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Eotaxin/CCL11 Suppresses IL-8/CXCL8 Secretion from Human Dermal Microvascular Endothelial Cells

Sara S. Cheng,* Nicholas W. Lukacs, † and Steven L. Kunkel 2*†

The CC chemokine eotaxin/CCL11 is known to bind to the receptor CCR3 on eosinophils and Th2-type lymphocytes. In this study, we demonstrate that CCR3 is expressed on a subpopulation of primary human dermal microvascular endothelial cells and is up-regulated by TNF-α. We found that incubation of human dermal microvascular endothelial cells with recombinant eotaxin/CCL11 suppresses TNF-α-induced production of the neutrophil-specific chemokine IL-8/CXCL8. The eotaxin/CCL11-suppressive effect on endothelial cells was not seen on IL-1β-induced IL-8/CXCL8 release. Eotaxin/CCL11 showed no effect on TNF-α-induced up-regulation of growth-related oncogene-α or IFN-γ-inducible protein-10, two other CXC chemokines tested, and did not affect production of the CC chemokines monocyte chemoattractant protein-1/CCL2 and RANTES/CCL5, or the adhesion molecules ICAM-1 and E-selectin. These results suggest that eotaxin/CCL11 is not effecting a general suppression of TNF-αR levels or signal transduction. Suppression of IL-8/CXCL8 was abrogated in the presence of anti-CCR3 mAb, pertussis toxin, and wortmannin, indicating it was mediated by the CCR3 receptor, G proteins, and phosphatidylinositol 3-kinase signaling. Eotaxin/CCL11 decreased steady state levels of IL-8/CXCL8 mRNA in TNF-α-stimulated cells, an effect mediated in part by an acceleration of IL-8 mRNA decay. Eotaxin/CCL11 may down-regulate production of the neutrophil chemoattractant IL-8/CXCL8 by endothelial cells in vivo, acting as a negative regulator of neutrophil recruitment. This may play an important biological role in the prevention of overzealous inflammatory responses, aiding in the resolution of acute inflammation or transition from neutrophilic to mononuclear/eosinophilic inflammation. The Journal of Immunology, 2002, 168: 2887–2894.

Chemokines are a large family of small, basic peptides that have mainly been characterized as leukocyte chemoattractants. They are divided into four subfamilies based on the number and spacing of conserved cysteines within the amino terminus of each peptide, with each subfamily having a limited specificity for different leukocyte subsets. Chemokines from the CXC family, in which the first two conserved cysteines are separated by a nonconserved amino acid, are chemoattractants for polymorphonuclear phagocytes, some T lymphocyte subsets, and NK cells (1). Members of the CC family, in which the cysteines are adjacent to one another, have a broader spectrum of action that includes monocytes, eosinophils, basophils, NK cells, and T lymphocytes (2). Lymphotactin/XCL1 and fractalkine/CX3CL1 are the sole members of the C and CX3C families, respectively. Lymphotactin is chemotactic for T lymphocytes (3), while fractalkine acts on both lymphoid cells and neutrophils (4). While chemokines are widely recognized as important chemotactic factors, some of these proteins have other varied functions, including immunoregulation (5–7), lymphocyte activation (8, 9), embryonic development (10, 11), and angiogenesis (12–14).

Eotaxin/CCL11 is a member of the CC chemokine family that has potent chemotactic activity for eosinophils (15, 16), basophils (17), mast cells (18), and Th2-type lymphocytes (19). Eotaxin/CCL11 protein is up-regulated in a variety of inflammatory diseases possessing an eosinophilic component, such as allergic asthma (20, 21), chronic sinusitis (22), and allergic rhinitis (23), and is thought to be a key player in the pathogenesis of these conditions. In addition, eotaxin/CCL11 mRNA is up-regulated in the lesions of patients with inflammatory bowel disease (24) and within lymphomas from patients with Hodgkin’s disease (25), suggesting that eotaxin/CCL11 may play a role in these diseases as well.

Early reports characterizing the tissue expression patterns of eotaxin/CCL11 in human, guinea pig, and mouse tissues demonstrate that eotaxin/CCL11 mRNA is constitutively expressed in a wide array of tissues, including the gut mucosa, lung, heart, testes, and endometrium (15, 26–28). Relatively few chemokines are expressed in a constitutive fashion. The constitutive expression of eotaxin/CCL11 in a wide variety of tissues, often in the absence of a significant eosinophil infiltrate, suggests that it may play a role in maintaining homeostasis in these tissues. Eotaxin/CCL11 exerts its chemotactic activity primarily through the chemokine receptor CCR3, a seven-transmembrane receptor coupled to heterotrimeric G proteins. The CCR3 has been found on human brain endothelial cells (29, 30), and recent studies indicate it may be involved in angiogenesis (31). To further study CCR3 function in endothelial cells in vitro, we investigated whether primary cultures of endothelial cells express CCR3, and we uncovered a novel regulatory role for eotaxin and CCR3 on endothelial cell chemokine production.

Materials and Methods

Cytokines and other reagents

Recombinant human IL-1β, TNF-α, eotaxin/CCL11, RANTES/CCL5, and monocyte chemoattractant protein-1 (MCP-1)3/CCL2 were purchased from R&D Systems (Minneapolis, MN). Abs against E-selectin, ICAM-1, MCP-1, IL-8/CXCL8, MCP-1/CCL2, RANTES/CCL5, and CCR3 were purchased from R&D Systems. Actinomycin D, pertussis toxin, and wortmannin were purchased from Sigma-Aldrich (St. Louis, MO) and stored at a

3 Abbreviations used in this paper: MCP-1, monocyte chemoattractant protein-1; Groα, growth-related oncogene-α; HDMEC, human dermal microvascular endothelial cell; IP-10, IFN-γ-inducible protein-10; PI3K, phosphatidylinositol 3-kinase.
concentration of 2.5 mg/ml, 100 ng/µl, and 10 mM, respectively, in DMSO at −20°C.

**Cell culture**

Human dermal microvascular endothelial cells (HDMEC) were obtained in single donor amnions from Clonetics. Cells were passaged by trypsinization and seeding at 1 × 105 cells/ml in endothelial cell growth medium-2 (Clonetics, Walkersville, MD) medium on plates or flask (Costar, Corning, NY) coated with 2% gelatin (Sigma-Aldrich). Cells were used at passages 2–6 for all experiments.

**RNase protection assay**

Total RNA was isolated using TRIzol reagent (Life Technologies, Rockville, MD), according to manufacturer’s instructions, and used in the standard BD PharMingen (San Diego, CA) RNase protection protocol, as follows. The multiprobe template sets include CR5 (containing DNA templates for CCR1, CCR3, CCR4, CCR5, CCR8, CCRα2b, CCR2α, CCR2b, L32, and GAPDH) and hCR6 (containing DNA templates for CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CX3CR1, L32, and GAPDH) were purchased from BD PharMingen. This template set was used to synthesize [α-32P]UTP (Anachemia, Little Chalfont, Buckinghamshire, U.K.)–labeled probes in the presence of a GACU pool using a T7 RNA polymerase. Probes were hybridized overnight with 10–15 µg target RNA, followed by RNase digestion and protamine K treatment. Samples were chloroform extracted, ethanol precipitated in the presence of ammonium acetate, and loaded on an acrylamide–urea sequencing gel made in TBE (0.53 M Tris–HCl, 0.53 M boric acid, and 0.02 M EDTA) buffer. After electrophoresis, the gel was adsorbed to glass test plates (Fisher Scientific, Pittsburgh, PA) and dried under vacuum, and exposed to [32P]phosphoglycerate for autoradiography (X-OMAT; Kodak, Rochester, NY) with intensifying screen at −70°C. Alternatively, the dried gel blots were exposed to a phosphor screen for phosphor imagery analysis using the Quantity One software application (Bio-Rad, Richmond, CA). The intensity of each band was normalized to the intensity of the housekeeping gene L32.

**Flow cytometry analysis of chemokine receptor expression**

Single cell suspensions were prepared by harvesting adherent cells with cold EDTA (0.05%, m/v), then pelleting and washing three times with flow buffer (Dulbecco’s PBS plus 0.5% BSA). Cells were resuspended in 25 µl flow buffer, mixed with 10 µl FITC-conjugated rat anti-human CCR3 Ab (50 µg/ml; R&D Systems) or appropriate isotype control Ab, and incubated at 4°C for 30 min. Cells were washed three times in flow buffer, fixed for 10 min in 2% paraformaldehyde, and stored in flow buffer at 4°C until FACS analysis. Fluorescent flow cytometric analysis was performed on a Coulter EPICS XL cytometer, with at least 5000 cells being counted per sample.

**ELISA analysis of chemokine protein in cell supernatants**

Human growth-related oncogene-α (GROα)/CXCL1, IFN-γ-inducible protein-10 (IP-10)/CXCL10, and stromal cellderived factor-1α (Cxcl12) protein levels were measured using Quantikine Immunoassay kits (R&D Systems), according to manufacturer’s instructions. Human IL-8/CXCL8, MCP-1/CCL2, and RANTES/CCL5 protein levels were determined in 50-µl samples from cell supernatants using a standard sandwich ELISA technique previously described in detail (5). In brief, Nunc-immuno ELISA plates (Fisher Scientific, Springfield, NJ) were coated with the appropriate polyclonal capture Ab (R&D Systems), washed, and blocked with 2% BSA/PBS. Plates were then washed and cell supernatants were added. After incubation at 37°C for 1 h, plates were washed and the appropriate biotinylated polyclonal detection Ab was added. After a 45-min incubation, plates were washed and streptavidin-peroxidase (1/5000 dilution; Bio-Rad) was added for 30 min. Plates were thoroughly washed, a chromagen substrate (3,3′,5′-tetramethylbenzidine, Sigma-Aldrich) was added to stop reaction, and chromophore development was determined by measuring OD450 using microplate reader. OD readings from samples stained with goat IgG were consistently indistinguishable from readings taken from unstained samples, indicating no nonspecific binding of the Ab was occurring.

**RNA isolation and cDNA synthesis**

For analysis of chemokine mRNA, total RNA was isolated using TRIzol reagent (Life Technologies), according to manufacturer’s instructions. Total RNA was used to synthesize first-strand cDNA using standard techniques. cDNA was stored at −20°C until RT-PCR analysis.

**Real-time RT-PCR analysis**

Relative levels of chemokine mRNA were analyzed using semi-quantitative real-time PCR analysis. Predeveloped probes for detection of human IL-8/CXCL8 and the housekeeping gene cyclophilin were purchased from PE Applied Biosystems (Foster City, CA) and stored at −20°C. Each reaction mixture contained primer/probe sets for detecting the amplification of the target gene of interest as well as the internal control cyclophilin, to control for intersample variation in the amount of cDNA added. Each reaction mixture was prepared by combining the following components: 12.5 µl TaqMan 2×PCR Mix (PE Applied Biosystems), 1 µl target probe, 1 µl control probe, 0.5 µl prepared cDNA, and 10 µl sterile water. Samples were amplified on a PRISM 7700 Sequence Detection System (PE Applied Biosystems) under the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C. The amount of a particular cDNA species within a sample is related logarithmically to the Ct number, which indicates the first cycle number at which amplification is detectable. Calculation of the relative amount of each cDNA species was done according to standard manufacturer’s protocol. In brief, the amplification of target genes in stimulated cells was calculated by first normalizing to the amplification of cyclophilin and then expressing these normalized values as a fold increase over the value obtained with unstimulated control cells.

**Statistical analysis**

Results are expressed as mean ± SEM of n experiments, unless otherwise stated. One-way ANOVA followed by a Bonferroni posttest between selected samples was performed using GraphPad Prism version 3.0a (GraphPad Software, San Diego, CA) for Macintosh (Apple Computer, Cupertino, CA). Results were deemed significant if p < 0.05.

**Results**

**CCR3 is expressed on the surface of HDMEC**

To assess whether unstimulated HDMEC express CCR3 protein, we performed flow cytometry analysis on single cell suspensions of HDMEC from five different donors that had been grown to 70–90% confluence (Fig. 1). A significant subpopulation of HDMEC showed positivity when stained with a mAb against CCR3 (Fig. 1). When results from five donors were pooled, 35.6 ± 3.6% stained positively with an anti-CCR3 Ab vs 7.4 ± 2% with control Ab (p < 0.001), indicating these results were statistically significant.

To study cytokine regulation of surface levels of CCR3, HDMEC monolayers were grown to confluence and stimulated with the proinflammatory cytokines TNF-α or IL-1β (5 ng/ml; 24 h). CCR3 protein expression was then analyzed by flow cytometry (Fig. 2A). In all donors tested (n = 6), TNF-α stimulation increased the number of CCR3-positive cells (Fig. 2A). However, parallel cultures stimulated with IL-1β showed no up-regulation in surface levels of

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C. CCR3, with donors showing no change or decreases in CCR3 surface levels (Fig. 2A). The basis of this variability is unclear, as cultures were all treated at the same growth stage and with freshly prepared aliquots of IL-1β. These data demonstrate that TNF-α stimulation consistently increases the frequency of CCR3-positive cells, while IL-1β does not.

To determine whether cytokine-induced changes in CCR3 protein were mirrored by similar changes in mRNA expression, we performed RNase protection analysis on RNA samples taken from HDMEC stimulated with IL-1β, TNF-α, or medium alone (5 ng/ml; 8 h) (Fig. 2B). When RNA probes for CC chemokine receptors were used, CCR2, CCR3, CCR4, and CCR8 mRNA were present in unstimulated HDMEC, while CCR1 and CCR5 mRNA were undetectable. Unexpectedly, cytokine stimulation did not affect the expression patterns of any of these chemokine receptor mRNAs. CXCR4 was the only CXC receptor message detected in unstimulated HDMEC, and as previously demonstrated, was down-regulated by both IL-1β and TNF-α (32) (gel not shown). Quantitative results were obtained by normalizing the band intensities of CCR3 or CXCR4 to that of the housekeeping gene L32; pooled results from three donors are depicted in Fig. 2C.

Recombinant eotaxin/CCL11 reduces IL-8/CXCL8 release induced by TNF-α

After we had established the presence of CCR3 protein on the surface of HDMEC, we then investigated whether interaction of CCR3 with its major ligand, eotaxin, affected parameters of endothelial cell activation. Because both TNF-α and IL-1β are potent stimulators of chemokine production by endothelial cells, we chose to test whether eotaxin/CCL11 was involved in modulating production of a CXC family chemokine, IL-8/CXCL8. As has been shown previously, we found that 48-h stimulation of HDMEC with TNF-α and IL-1β (5 ng/ml) strongly up-regulated IL-8/CXCL8 production, as measured by ELISA analysis of cell-free supernatants (Fig. 3). Surprisingly, the addition of recombinant eotaxin/CCL11 (20 ng/ml) during the period of cytokine stimulation partially suppressed TNF-α induction of IL-8/CXCL8 production (Fig. 3). Eotaxin/CCL11 suppression of TNF-α-induced IL-8/CXCL8 was concentration dependent, with a suppressive effect being found at eotaxin/CCL11 concentrations from 20 to 200 ng/ml (Fig. 3A). Eotaxin had no effect on IL-1β-induced chemokine levels (Fig. 3A), which is consistent with the variable effects of IL-1β on CCR3 expression in these cells. The magnitude of suppression varied with the concentration of TNF-α stimulation, with the greatest suppression being obtained at a TNF-α concentration of 0.5 ng/ml (Fig. 3B).

Recombinant eotaxin/CCL11 does not suppress TNF-α induction of other chemokines and adhesion molecules

Endothelial cells elaborate a number of other CXC-type chemokines in addition to IL-8/CXCL8. When cell-free supernatants from the same experiment were assayed in parallel for IL-8/CXCL8 content as well as two other CXC chemokines, Groα/CXCL1 and IP-10/CXCL10, eotaxin suppressed the TNF-α-induced secretion of IL-8/CXCL8, but not the latter two chemokines (Fig. 4). An additional CXC chemokine, stromal cell-derived factor-1α, was not detectable under any of the stimulatory conditions.
tested. TNF-α is also a strong inducer of the CC chemokines MCP-1/CCL2 and RANTES/CCL5, as well as the adhesion molecules E-selectin and ICAM-1. Recombinant eotaxin/CCL11 had no effect on TNF-α up-regulation of these molecules (Fig. 5), suggesting that eotaxin/CCL11 is exerting a very specific effect on the glutamine-leucine-arginine (ELR)-positive CXC chemokines IL-8/CXCL8 rather than a broad effect on TNF-αR levels or signaling. We also used an MTT assay to demonstrate that eotaxin had no effect on cellular viability (data not shown), a result consistent with the lack of effect on molecules other than IL-8/CXCL8.

Anti-CCR3 Ab, wortmannin, and pertussis toxin block the effect of eotaxin/CCL11 on IL-8/CXCL8 secretion

Eotaxin/CCL11 suppression of IL-8/CXCL8 production was blocked by a neutralizing mAb against CCR3, indicating that the

FIGURE 3. Recombinant eotaxin suppresses IL-8/CXCL8 release from TNF-α-stimulated HDMEC. Significant variation was found in basal (0.02–3.98 ng/ml) and TNF-α-elicted (2.16–84.14 ng/ml) levels of IL-8 from cells from different donors. To pool results from different experiments, the data were normalized by setting the IL-8 level induced by 5 ng/ml TNF-α in each experiment as 1 and then calculating the fraction with respect to 1 for each condition within that experiment (Fraction of maximum). These normalized values from each experiment were then pooled and shown as the mean ± SEM of four to six experiments done in triplicate. A, Cells were stimulated with TNF-α or IL-1β (5 ng/ml) with or without eotaxin (2–200 ng/ml) for 48 h and analyzed for immunoreactive IL-8/CXCL8 content by ELISA. B, Cells were stimulated with TNF-α (0.05–5 ng/ml) with or without eotaxin (20 ng/ml) for 48 h. Cell-free supernatants were collected and analyzed for immunoreactive IL-8/CXCL8 content by ELISA. *, p < 0.05.

FIGURE 4. Eotaxin/CCL11 does not affect TNF-α-induced Groα/CXCL1 or IP-10/CXCL10. Cells were stimulated with TNF-α or IL-1β (5 ng/ml) with or without eotaxin (2–200 ng/ml) for 48 h and analyzed in parallel for immunoreactive IL-8/CXCL8, Groα/CXCL1, or IP-10/CXCL10 content by ELISA.

FIGURE 5. Eotaxin/CCL11 does not affect TNF-α-induced MCP-1/CCL2 and RANTES/CCL5 release, or E-selectin and ICAM-1 expression. A, Cells were stimulated with TNF-α (0.05–5 ng/ml) with or without eotaxin (20 ng/ml) for 48 h. Cell-free supernatants were collected and analyzed for immunoreactive IL-8/CXCL8, MCP-1/CCL2, and RANTES/CCL5 content by ELISA. Results shown are the means ± SE of data from triplicate wells of one experiment and are representative of data obtained in two separate experiments. For each chemokine, the results were normalized by setting the level induced by 5 ng/ml TNF-α as 100% and calculating all other values in the experiment as a fraction of this value. B, Cells were stimulated with TNF-α (5 ng/ml) with or without eotaxin (2–200 ng/ml) for 4 h and analyzed for cell surface expression of E-selectin using a modified ELISA format. C, Cells were stimulated with TNF-α (5 ng/ml) with or without eotaxin (2–200 ng/ml) for 24 h and analyzed for cell surface expression of ICAM-1 using a modified ELISA format. *, p < 0.05.
suppressive effect of eotaxin/CCL11 is mediated via binding to CCR3 (Fig. 6A). Eotaxin suppression of IL-8/CXCL8 production was also abrogated in the presence of pertussis toxin and wortmannin (Fig. 6B), indicating a dependence on $G_\text{i}$ proteins and phosphatidylinositol 3-kinase (PI3K). This is consistent with previous reports demonstrating these mediators are important in CCR3 signaling in eosinophils (33).

Recombinant eotaxin/CCL11 reduces steady state levels of IL-8/CXCL8 mRNA via effects on mRNA stability

Because we found that eotaxin/CCL11 was exerting a specific effect on IL-8/CXCL8 protein production, we investigated whether it was reducing steady state levels of IL-8 mRNA. Confluent cells were stimulated with TNF-$\alpha$ (5 ng/ml) with or without eotaxin (20–200 ng/ml), and total RNA was harvested at 48 h. This RNA was used to synthesize cDNA, which was analyzed for IL-8 expression using real-time PCR (TaqMan) analysis. IL-8/CXCL8 mRNA was elevated in TNF-$\alpha$-stimulated samples ~5.5-fold over control levels (Fig. 7). This elevation was suppressed in samples stimulated with TNF-$\alpha$ and eotaxin, with a concentration-dependent effect being found at eotaxin concentrations of 20 and 200 ng/ml. Eotaxin did not affect the steady state levels of IL-1$\beta$-induced IL-8 mRNA (data not shown), which is consistent with its lack of effect on IL-1$\beta$-induced IL-8 release.

IL-8/CXCL8 production is controlled at the level of transcription and mRNA stability (34, 35). Because eotaxin did not affect cytokine induction of adhesion molecules or other chemokines, we reasoned that the mechanism of eotaxin/CCL11 suppression of IL-8/CXCL8 release was likely to be posttranscriptional. To test whether eotaxin/CCL11 affects mRNA stability, we stimulated HDMEC with TNF-$\alpha$ (5 ng/ml) with or without eotaxin/CCL11 (100 ng/ml) for 24 h. At this time, actinomycin D (5 $\mu$g/ml) was added to inhibit transcription. Total RNA was harvested at appropriate time points after actinomycin D addition, cDNA was synthesized, and samples were assayed for steady state levels of IL-8/CXCL8 mRNA using real-time RT-PCR analysis. Results are expressed as fold increase in IL-8 mRNA levels over cells grown in medium alone. Please refer to Materials and Methods for further details on the types of calculations involved. Data shown are from one experiment done in triplicate and are representative of three experiments done with cells from different donors.

8/CXCL8 release was likely to be posttranscriptional. To test whether eotaxin/CCL11 affects mRNA stability, we stimulated HDMEC with TNF-$\alpha$ (5 ng/ml) with or without eotaxin/CCL11 (100 ng/ml) for 24 h. At this time, actinomycin D (5 $\mu$g/ml) was added to inhibit transcription. Total RNA was harvested at appropriate time points after actinomycin D addition, cDNA was synthesized, and samples were assayed for steady state levels of IL-8/CXCL8 mRNA using real-time PCR analysis. Samples stimulated with TNF-$\alpha$ (5 ng/ml) alone showed steady degradation of IL-8 mRNA, with ~40% remaining at 4 h after actinomycin D addition (Fig. 8). The rate of degradation was significantly increased in samples stimulated with TNF-$\alpha$ and eotaxin (100 ng/
ml), with levels dropping to ~40% within 30 min after actinomycin D addition. IL-8 mRNA levels did not drop significantly below 40% under either treatment condition.

**Discussion**

Endothelial cells act as physical and functional barriers between the vascular space and the tissue. Rather than being passive bystanders to leukocyte transmigration, endothelial cells are important participants in the development and maintenance of inflammation (36). Upon activation by soluble mediators or physical trauma, they express a number of leukocyte chemoattractants and adhesion molecules that work together to cause leukocyte adhesion and diapedesis into tissues (37). While endothelial cell activation is part of a typical immunoinflammatory response, inappropriate or overzealous activation can lead to tissue damage by infiltrating leukocytes. Thus, a better understanding of the factors regulating endothelial cell activation may provide insights into possible antiinflammatory therapies.

A number of chemokines are active on endothelial cells (13, 14, 32, 38–40). Several groups have documented expression of CXC-type receptors by these cells (12, 32, 39, 41, 42), CXCR1, CXCR2, and CXCR4 function as angiogenic receptors (32, 39), while CXCR3 has an angiostatic function (43–45). In addition, the CC-type receptors CCR2 (46) and CCR3 (31) have also been documented on endothelial cells. The expression of CXCR4 is downregulated by TNF-α (32) and up-regulated by basic fibroblast factor and vascular endothelial growth factor (42), while CCR2 is up-regulated by IL-1β (47), suggesting that regulation of these endothelial cell chemokine receptors by cytokines or growth factors may be important in various disease or physiological processes.

To study the regulation and function of CCR3 on endothelial cells in vitro, we first established the expression of this receptor on HDMEC. We found that a subpopulation of proliferating HDMEC has significant levels of surface-expressed CCR3, as assayed by flow cytometry. CCR3 was only expressed on a subpopulation of proliferating HDMEC. This finding differs slightly from results obtained by Salcedo et al. (31), which demonstrate low levels of CCR3 expression across the entire cell population in human microvascular endothelial cells. The basis for these differences is unclear, but is likely to involve differences in source tissue, culture conditions, or harvesting techniques. Interestingly, a recent report shows that the CXC-type chemokine receptor CXCR3 exhibits a cell cycle-specific expression on endothelial cells, which explains why its expression is detectable on a small subpopulation of cells from proliferating cultures (43). A similar cell cycle-restricted expression pattern may exist for CCR3 on HDMEC.

The level of surface-expressed CCR3 protein on HDMEC could be up-regulated by TNF-α, a cytokine that has potent proinflammatory effects on these cells. Surprisingly, IL-1β did not have consistent effects on surface levels of CCR3. While TNF-α and IL-1β are generally quite similar in the signaling molecules they use (47, 48), these cytokines also activate nonoverlapping signaling pathways (49–51). TNF-α up-regulation of CCR3 protein expression is probably dependent on a TNF-α-specific pathway that is not activated by IL-1β. Because TNF-α does not affect CCR3 mRNA levels, up-regulation of CCR3 surface protein by TNF-α is most likely dependent on posttranscriptional mechanisms such as decreased receptor internalization or altered receptor trafficking. Posttranscriptional control of chemokine receptor levels has already been demonstrated for other receptor types (52).

Next we demonstrated that eotaxin/CCL11 suppressed the production of IL-8/CXCL8 by TNF-α-stimulated endothelial cells. Suppression of IL-8/CXCL8 was abrogated in the presence of a neutralizing Ab against CCR3, indicating that eotaxin/CCL11 was exerting its effects via CCR3. In addition, treatment with pertussis toxin and wortmannin also blocked the suppressive effect of eotaxin/CCL11, indicating the involvement of G proteins and PI3K. Previous reports have indicated the activity of these mediators in CCR3-mediated chemotaxis and respiratory burst in eosinophils (33). This study now demonstrates that a novel link exists between a Gαi-PI3K-dependent pathway and chemokine production in HDMEC.

The mechanism by which eotaxin/CCL11 suppresses production of IL-8/CXCL8 appears to be via an eotaxin/CCL11-induced acceleration of IL-8/CXCL8 mRNA decay. Previous reports have indicated that cellular control of IL-8/CXCL8 production is largely regulated at the posttranscriptional level (35, 53, 54). Experimental evidence suggests that binding of certain proteins to AU-rich regions in the 3′-untranslated regions of chemokine mRNAs modulates the stability of these mRNAs (55). At present, it is not known, however, whether eotaxin regulates the expression of any known AU-binding factors.

Disparate results between TNF-α- and IL-1β-stimulated cells were observed in that eotaxin/CCL11 did not affect the production of IL-8/CXCL8 by IL-1β-stimulated endothelial cells nor the degradation rate of IL-1β-stimulated IL-8/CXCL8 mRNA. We hypothesize that this is related to the specific mechanism of eotaxin action. Eotaxin most likely activates an mRNA-destabilizing pathway in endothelial cells. It has already been shown that this pathway is countered in IL-1β-stimulated cells, but not in TNF-α-stimulated cells, in that IL-1β, but not TNF-α, activates a signaling pathway that leads to protein-dependent stabilization of an IL-8 homolog, the murine chemokine KC (49). The rate of mRNA degradation is also not the main determinant of the basal “setpoint” of IL-8 release in unstimulated endothelial cells, which is controlled at the level of IL-8 basal promotor activity (56). This mechanism of eotaxin action would explain its lack of effect on IL-8 secretion from IL-1β-stimulated and resting endothelial cells.

Other factors with antiinflammatory effects on vascular inflammatory responses include TGF-β, IL-1R antagonist, and IL-10 (57). The mechanism of action of each of these antiinflammatory cytokines results in inhibition of a broad array of activation parameters, including multiple chemokines and adhesion molecules. Unlike these cytokines, eotaxin/CCL11 specifically inhibits the production of the CXC chemokine IL-8/CXCL8 but does not affect expression of other chemokines or adhesion molecules. The biological significance of this specificity is unknown; however, we speculate that eotaxin may play a role in specifically dampening neutrophil influx via down-regulation of IL-8/CXCL8 in vivo. IL-8/CXCL8 is generally regarded as the most robust and specific neutrophil chemoattractant known. In contrast, IP-10/CXCL10 is known as a lymphocyte chemoattractant (58), while MCP-1/CCL2 and RANTES/CCL5 attract primarily mononuclear cells. Therefore, a specific reduction in IL-8/CXCL8 might affect neutrophil influx without affecting the trafficking of other leukocyte subtypes. It is interesting to speculate whether eotaxin may be a key effector of the switch from granulocytic to mononuclear inflammation observed in vivo during the time course of acute infection.

Surprisingly, eotaxin/CCL11 did not suppress release of Groa/CXCL1, another CXC chemokine that acts as a neutrophil chemoattractant in vitro. However, a recent report indicates that activated neutrophils do not respond to Groa (59), although they give robust responses to IL-8/CXCL8. Therefore, IL-8/CXCL8 may be more relevant to neutrophil influx in vivo.

A recent report supports a regulatory role for endogenous eotaxin/CCL11 in vivo. Guo et al. (60) have demonstrated that
endoogenous eotaxin/CCL11 is expressed during acute lung inflammation and negatively regulates neutrophil influx into the lungs. Furthermore, this study shows that eotaxin/CCL11-induced decreases in neutrophil content correlate with decreased lung damage. This study also demonstrated that recombinant eotaxin specifically decreases levels of the neutrophil-specific CXC chemokines macrophage-inflammatory protein 2 and cytokine-induced neutrophil chemotactant in stimulated rat alveolar macrophages. Our studies suggest that the mechanism of the effect of eotaxin/CCL11 may also involve altering levels of endothelial cell-derived IL-8/CXCL8. Taken together, these two studies suggest that eotaxin/CCL11 can affect neutrophil trafficking in vivo via effects on several different cell types.

We have demonstrated a novel link between CCR3 chemokine receptor activation and a pertussis toxin-sensitive, PI3K-dependent pathway that leads to an acceleration in IL-8/CXCL8 mRNA decay and a decrease in IL-8/CXCL8 protein release. Endothelial-derived IL-8/CXCL8 is most likely a key effector of neutrophil elicitoin in vivo. Eotaxin may act as an important negative regulator that, in summation with proinflammatory factors, regulates the net output of IL-8/CXCL8 in vivo. Eotaxin/CCL11 down-regulator that, in summation with proinflammatory factors, may also involve altering levels of endothelial cell and umbilical vein endothelial cells, aiding in the resolution of acute inflammation, or transition from neutrophilic to monocellular/eosinophilic inflammation.

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