Production of MDC/CCL22 by human intestinal epithelial cells

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Berin, M. Cecilia, Michael B. Dwinell, Lars Eckmann, and Martin F. Kagnoff. Production of MDC/CCL22 by human intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 280: G1217–G1226, 2001.—The intestinal mucosa contains a subset of lymphocytes that produce Th2 cytokines, yet the signals responsible for the recruitment of these cells are poorly understood. Macrophage-derived chemokine (MDC/CCL22) is a recently described CC chemokine known to chemoattract the Th2 cytokine producing cells that express the receptor CCR4. The studies herein demonstrate the constitutive and regulated production of MDC/CCL22 in vivo by human colon epithelium and by epithelium of human intestinal xenografts. MDC/CCL22 mRNA expression and protein secretion were upregulated in colon epithelial cell lines in response to proinflammatory cytokines or infection with enteroinvasive bacteria. Inhibition of nuclear factor (NF)-κB activation abolished MDC/CCL22 expression in response to proinflammatory stimuli, demonstrating that MDC/CCL22 is a NF-κB target gene. In addition, tumor necrosis factor-α-induced MDC/CCL22 secretion was differentially modulated by Th1 and Th2 cytokines. Supernatants from the basal, but not apical, side of polarized epithelial cells induced a MDC/CCL22-dependent chemotaxis of CCR4-positive T cells. These studies demonstrate the constitutive and regulated production by intestinal epithelial cells of a chemokine known to function in the trafficking of T cells that produce anti-inflammatory cytokines.

THE INTESTINAL EPITHELIUM forms a barrier that restricts entry of intestinal luminal contents and microbes into the host. Nonetheless, the lamina propria underlying the surface epithelium is “physiologically inflamed” in that it contains abundant numbers of B and T cells, monocytes, and dendritic cells, as well as variable numbers of eosinophils and mast cells. T cells that encounter luminal derived antigens within the organized lymphoid tissue of the Peyer’s patches can exit from those structures, recirculate, and eventually home to the intestinal lamina propria. T cells in the lamina propria are mostly CD45RO+, consistent with a memory phenotype, and also express the mucosal homing α4β7-integrin as well as human leukocyte antigen-DR and CD25, reflecting an activated state compared with T cells in other peripheral sites (27).

Lamina propria T cells play an important role in host defense but can also contribute to the pathophysiology of intestinal inflammation. The cytokine profile of lamina propria lymphocytes is predominantly Th1 in nature [i.e., interferon (IFN)-γ producing] under physiological conditions (21) as well as in Crohn’s disease (18) and most murine models of intestinal inflammation (5). Nonetheless, interleukin (IL)-4-producing Th2 cells are also present in the normal human intestinal mucosa and are increased in certain murine models of colitis, such as the TCR αα mutant mouse (25, 35) and the oxazolone-induced colitis model (6). Despite the association of those models of colitis with elevated Th2 cytokines, most evidence supports the hypothesis that Th2 cytokines, primarily IL-4 and IL-10, are anti-inflammatory in the intestinal mucosa through their actions on macrophages and Th1 cells (3, 23).

Little is known about the signals responsible for the recruitment of T cells within the intestinal mucosa. Although an initial interaction between α4β7-integrin on T cells and mucosal addressin cell adhesion molecule on venules is required for homing of T cells to the gut, additional activation signals provided by chemokines are also thought to be necessary (8, 31). T cells express a number of cell surface chemokine receptors, and different subsets of T cells express different chemokine receptors, allowing for differential recruitment of those subsets. For example, Th2-type cells (IL-4, IL-5, IL-10, and IL-13 producing) express the receptor CCR4 on their surface (11, 37), whereas Th1-type cells (IFN-γ and IL-2 producing) express the receptor CXCR3.

Intestinal epithelial cells are an important source of chemokines in the intestinal mucosa. Epithelial cells are the first line of defense against luminal pathogens and, in response to bacterial invasion, can secrete an array of neutrophil and macrophage chemotaxants (e.g., IL-8/CXCL8, ENA-78/CXCL5, GROα/CXCL1, MCP-1/CCL2, and MIP-1α/CCL3) (14, 42). Human intestinal epithelium can also produce IP-10/CXCL10, Mig/CXCL9, and I-TAC/CXCL11 (13, 40), chemokines that are known to chemoattract CXCR3-expressing T cells that have a Th1 phenotype (i.e., IFN-γ-producing

mucosa; cytokines; Th1/Th2; chemotaxis
CD4+ T cells) and MIP-3α/CCL20, a chemoattractant for CD45RO+ T cells and immature dendritic cells (26). These findings suggest that epithelial cells may have a role not only in the acute host response to infection but also in T cell trafficking in the intestine.

The CC chemokine receptor CCR4 is preferentially expressed by T lymphocytes that produce Th2 cytokines (11, 37). The production of a chemokine specific for CCR4 by the intestinal epithelium could provide a signal for the specific recruitment of Th2 cells. Macrophage-derived chemokine (MDC/CCL22) is a recently identified chemokine of the CC chemokine family that uses CCR4 as its receptor and is known to selectively chemoattract Th2 cytokine-producing cells (2, 20). MDC/CCL22 is constitutively expressed by macrophages, mature dendritic cells, and B cells, and up-regulated expression of MDC/CCL22 has been noted in T lymphocytes that concomitantly produce the Th2 cytokines IL-4, IL-5, and IL-6 and in monocytes stimulated with the Th2 cytokines IL-4 and IL-13 (2, 19).

We hypothesized that human intestinal epithelial cells may have the capacity to signal Th2 cells through the production of the CCR4 ligand MDC/CCL22. In the studies herein, we report on the constitutive expression of MDC/CCL22 by epithelium in normal human colon and in human intestinal xenografts in vivo and the regulated expression of MDC/CCL22 by cultured human intestinal epithelial cells. These data suggest the notion that intestinal epithelial cells have the capacity to regulate mucosal T cell trafficking through the release of specific cytokine-producing Th2 cell subsets.

MATERIALS AND METHODS

Reagents. Recombinant human (rh) tumor necrosis factor (TNF)-α, IL-1α, MDC/CCL22, and transforming growth factor-β1 were from R&D Systems (Minneapolis, MN), rhIFN-γ was from Bioisoce International (Camarillo, CA), and rhIL-4 and IL-13 were from PeproTech (Rocky Hill, NJ). Mouse anti-human MDC/CCL22 monoclonal antibody (MAb) (IgG2b) and affinity-purified goat anti-human TNF-α were from R&D Systems, and rabbit anti-human MDC/CCL22-IgG was from PeproTech. MG-132 was from Sigma Chemical (St. Louis, MO).

Cell culture. The human colon adenocarcinoma cell lines HT-29 (ATCC HTB 38, Rockville, MD) and HCA-7 colony 29 (a gift from S. C. Kirkland) were grown in DMEM supplemented with 10% heat-inactivated FCS and 2 mM l-glutamine. Epithelial cells were grown to confluence in six-well plates before stimulation with cytokines or bacterial infection. To obtain polarized monolayers, HCA-7 cells were seeded onto tissue culture-treated transwell filters (0.4 μm pore size, 1.2 cm² surface area; Costar, Cambridge, MA) and allowed to grow for ~7 days, at which time a mean resistance of ~500 Ω·cm² was established. Peripheral blood monocytes were prepared by separation of whole blood on a Ficoll gradient, followed by adherence of the harvested buffy coat to tissue culture plastic wells overnight. Monocyte-derived macrophages were prepared as previously described (32).

Bacterial infection. Enteroinvasive Escherichia coli strain 029:NM was obtained from the ATCC (43892), and Salmonella dublin was provided by Dr. J. Fierer (University of California, San Diego). For infection, HT-29 cells were grown to confluence in six-well plates and incubated with 10⁶ S. dublin or 5 × 10⁶ enteroinvasive E. coli for 1 h to allow invasion to occur, after which the extracellular bacteria were removed by washing and cells were incubated for an additional 24 h in the presence of 50 μg/ml of the non-membrane-permeant antibiotic gentamicin to kill remaining extracellular bacteria (15). Culture supernatants were removed 24 h after infection and stored at −20°C before measurement of MDC/CCL22 by ELISA.

Adenovirus constructs and adenovirus infection. Recombinant adenovirus 5 (Ad5) containing an IκB-α superrepressor (Ad5IκB-α32/36) or the E. coli β-galactosidase gene (Ad5LacZ) was constructed as described before (17). Ad5IκB-α32/36 expresses a hemagglutinin (HA) epitope-tagged mutant form of IκBα in which serine residues 32 and 36 are replaced by alanine residues. The mutant IκBα cannot be phosphorylated at positions 32 and 36 and acts as a superrepressor of nuclear factor (NF)-κB activation (17). The HA epitope tagging enables identification of superrepressor with anti-HA antibodies. Viral titers were determined by plaque assay. Recombinant virus was stored in PBS containing 10% (vol/vol) glycerol at −80°C.

HT-29 cells grown to confluence in six-well tissue culture plates were infected with Ad5IκB-α32/36 or Ad5LacZ in serum-free medium at a multiplicity of infection (MOI) of 100 for 16 h as described before (17). At this MOI, Ad5IκB-α32/36 or Ad5LacZ infected >80% of HT-29 cells, and infected cells expressed IκB-α32/36 and β-galactosidase, respectively, at high levels, as assessed by staining for β-galactosidase and immunostaining for HA-tagged IκBα-α32/36 (data not shown). After infection, adenovirus was removed by washing, fresh medium containing serum was added, and cells were incubated for an additional 12 h before bacterial infection or IL-1α or TNF-α stimulation. The IκB-α superrepressor inhibited NF-κB activation in HT-29 cells, as assessed by EMSA, and inhibited cytokine-induced and bacteria-induced upregulation of IL-8 and intracellular adhesion molecule-1 expression in HT-29 cells but did not alter β-actin mRNA levels in the same cells (17).

RT-PCR. Total cellular RNA was extracted with TRIzol reagent (GIBCO BRL, Gaithersburg, MD), treated with RNase-free DNase (Stratagene, La Jolla, CA) to remove contaminating genomic DNA, and reverse transcribed using 1 μg of total RNA and 2.5 units Superscript II RT (GIBCO BRL). Sequences were amplified from cDNA with primers specific for human MDC/CCL22 and β-actin. Primer sequences for MDC/CCL22 were 5’-GAGACATAACAGCA-GAGCATGGC T-3’ (sense) and 5’-ATGGAGATCAGGGAAT-AGACATACAGGAC-GGATGC-3’ (antisense), and those for β-actin were 5’-TGACGGGGTACCCCACTGTGCCATCTA-3’ (sense) and 5’-CTAGAGAAGCTTGCGTGACAGATGAGG-3’ (antisense). Five microliters of RT reaction were used for PCR amplification in a volume of 50 μl of PCR buffer (GIBCO BRL), including 25 μM of each primer, 1.5 mM MgCl₂, and 200 μM of each dNTP. After an initial hot start at 95°C for 5 min, 40 units Taq polymerase (GIBCO BRL) was added. The amplification profile consisted of 45 s denaturation at 95°C, 2 min annealing, and extension at 64°C (MDC/CCL22) or 72°C (β-actin) for 38 (MDC/CCL22) or 28 (β-actin) cycles. Each experiment included negative controls in which RNA was omitted from the RT mixture and cDNA was omitted from the PCR reactions. RNA from human monocyte-derived macrophages was used as a positive control for MDC/CCL22.

Real-time PCR. Real-time PCR was performed using an ABI Prism 7700 sequence detection system (PE Applied Bio-
systems, Foster City, CA). Each reaction contained 25 μl of 2×TaqMan Universal Master Mix (containing 200 nM dATP, dGTP, and dCTP, 400 nM dUTP, 2 mM MgCl₂, 0.25 units uracil N-glycosylase, and 0.625 units AmpliTaq Gold DNA polymerase), 25 pmol of the sense and antisense primers, and 2 μl of cDNA in a final volume of 50 μl. MDC/CCL22 primers used were described above, and β-actin primers were as follows: sense, 5'-CAACGACCTCTGACGGCCACA-3'; antisense, 5'-CATACTCTCGTTGCTGATCC-3'. Reactions were incubated at 50°C for 2 min followed by 95°C for 10 min. The amplification profile was 95°C for 15 s and 62°C for 1 min for 40 cycles. Amplification of the expected single PCR product was confirmed by electrophoresis of the product on a 1% agarose gel that was stained with ethidium bromide. Data analysis was performed using PE Biosystems 7700 sequence detection system software. Real-time PCR data were plotted as the ΔRQ, fluorescence signal vs. the cycle number. ΔRQ was calculated using the equation ΔRQ = RQq - RQr, where RQq is the fluorescence signal of the product and RQr is the fluorescence signal of the baseline emission. Ct is the PCR cycle at which an increase in reporter fluorescence above the baseline signal can first be detected, as determined by the sequence detection system software. The fold increase in MDC/CCL22 signal can first be detected, as determined by the sequence detection system software. The fold increase in MDC/CCL22 signal can first be detected, as determined by the sequence detection system software. The fold increase in MDC/CCL22 signal can first be detected, as determined by the sequence detection system software. The fold increase in MDC/CCL22 signal can first be detected, as determined by the sequence detection system software. The fold increase in MDC/CCL22 signal can first be detected, as determined by the sequence detection system software. The fold increase in MDC/CCL22 signal can first be detected, as determined by the sequence detection system software.

MDC/CCL22 ELISA. Polystyrene 96-well plates (Immulon-4; Dynex Technologies, Chantilly, VA) were coated with mouse anti-human MDC/CCL22 MAb (R&D Systems), diluted in carbonate buffer, as the capture antibody. Affinity-purified rabbit polyclonal anti-human MDC/CCL22 (PeproTech) was diluted in PBS, 1.0% BSA, and 0.1% Tween 20 and used as the detection antibody. The second step reagent was horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Life Science, Arlington Heights, IL). Bound horseradish peroxidase was visualized with tetramethylbenzidine and H₂O₂ diluted in sodium acetate buffer, pH 6.0. The color reaction was stopped by addition of 1.2 M H₂SO₄, and absorbance was measured at 450 nm. MDC/CCL22 concentrations were calculated from standard curves using rhMDC/CCL22 (R&D Systems). The MDC/CCL22 ELISA was sensitive to 50 pg/ml.

Human intestinal xenografts. Human fetal intestine, gestational age 14–20 wk (Advanced Biosciences Resources, Alameda, CA), was transplanted subcutaneously onto the backs of C57BL/6 SCID mice as described before (16, 24). Human fetal intestinal xenografts were allowed to develop for at least 10 wk following transplantation, at which time the epithelium, which is strictly of human origin, is fully differentiated (38). Intestinal xenografts were removed, and segments were embedded in optimum cutting temperature (OCT) compound and frozen in isopentane/dry ice for immunohistochemical analysis.

Immunohistochemical detection of MDC/CCL22 in human colon and human intestinal xenografts. Sections of histologically normal human colon from surgical specimens from individuals undergoing partial colectomy were imbedded in OCT and snap frozen in isopentane/dry ice. Cryostat sections (5 μm) of human colon or intestinal xenografts were fixed in acetone for 10 min and blocked in PBS-1% BSA with 10% goat serum for 1 h at room temperature. Sections were incubated overnight at 4°C with 2.5 μg/ml mouse anti-human MDC/CCL22 MAb, an isotype-matched control MAb (mouse IgG2b, 2.5 μg/ml, Sigma), or anti-MDC/CCL22 antibody that was preabsorbed with rhMDC/CCL22 (2.5 μg/ml), and sections were subsequently stained with Cy3-labeled goat anti-mouse IgG. Sections were counterstained with the nuclear dye Hoechst 33258 (Calbiochem, San Diego, CA).

Chemotaxis assay. To determine whether epithelial cell-derived MDC/CCL22 chemoattracts T lymphocytes, chemotaxis assays were performed using the CEM T cell line, which expresses CCR4 as we verified by RT-PCR using previously described primers (12). Supernatants were obtained from the apical or basolateral chambers of polarized HCA-7 cells cultured in Transwells in RPMI-0.1% BSA 24 h after stimulation with TNF-α or IL-1α plus IFN-γ. Recombinant human MDC/CCL22 or HCA-7 supernatants were added to the bottom well of ChemoTx chemotaxis microplates (Neuroprobe, Gaithersburg, MD), and a 5-μm-pore filter plate was placed over the bottom wells. To assess chemotaxis, 1.25 × 10⁶ CEM cells labeled with the fluorescent dye calcine-AM (Molecular Probes, Eugene, OR) in a total volume of 25 μl were added to the top of the filter. After 2 h incubation, fluorescence in the bottom wells was measured with a fluorescence microplate reader and number of migrated cells were calculated from a standard curve of labeled cells. To determine the role of MDC/CCL22 in supernatant-induced chemotaxis, 5 μg/ml of a mouse anti-human MDC/CCL22 antibody (R&D Systems) or an equivalent concentration of control IgG (Jackson ImmunoChemical Laboratories, West Grove, PA) was added to the supernatants before addition to the chemotaxis plate.

All studies involving human and animal protocols were approved by the UCSD Human Subjects Committee and the Animal Subjects Program.

RESULTS

Constitutive MDC/CCL22 expression by epithelial cells in human colon and human intestinal xenografts. To determine if human intestinal epithelium in vivo expresses MDC/CCL22, we first immunostained sections of normal human colon for that chemokine. As shown in Fig. 1A, MDC/CCL22 was constitutively expressed by epithelial cells in normal human colon. Sections stained with an isotype-matched control antibody at the same concentration (data not shown) or anti-MDC/CCL22 antibody preabsorbed with rhMDC/CCL22 (Fig. 1B) did not demonstrate any specific immunoreactivity. Surface epithelium as well as crypt epithelium stained with the anti-MDC/CCL22 antibody.

To determine if MDC/CCL22 production in vivo depends on the exposure of the intestinal epithelium to a conventional luminal bacterial flora, we assessed MDC/CCL22 expression by the epithelium of human intestinal xenografts grown subcutaneously in SCID mice, since the xenograft lumen is sterile. As shown in Fig. 2, like normal human colon epithelium in vivo, epithelium in the xenografts produced MDC/CCL22, indicating that the normal colon flora was not required for constitutive cellular MDC/CCL22 expression.

Constitutive and regulated MDC/CCL22 mRNA expression in human intestinal epithelial cells. To determine if the expression of MDC/CCL22 by intestinal epithelial cells is regulated, we took advantage of cultured human colon epithelial cell lines. As shown in Fig. 3A, MDC/CCL22 mRNA expression was upregulated in the human colon epithelial cell line (HT-29) by 3 h and continued to increase up to 12 h after stimulation with TNF-α or IL-1α. MDC/CCL22 mRNA expression also increased after IFN-γ stimulation, but
increased expression was delayed for up to 12 h after IFN-\(\gamma\) stimulation. Furthermore, costimulation of HT-29 cells with a combination of IFN-\(\gamma\) plus TNF-\(\alpha\) or IFN-\(\gamma\) plus IL-1\(\alpha\) resulted in a synergistic increase in MDC/CCL22 mRNA expression (Fig. 3). Similar data were obtained using HCA-7 cells (data not shown). Real-time PCR was performed to quantify the increase in MDC/CCL22 mRNA expression after cytokine stimulation. These data confirmed that costimulation of HT-29 cells with TNF-\(\alpha\) or IL-1\(\alpha\) plus IFN-\(\gamma\) induced a synergistic increase in MDC/CCL22 mRNA expression (Fig. 3, B and C). Thus IFN-\(\gamma\) and TNF-\(\alpha\) costimulation induced a 123-fold increase in MDC/CCL22 expression over unstimulated controls, compared with a 35-fold increase after TNF-\(\alpha\) stimulation and an 8-fold increase after IFN-\(\gamma\) stimulation.

**Cytokine-stimulated MDC/CCL22 secretion.** To determine if agonist-stimulated MDC/CCL22 mRNA expression was accompanied by increased MDC/CCL22 protein secretion, HT-29 cells were left unstimulated or were stimulated for up to 24 h with TNF-\(\alpha\), IL-1\(\alpha\), or IFN-\(\gamma\), after which MDC/CCL22 levels in culture supernatants were assayed by ELISA. As shown in Fig. 4A, TNF-\(\alpha\) stimulation markedly increased MDC/CCL22 secretion (130-fold increase over unstimulated cells at 24 h), and this was also the case, although to a lesser extent, for IL-1\(\alpha\) stimulation (36-fold increase). In contrast, MDC/CCL22 secretion in response to IFN-\(\gamma\) stimulation was significantly less than that induced by TNF-\(\alpha\) or IL-1\(\alpha\) (10-fold increase over control at 24 h).

As shown in Fig. 4B, IFN-\(\gamma\) synergistically increased TNF-\(\alpha\)-stimulated MDC/CCL22 secretion. IFN-\(\gamma\) induced a 10- to 15-fold increase in TNF-\(\alpha\)-induced MDC/CCL22 secretion at TNF-\(\alpha\) concentrations ranging from 0.1 to 10 ng/ml and a lesser 3-fold increase at 100 ng/ml, the highest concentration of TNF-\(\alpha\) tested. Synergy between IFN-\(\gamma\) and TNF-\(\alpha\) or IL-1\(\alpha\) was not ob-
served 3 h after stimulation but was present at each
time point examined thereafter (8, 12, 16, and 24 h)
(data not shown).

MDC/CCL22 secretion by peripheral blood mono-
cytes was determined to compare levels of MDC/CCL22
production between epithelial cells and monocytes,
which had previously been described to produce MDC/
CCL22 (2). Monocytes cultured at the same cell density
as intestinal epithelial cells constitutively produced
slightly higher levels of MDC/CCL22 than intestinal
epithelial cells (1.5 ng/ml for monocytes compared with
0.5 ng/ml for intestinal epithelial cells). However, max-
imal MDC/CCL22 production by monocytes, which was
observed after TNF-α plus IL-4 stimulation, increased
by only fourfold over constitutive levels (up to 5.6
ng/ml), whereas HT-29 cells stimulated with TNF-α
plus IFN-γ produced >300 ng/ml MDC/CCL22.

In contrast to MDC/CCL22, the CCR4 ligand, TARC/
CCL17, which has been shown by us (4) and others (39)
to be expressed by airway epithelial cells, was not
detected in supernatants from intestinal epithelial
cells after stimulation with proinflammatory cytokines
(TNF, IL-1α, IL-4, IL-13) (data not shown).

Polarized secretion of MDC/CCL22. Intestinal
epithelial cells in vivo are polarized with functionally
distinct apical and basolateral domains. For epithelial
MDC/CCL22 to act as a physiological chemoattractant
for target cells in the lamina propria, it predictably would be secreted in a polarized manner from the basolateral epithelial cell membrane rather than apically into the intestinal lumen. To determine if this was the case, HCA-7 cells were grown as polarized monolayers and stimulated with TNF-α or IL-1α either alone or in combination with IFN-γ. As shown in Table 1, for each stimulus, ~90% of MDC/CCL22 was secreted into the basal compartment.

### Table 1. Polarized MDC/CCL22 secretion by HCA-7 cells

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>MDC/CCL22 Secretion, ng/well</th>
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<tbody>
<tr>
<td></td>
<td>Apical</td>
</tr>
<tr>
<td>None</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.06±0.01(10)</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0.08±0.02(11)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.20±0.02(9)</td>
</tr>
<tr>
<td>TNF-α + IFN-γ</td>
<td>0.58±0.03(6)</td>
</tr>
<tr>
<td>IL-1α + IFN-γ</td>
<td>0.49±0.02(7)</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 independent experiments. Values in parentheses are % of total. Supernatants were collected 24 h after stimulation with 20 ng/ml of each of the indicated cytokines, and MDC/CCL22 concentrations were determined by ELISA. MDC, macrophage-derived chemokine; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon.

Epithelial cell-derived MDC/CCL22 induces T cell chemotaxis. To determine if MDC/CCL22 secreted by agonist-stimulated epithelial cells could contribute to the recruitment of T cells, chemotaxis assays were performed using supernatants obtained from polarized HCA-7 cells (Fig. 5). rhMDC/CCL22 induced a dose-dependent increase in chemotaxis of the CCR4-expressing T cell line CEM. Supernatants taken from the basal chamber of Transwell-grown HCA-7 cells stimulated with IL-1α and IFN-γ induced an increase in T cell chemotaxis compared with media from unstimulated monolayers (18.6 vs. 8.3% of input cells migrated). This was comparable to the level of chemotaxis induced by similar concentrations of rhMDC/CCL22. In contrast, supernatants from the apical chamber of filter-grown, stimulated HCA-7 cells did not induce any increase in chemotaxis (7.7% of input cells migrated). IL-1 and IFN-γ at a concentration of 20 ng/ml in RPMI did not affect chemotaxis of CEM cells (7.3% of input cells migrated). Incubation of the basal chamber super-
intestinal epithelial cells in response to stimulation of several genes that are upregulated in transcription factor NF-$\kappa B$. Values are means of 2 independent experiments.

Fig. 6. MDC/CCL22 secretion in response to infection with enteroinvasive bacteria. HT-29 cells were grown to confluence and were infected with E. coli O29:NM or S. dublin at a multiplicity of infection of 500 and 100, respectively. After infection, cells were incubated for a further 24 h with media alone or IFN-γ (20 ng/ml). Values are means ± SE of 3 or more independent experiments.

Epithelial MDC/CCL22 secretion in response to infection with enteroinvasive bacteria. Epithelial cells can function as sensors for microbial infection and, in response to bacterial infection, secrete chemokines that can chemoattract neutrophils and monocyte/macrophages within the intestinal mucosa (22, 29, 34). To determine if epithelial cell infection with enteroinvasive bacteria upregulates MDC/CCL22 secretion, HT-29 cells were infected with S. dublin or enteroinvasive E. coli O29:NM. As shown in Fig. 6, infection with those bacteria increased MDC/CCL22 secretion. To determine if IFN-γ synergized with bacterial infection to increase MDC/CCL22 production, HT-29 cells were stimulated with IFN-γ and enteroinvasive S. dublin or enteroinvasive E. coli O29:NM. MDC/CCL22 secretion was markedly inhibited in Ad5IκB-A32/36-infected cells, but not in cells infected with control adenovirus, in response to TNF-α or IL-1α stimulation or S. dublin or E. coli O29:NM infection. Real-time RT-PCR was performed on mRNA isolated 12 h after cytokine stimulation to examine the effect of inhibiting NF-κB activation at a time when maximal changes were observed in MDC/CCL22 expression.

Fig. 7. Effect of NF-κB inhibition on MDC/CCL22 expression. A: HT-29 cells were pretreated with 10 μM of the proteasome inhibitor MG-132 for 60 min before replacement of the media and stimulation with TNF-α for 8 h. MDC/CCL22 and β-actin mRNA expression were assessed by RT-PCR as described in MATERIALS AND METHODS. B: HT-29 cells were left uninfected (open bars) or infected with an adenovirus encoding an IκBα superrepressor (Ad5IκB-A32/36; hatched bars) or β-galactosidase (Ad5LacZ, black bars) as a virus control. Cells were then stimulated with cytokines (20 ng/ml) or infected with S. dublin or E. coli O29:NM. MDC/CCL22 in supernatants was determined by ELISA 6 h after cytokine stimulation or bacterial infection. Values are means of 2 independent experiments.
CCL22 mRNA expression. TNF-α induced a 15-fold increase in MDC/CCL22 mRNA expression in HT-29 cells infected with control virus compared with a 4-fold increase in cells infected with virus containing the Ad5IκB-A32/36 superrepressor. At 12 h after stimulation, MDC/CCL22 protein secretion induced by TNF-α was reduced by 91% in Ad5IκB-A32/36-infected HT-29 cells compared with cells infected with the control virus. Similar results were obtained in HT-29 cells stimulated with TNF-α plus IFN-γ at 12 h. Inhibition of NF-κB had no effect on the induction of MDC/CCL22 expression in response to stimulation of cells with IFN-γ alone (data not shown), which is consistent with the lack of NF-κB activation in these cells after IFN-γ stimulation.

Modulation of MDC/CCL22 secretion by Th2 cytokines. IL-4 and IL-13 increase MDC/CCL22 expression by monocytes and macrophages (2, 7). To determine if this is the case also for MDC/CCL22 production by intestinal epithelial cells, HT-29 cells were stimulated with IL-4 or IL-13, alone or in the presence of TNF-α. IL-4 or IL-13 alone had no significant effect on constitutive MDC/CCL22 secretion. However, IL-4 and IL-13 inhibited TNF-α-stimulated MDC/CCL22 secretion by ~60% (Table 2).

DISCUSSION

The normal adult human intestine is considered to be “physiologically inflamed.” Thus the normal intestinal mucosa contains B and T lymphocytes, mononuclear phagocytes, and dendritic cells, as well as smaller numbers of eosinophils and mast cells that, if present in other tissues, would be regarded as an abnormal chronic inflammatory cell infiltrate. A requirement for the presence of cells producing downregulatory immune mediators in the intestinal mucosa is evident in the IL-10 knockout mouse, which develops spontaneous inflammation of the colonic mucosa (30). We hypothesized that the intestinal epithelium may play a role in regulating physiological mucosal inflammation by producing signals with the capacity to chemoattract T cells that can, in turn, produce anti-inflammatory cytokines (e.g., IL-4, IL-10) capable of downregulating mucosal inflammation (3, 23). As we demonstrate herein, normal human intestinal epithelial cells produce MDC/CCL22, a CC chemokine that could fulfill that role.

Using cultured human intestinal epithelial cells, we demonstrated that the regulation of MDC/CCL22 production differs between intestinal epithelial cells and macrophages and monocytes. Whereas TNF-α and IL-1 were potent agonists for MDC/CCL22 protein production by cultured human intestinal epithelial cells, this was not the case for other MDC/CCL22-expressing cells (including monocytes, macrophages, and B cells from human peripheral blood) in vitro (2), although one report described small increases of MDC/CCL22 mRNA expression in TNF-α- or IL-1-stimulated macrophages (36). In contrast, IL-4 and IL-13 increased MDC/CCL22 production by macrophages (2, 7) but had no effect by themselves on MDC/CCL22 secretion by cultured intestinal epithelial cells and modestly inhibited TNF-α-stimulated MDC/CCL22 secretion. Whereas IFN-γ alone stimulated low levels of MDC/CCL22 production in human intestinal epithelial cells and synergistically increased TNF-α- and IL-1α-stimulated MDC/CCL22 production in those cells, IFN-γ inhibited MDC/CCL22 secretion by macrophages and monocytes (7). Together, these findings suggest that MDC/CCL22 production by different cell types is dependent on the cytokine milieu in their microenvironment, with inflammatory stimuli appearing to favor epithelial but not macrophage-derived MDC/CCL22 production. The predicted outcome of this epithelial cell response would be an influx of T cells known to produce cytokines that can potentially down-regulate mucosal inflammation. The observation of constitutive MDC/CCL22 protein expression in the human intestinal xenograft tissue demonstrates that epithelial-derived MDC/CCL22 is not completely dependent on the presence of an inflammatory state and may be stimulated by low basal levels of cytokines released by resident immune cells or by other factors that remain to be identified.

Expression of the chemoattractants MDC/CCL22 and TARC/CCL17, which are both ligands for CCR4, appears to differ between different mucosal sites. We show herein that MDC/CCL22, but not TARC/CCL17, is produced by intestinal epithelial cells in response to proinflammatory stimuli. In addition, chemotactic activity for the CCR4-expressing T cell line CEM in supernatants of intestinal epithelial cells was completely abolished by neutralizing anti-MDC/CCL22 antibodies. This finding indicates that MDC/CCL22 may be the sole intestinal epithelial-derived factor responsible for recruiting CCR4-positive T cells. In contrast to intestinal epithelial cells, bronchial epithelial cells produce TARC/CCL17, but not MDC/CCL22, after stimulation with the same proinflammatory cytokines (4, 39). The significance of this regional compartmentalization of chemokine expression is unclear since both chemokines are known to signal through CCR4 as their receptor and both can act as Th2-type T cell chemoattractants. Nonetheless, these findings suggest that there may be divergent functions between MDC/CCL22 and TARC/CCL17.

The regulated production of MDC/CCL22 by intestinal epithelial cells shares similarities with several

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Table 2. Th2 cytokines inhibit TNFα-stimulated MDC/CCL22 secretion by HT-29 cells

<table>
<thead>
<tr>
<th>Cytokines Added</th>
<th>MDC/CCL22 Secretion, ng/ml</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No TNF-α</td>
</tr>
<tr>
<td>None</td>
<td>0.30 ± 0.12</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.48 ± 0.25</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.37 ± 0.11</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 independent experiments. IL-4, IL-13, and TNF-α were each used at a concentration of 20 ng/ml. Supernatants were collected after 24 h of culture. MDC/CCL22 concentrations were assayed by ELISA.
other chemokines that are expressed by the intestinal epithelium. Like MDC/CCL22, the CXC chemokines IL-8/CXCL8 and GROα/CXCL1 and the CC chemokines MCP-1/CCL2 and MIP-3α/CCL20 are upregulated in intestinal epithelial cells in response to TNF-α and IL-1α stimulation or bacterial infection (26, 42) and, like the genes that encode those other chemokines (17), we show that MDC/CCL22 is a NF-κB target gene. Furthermore, synergy between IFN-γ and TNF-α or IL-1α was observed for MDC/CCL22 regulation, similar to that noted for IL-8 (14), RANTES, MCP-1 (41), IP-10, and I-TAC (13). Therefore, when the cytokine milieu includes the proinflammatory stimuli TNF-α or IL-1 together with IFN-γ, chemoattractors for both CCR4-expressing (i.e., MDC/CCL22) and CXCR3-expressing (i.e., IP-10, Mig, and I-TAC) cells could be secreted by the intestinal epithelium.

The constitutive production of MDC/CCL22 by intestinal epithelium in vivo suggests that this chemokine may contribute to the trafficking of CCR4-expressing CD4+ T lymphocytes within the microenvironment of the normal intestinal mucosa. Nonetheless, the cytokine milieu in the normal human intestinal mucosa is thought to be predominantly Th1 in nature, with 10-fold more IFN-γ-producing than IL-4-producing T cells (21). This may reflect the low expression of CCR4, relative to CXCR3, on αβ7-integrin mucosal homing T cells (1, 9). A recent study demonstrated that ~7–10% of αβ7-integrin-positive peripheral blood T cells express CCR4 (9), which approximately corresponds with the fraction of Th2 cells found within the intestinal mucosa (21). It is clear that Th2 cytokine-producing cells are present in the intestinal mucosa, under both basal conditions (21) and in sufficient numbers to induce pathology, as seen in the TCR basal conditions (21) and in sufficient numbers to induce pathology, as seen in the TCR basal conditions (21). Alternatively, the relative paucity of CCR4-expressing T cells in the intestinal mucosa could suggest that epithelial cell-derived MDC/CCL22 can also act on a chemokine receptor other than CCR4.

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