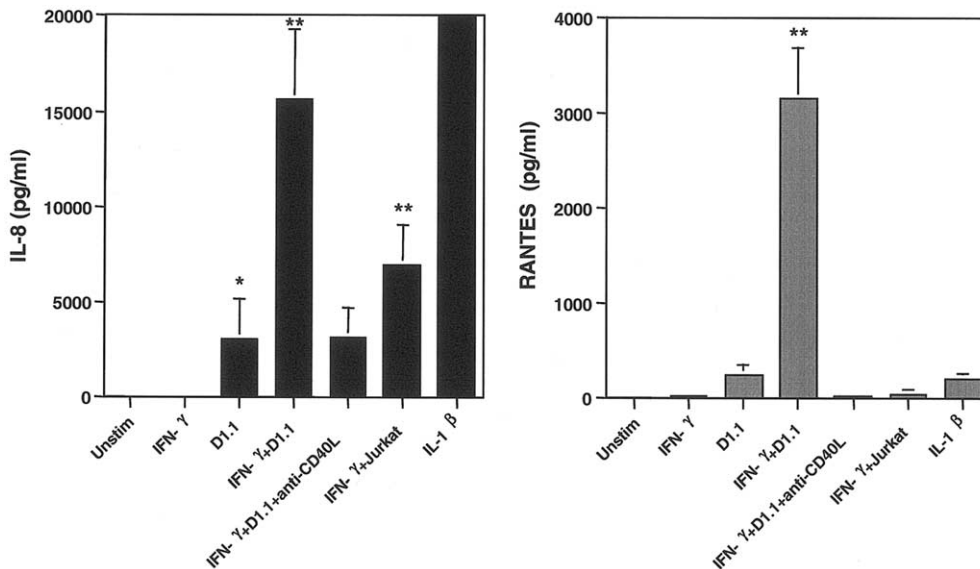


Figure 9. CD40-dependent IL-8 and RANTES production by control HIF. Unstimulated and IFN- γ -pretreated HIF monolayers were exposed to D1.1 cells (D1.1/HIF ratio of 10:1) for 72 hours, after which supernatants were harvested and chemokine content measured by ELISA. The M90 blocking antibody and Jurkat cells were used for specificity control and IL-1 β as a positive control for CD40-independent chemokine production. The data represent the mean \pm SEM of 8–14 separate experiments. * P < 0.05 for D1.1 cell addition compared with unstimulated or IFN- γ -pretreated HIF; ** P < 0.001 for D1.1 cell addition to IFN- γ -pretreated HIF compared with unstimulated or IFN- γ -pretreated HIF and for Jurkat cell addition to IFN- γ -pretreated HIF compared with IFN- γ -pretreated HIF.

(Figure 14). When lipopolysaccharide was used to stimulate HIF, the resulting supernatants induced a degree of T-cell transmigration similar to that obtained with medium alone or unstimulated HIF supernatants and significantly lower (P < 0.001) than that induced by D1.1

cell conditioning. IP-10 and RANTES were used as positive controls for induction of T-cell transmigration and both generated a degree of transmigration comparable to that of unstimulated HIF supernatants, which was significantly lower (P < 0.001) than that induced by

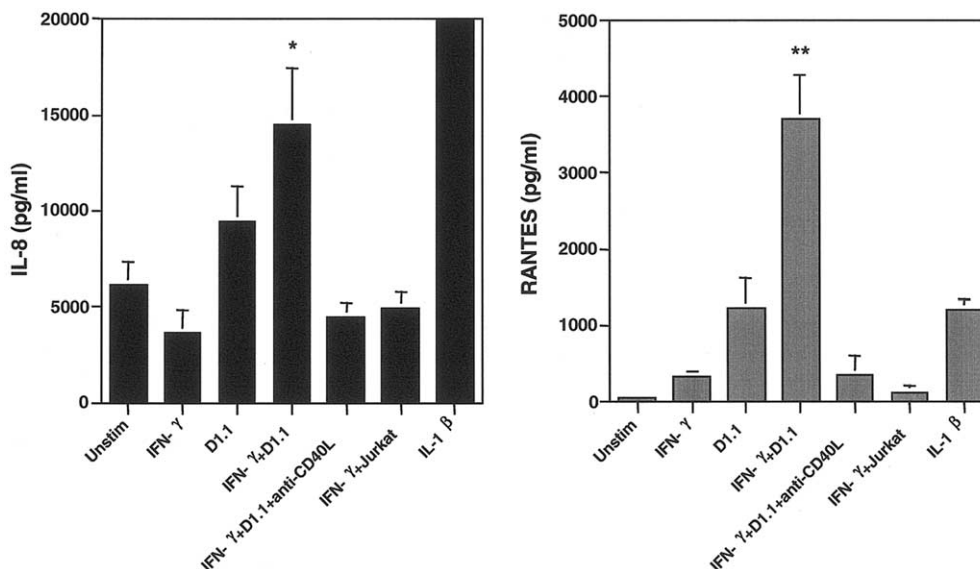
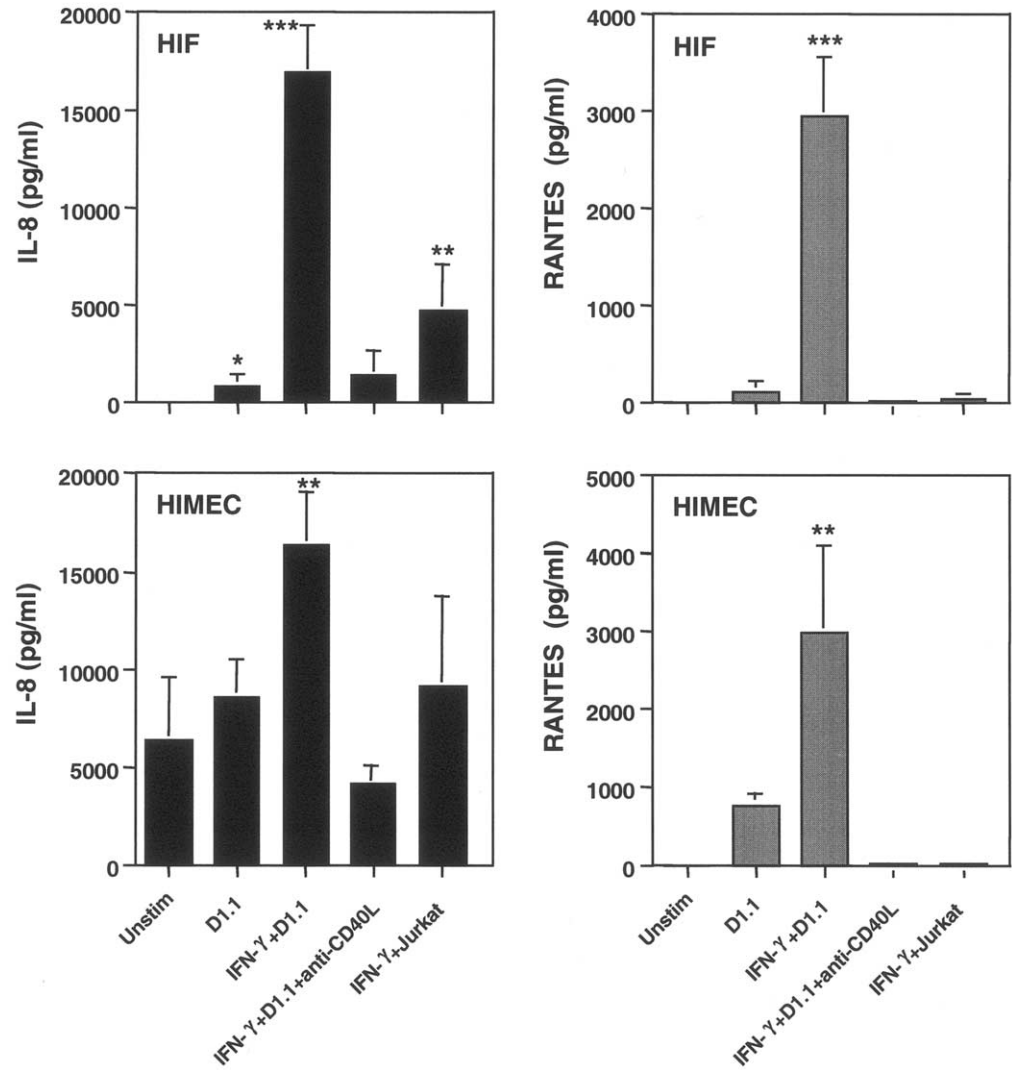


Figure 10. CD40-dependent IL-8 and RANTES production by control HIMEC. Unstimulated and IFN- γ -pretreated HIMEC monolayers were exposed to D1.1 cells (D1.1/HIF ratio of 10:1) for 72 hours, after which supernatants were harvested and chemokine content measured by ELISA. The M90 blocking antibody and Jurkat cells were used for specificity control and IL-1 β as a positive control for CD40-independent chemokine production. The data represent the mean \pm SEM of 6–16 separate experiments. * P < 0.01 for D1.1 cell addition to IFN- γ -pretreated HIMEC; ** P < 0.001 for D1.1 cell addition to IFN- γ -pretreated HIMEC compared with unstimulated, IFN- γ -pretreated HIMEC.

Figure 11. CD40-dependent IL-8 and RANTES production by IBD HIF and HIMEC. Unstimulated and IFN- γ -pretreated cell monolayers were exposed to D1.1 cells (D1.1/HIF or HIMEC ratio of 10:1) for 72 hours, after which supernatants were harvested and chemokine content measured by ELISA. The M90 blocking antibody and Jurkat cells were used for specificity control. The data represent the mean \pm SEM of 4–9 separate experiments. * $P < 0.05$ for D1.1 cell addition compared with unstimulated HIF; ** $P < 0.01$ for D1.1 cell addition to IFN- γ -pretreated HIMEC compared with unstimulated or D1.1 cell addition to HIMEC and for Jurkat cell addition compared with unstimulated HIF; *** $P < 0.001$ for D1.1 cell addition to IFN- γ -pretreated HIF compared with unstimulated HIF or D1.1 cell addition to HIF.



D1.1 cell-conditioned HIF supernatants. As seen in the T-cell adhesion experiments, comparable degrees of T-cell transmigration were obtained using chemoattractants produced by control or IBD HIF.

Discussion

The results of this study show that the cognate interaction of mucosal CD40-positive nonimmune cells with CD40L-positive T cells results in the up-regulation of CAM by HIF, which increase their T-cell binding capacity and secretion of biologically active chemoattractants able to induce migration of T cells through a HIMEC monolayer. This series of events is likely to recapitulate what occurs in vivo given the enhanced expression of CD40 and CD40L by nonimmune and immune cells, respectively, in IBD mucosa. Enhanced in situ expression of CD40 by mesenchymal, endothelial, and epithelial cells and CD40L by lymphoid cells in inflamed tissues is a phenomenon shared by various

chronic conditions including rheumatoid arthritis, autoimmune thyroiditis, polymyositis and dermatomyositis, leprosy, and primary biliary cirrhosis.^{44–48} Whereas there is agreement that, in IBD, expression of CD40L is

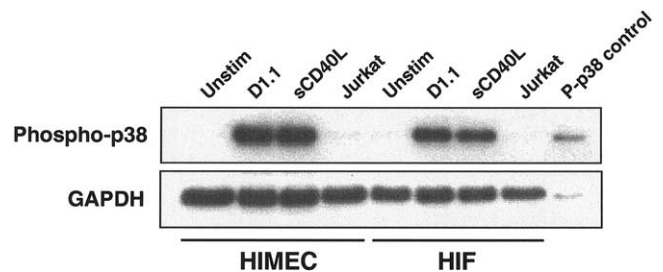


Figure 12. Immunoblotting of CD40-dependent p38 phosphorylation in HIF and HIMEC. IFN- γ -pretreated HIF and HIMEC monolayers were left alone or cocultured with D1.1 cells, sCD40L, or Jurkat cells. After 15 minutes, the monolayers were extensively washed to remove D1.1 and Jurkat cells, lysed, and total proteins extracted for hybridization with a phospho-p38-specific polyclonal antibody. This figure is representative of 4 separate experiments with 2 control and 2 IBD cell lines.

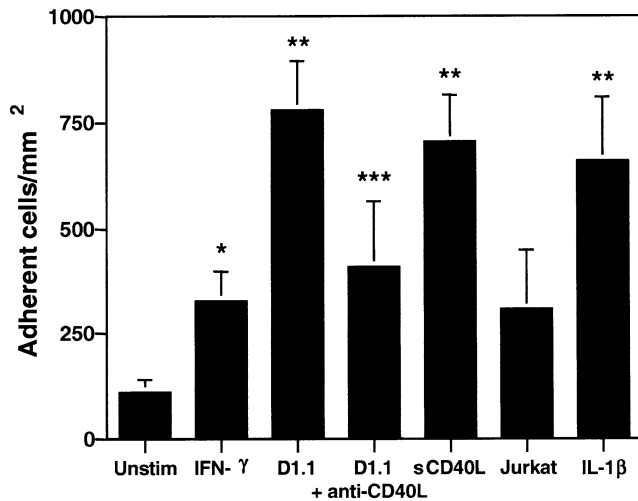


Figure 13. Effect of cell-bound CD40L and sCD40L on adhesiveness of HIF for T cells. Unstimulated or IFN- γ -treated control HIF monolayers were cultured overnight with D1.1 cells or sCD40L before an adhesion assay with calcein-labeled MOLT-4 T cells. A CD40L blocking antibody was used for specificity control, Jurkat cells as a negative control for D1.1 cells, and IL-1 β as a positive control for T-cell adhesion. The data represent the mean \pm SEM of 3 separate experiments. * $P < 0.05$ compared with unstimulated HIF; ** $P < 0.001$ and *** $P < 0.01$ compared with IFN- γ -treated and -untreated HIF.

restricted to T cells and CD40 is found mainly on B and monocytic cells,^{24–26} one study detected CD40-positive endothelial cells in CD²⁵ whereas another stated that epithelial and endothelial cells were negative for CD40 in CD or UC²⁴ with no mention of mesenchymal cells. Our immunohistochemistry results show, in addition to CD40- and CD40L-positive lymphoid cells, unambiguous expression of CD40 by both mesenchymal and microvascular cells in actively inflamed CD and UC tissue, similar to rheumatoid synovial and thyroiditis tissue.^{44,45}

CD40 is expressed by cultured human umbilical endothelial cells^{49–51} and fibroblasts or muscle cells from a variety of tissues, including skin, lung, eye, synovium, and liver.^{37,52–55} We found that, in addition to spontaneous in vivo expression, CD40 is also abundantly expressed in cultures of intestinal mucosal microvascular endothelial and mesenchymal cells exposed to IFN- γ , both at the messenger RNA and protein level. Several proinflammatory cytokines can augment CD40 expression by a variety of nonimmune cells,^{49,54} but IFN- γ was singularly effective in HIF and HIMEC, inducing a protracted up-regulation of CD40 that lasted at least 6 days even after IFN- γ was removed. The importance of this observation is 2-fold. First, these results are fully compatible with the particularly strong and diffuse expression of CD40 we found in mucosa involved by CD, a Th1-like condition associated with increased production of IFN- γ .^{56,57} Second, and most important from a

functional standpoint, the long-lasting expression of CD40 on the surface of HIMEC and HIF enables these nonimmune cells to interact with CD40L-bearing T cells for prolonged periods, inducing a sustained production of chemokines and other mediators.

Several cell types can potentially express CD40L,²⁰ including endothelial and muscle cells, and induce autocrine or paracrine activation pathways as proposed in atherosclerosis.⁵⁸ We failed to observe CD40L expression by HIF and HIMEC in vivo or in vitro (not shown), but CD40L-positive lymphocytes, most likely activated T cells that are common in IBD mucosa,⁵⁹ were readily observed in inflamed mucosa by confocal microscopy. In contrast, we and others failed to detect CD40L-positive T cells in the peripheral blood of patients with IBD,^{24,60} even though they have been described in rheumatoid arthritis and multiple sclerosis.^{61,62} It is possible that the numerous CD40-positive cells present in extensive areas of inflamed gut sequester CD40L-positive T cells into the mucosa. Alternatively, peripheral T cells express CD40L only after recruitment to the highly stimulatory milieu of the IBD mucosa. In either case, the combination of numerous CD40-positive nonimmune cells and CD40L-positive T cells sets the stage for a persistent activation of the CD40 pathway in the mucosa. The

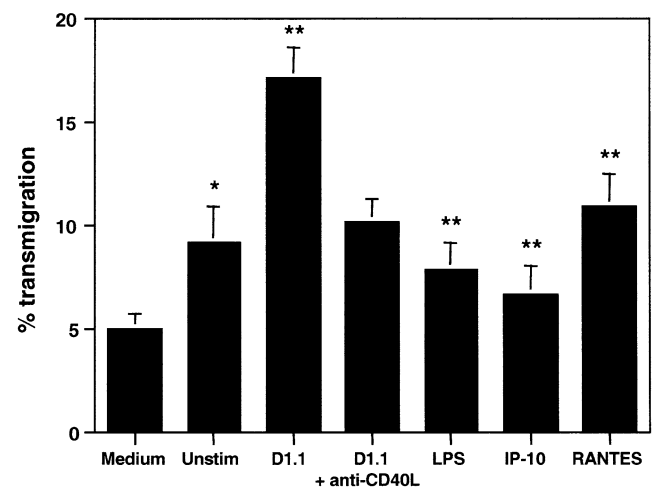


Figure 14. Effect of D1.1 cell-conditioned medium on transmigration of T cells through a HIMEC monolayer. Unstimulated, D1.1 cell-conditioned, or lipopolysaccharide-conditioned control HIF supernatants were placed in the lower chamber of a Transwell system. Calcein-labeled IL-2-derived PBT cell lines were placed in the upper chamber and allowed to transmigrate during a 4-hour period. A CD40L blocking antibody was used for specificity control and the chemokines IP-10 and RANTES as positive controls for T-cell transmigration. The data represent the mean \pm SEM of 8 separate experiments. * $P < 0.01$ compared with medium alone; ** $P < 0.001$ for D1.1 cell-conditioned supernatants compared with medium alone and unstimulated HIF supernatants and for lipopolysaccharide-conditioned supernatants, IP-10, and RANTES compared with D1.1 cell-conditioned HIF supernatants.

consequences of such activation are multiple,^{19,20} but we focused on 2 key events relevant to leukocyte recruitment and chronicity of inflammation (e.g., up-regulation of CAM and chemokine production).^{49,51,53,55,63}

Up-regulation of both ICAM-1 and VCAM-1 expression by HIF and HIMEC occurred after CD40 engagement by CD40L-positive T cells or sCD40L. This effect was specific, as shown by its inhibition with blocking CD40L antibodies. Thus, enhanced expression of ICAM-1 in IBD tissues⁶⁴ is probably induced at least in part by local CD40/CD40L interactions. Such an effect may prolong mucosal inflammation because, as we have previously shown, ICAM-1 is a major mediator of T-cell adhesion to HIF.³⁹ Because HIMEC express ICAM-1,³² it is likely that binding of CD40 expressed by the IBD mucosa microvasculature leads to increased ICAM-1 expression, causing enhanced lymphocyte recruitment.

In addition to CAM up-regulation, ligation of CD40 in HIF and HIMEC led to production of IL-8 and RANTES, chemokines that mediate neutrophil and T-cell chemoattraction, respectively, and are involved in innate and adaptive immunity.⁶⁵ CD40-mediated production of IL-8 and RANTES was differentially regulated in HIF and HIMEC. Production of IL-8 by HIF was specifically but not exclusively induced by CD40L-positive T cells because CD40-negative Jurkat cells could also induce IL-8, although to a lesser degree. In HIMEC, Jurkat cells failed to up-regulate IL-8 production, but both HIF and HIMEC produced large amounts of IL-8 in response to IL-1 β . In contrast, significant production of RANTES by these nonimmune cells was only achieved through binding of CD40L-positive T cells, with CD40L-negative cells and IL-1 β having no or little effect. These differences suggest that the contribution of the mucosal CD40 pathway to inflammation may vary depending on the relative availability of soluble or cell-bound CD40L and the type of nonimmune cell expressing CD40. It is also possible that the CD40-dependent production of IL-8 and RANTES may act differentially in acute or chronic gut inflammation, considering the key role of IL-8 in neutrophil recruitment and of RANTES in granuloma formation⁶⁶ and progression from acute to chronic inflammation in experimental colitis.⁶⁷

The previously mentioned functional outcomes of the interaction of CD40 with CD40L are mediated through complex signaling pathways that depend on the type and differentiation state of the cells involved, the expression level of each receptor, and the microenvironment where the interaction occurs.⁴³ Activity of p38 mitogen-activated protein kinase is enhanced in IBD mucosa,⁶⁸ and the strong induction of p38 phosphorylation in HIF and

HIMEC by either cell-bound or soluble CD40L probably contributes to its increase. p38 phosphorylation is a major CD40-dependent signaling event leading to up-regulation of CAM expression and cytokine production,^{69,70} both of which occurred in HIF and HIMEC after CD40 engagement. Of note, all previously discussed events, including up-regulation of CAM, chemokine production, and mitogen-activated protein kinase pathway signaling, were not exclusively induced by the cell surface-bound form of CD40L but also the soluble form of CD40L. This is important because elevated levels of biologically active sCD40L are found in several autoimmune and chronic inflammatory conditions, including IBD. In a recent study, plasma levels of sCD40L were reported as significantly higher in patients with CD than in healthy subjects.⁷¹ In another study, regardless of the clinical activity of IBD, sCD40L plasma levels were significantly higher in both patients with CD and UC than in normal controls and proportional to the anatomic extent of mucosal inflammation.⁶⁰ The second study also established that circulating sCD40L is of platelet origin, an intriguing observation that further amplifies the role of the CD40/CD40L system in IBD pathogenesis, especially considering the increased number of platelets in patients with IBD and their enhanced state of activation.⁷²

To understand the impact of CD40 activation in nonimmune cells on gut inflammation, we performed experiments to integrate CAM up-regulation and enhanced chemokine production by HIF and HIMEC and show that these 2 events may mediate excessive accumulation of leukocytes in IBD mucosa.⁷³ Cognate ligation of T-cell bound or soluble CD40L caused HIF to increase their T-cell binding capacity to a degree similar or greater than that induced by IL-1 β and IFN- γ . Considering that these 2 cytokines are among the most potent inducers of HIF T-cell binding,³⁹ activation of mesenchymal cell CD40 emerges as an extremely efficient mechanism to retain lymphocytes in the mucosa and thus perpetuate inflammation. Even more impressive were the results of the T-cell transmigration induced by HIF-conditioned medium. Unstimulated HIF supernatants significantly increased T-cell HIMEC transmigration, and CD40-conditioned medium was more potent than IP-10 or RANTES, 2 of the most effective T-cell chemokines.^{41,42} This shows that HIF spontaneously secrete biologically active chemoattractants and this capacity is substantially augmented by CD40 signaling, suggesting that HIF contribute to T-cell chemoattraction under both physiologic and pathologic conditions. The particularly

strong capacity of CD40-conditioned medium may be due to the production of multiple chemokines by HIF, mimicking what occurs *in vivo* in IBD mucosa.^{74,75} In addition, the mucosal microvasculature probably also contributes to attraction and binding of T cells not solely through production of its own chemokines⁷⁶ but also the transport and presentation of mesenchymal cell-derived chemokines,⁷⁷ as we recently showed.⁷²

In summary, the results of this study suggest that the CD40/CD40L system can effectively amplify intestinal inflammation by serving as a biological bridge linking activated T cells and nonimmune cells that in turn up-regulate CAM and secrete soluble mediators that recruit more T cells and sustain a cycle of immune-nonimmune cell interactions.⁷⁸ This conclusion is partly based on *in vitro* experiments, and to what extent it reflects the *in vivo* situation remains to be established. In addition, other CD40-bearing cells are also present in the mucosa, such as macrophages and dendritic cells, whose interaction with CD40L-positive T cells certainly results in similar proinflammatory events. Nevertheless, the conclusion provides a mechanistic framework for an antigen-independent influx of T cells to the inflamed intestine and highlights nonimmune cells as potential contributors to the chronicity of IBD. This framework resembles that proposed for other chronic inflammatory diseases, particularly atherosclerosis, where the CD40/CD40L system is now believed to mediate key interactions not only between classic immune cells but also between immune and structural cells and between structural cells themselves.⁷⁹ Disruption of CD40 signaling pathways in immune cells is immunosuppressive and anti-inflammatory, and the same should be true for immune-nonimmune interactions. Therapies that block the CD40/CD40L system offer distinctive advantages because they do not require knowledge of potential autoantigens and are relatively specific because CD40L is expressed on antigen-activated T cells, which constitute the majority of autoreactive pathogenic T cells.²³ Clinical trials using IDEC-131 (E6040), a humanized monoclonal antibody against CD40L, are currently underway, including for CD.⁸⁰

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