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Analysis of the Gene Expression Profile Activated by the CC Chemokine Ligand 5/RANTES and by Lipopolysaccharide in Human Monocytes

Massimo Locati,* Ullrich Deuschle,‡ Maria L. Massardi,‡ Fernando O. Martinez,* Marina Sironi,§ Silvano Sozzani,§§ Tamas Bartfai,† and Alberto Mantovani 4§

The gene expression profile induced by the CC chemokine ligand (CCL) 5/RANTES in human monocytes was examined using the oligonucleotide array technology. Of 5600 transcripts examined, 42 were consistently induced by CCL5, and none were suppressed. Chemokine-inducible transcripts could be clustered in functional groups, including selected cytokines and receptors (e.g., IL-1β, CCL2/monocyte chemotactic protein-1, and the CCL5 receptor CCR1) and molecules involved in extracellular matrix recognition and digestion (e.g., CD44 splice transcripts, urokinase-type plasminogen activator receptor, matrix metalloproteinase (MMP)-9, and MMP-19). Transcript expression, confirmed by quantitative real-time PCR analysis for selected genes, was associated with protein induction for some (e.g., CCL2), but not all (e.g., IL-1β), transcripts examined. The chemokine-induced gene profile was distinct from that activated by LPS, a prototypic phagocyte activator. Although certain transcripts were stimulated by both agonists (e.g., IL-1β and CCL2), others were induced only by either LPS (e.g., TNF-α and IL-6) or CCL5 (e.g., MMP-19) or were divergently regulated (e.g., CCR1). Thus, CCL5, a prototypic CC inflammatory chemokine, activates a restricted transcriptional program in monocytes distinct from that induced by the prototypic pathogen-derived proinflammatory stimulant LPS. Chemokine-induced chemokines production could represent a novel amplification loop of leukocyte recruitment, while a subset of chemokine-inducible transcripts could be involved in monocyte extravasation and tissue invasion. The Journal of Immunology, 2002, 168: 3557–3562.

L euocyte extravasation is a multistep process involving many different signals, including chemoattractants, adhesion molecules, and proteases. This process leads to monocyte recruitment into tissues, where they further differentiate and exert biological functions. The role of the chemokines in this process is to increase adhesion to the endothelium and attract leukocytes into the tissue. Chemokines are a large superfamily of secreted proteins, including >40 members distributed into four different families according to structural features related to the relative positions of cysteine residues. In general, chemokines are functionally divided into inducible or inflammatory chemokines, not expressed under resting conditions and rapidly induced in response to homeostasis perturbation, and constitutive chemokines, physiologically expressed in defined tissue compartments (1, 2). These two subsets subserve different functions, the first mainly involved in inflammatory reactions, and the second most relevant for secondary lymphoid organ organogenesis (2, 3).

Chemokines exert their biological functions through interaction with seven-transmembrane G protein-coupled specific receptors differentially expressed on leukocyte populations (1). Agonist binding causes activation of incompletely defined signaling events, leading to cytoskeleton reorganization and cell movement. There is also evidence that chemokines can regulate functions other than cell adhesion and migration, including cell proliferation and death (4–8) and cytokine production (9–11). However, available information on the mode by which chemokines regulate gene expression-dependent functions is fragmentary and conflicting, possibly depending on the presence of costimulatory signals (4, 5, 9, 10).

The aim of the present study was to define the gene expression profile of human monocytes exposed to the prototypic inflammatory CC chemokine ligand (CCL) 5/RANTES and to compare it with a prototypic primary proinflammatory pathogen-derived signal represented by LPS.

Materials and Methods

Reagents

Recombinant human CCL5 was obtained from PeproTech (Rocky Hill, NJ). LPS from Escherichia coli (serotype 055:B5), and all other chemicals not specified were obtained from Sigma-Aldrich (St. Louis, MO).

*Abbreviations used in this paper: CCL, CC chemokine ligand; MES, 2-(N-morpholino)ethanesulfonic acid; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; CXCL, CXC chemokine ligand; uPA-R, urokinase-type plasminogen activator receptor.
Human monocytes were obtained fromuffy coats of blood healthy donors through the courtesy of the Centro Trasfusionale, Spedali Civili (Brescia, Italy). Blood was washed twice in saline at 200 × g to remove plasma and platelets and stratified on isotonic Ficoll separating solution (Biochrom, Berlin, Germany) at 400 × g for 30 min at room temperature. Mononuclear cells were collected, and monocytes were further purified (>90% pure) by centrifugation at 600 × g for 30 min at room temperature on 46% isosmotic Percoll (Amersham Biosciences, Uppsala, Sweden) gradient as previously described (12). Monocytes were resuspended at 5 × 10^6/ml in RPMI 1640 medium (Biochrom) supplemented with 2% FCS (HyClone Laboratories, Logan, UT), plated in hydrophobic petriperm dishes (Sigma-Aldrich), and stimulated for indicated time.

**Analysis of gene expression profile using oligonucleotide array**

Analysis of gene expression was performed with commercial human gene probe arrays displaying 5600 human known genes (HuGeneFL array; Affymetrix, Santa Clara, CA). After cell stimulation, total RNA was prepared using TRIzol reagent (Life Technologies, Gaithersburg, MD) following the manufacturer’s instructions. Sample labeling and processing were performed according to the supplier’s instructions essentially as previously described (13), except that hybridization was performed in 1 × 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer (MES (pH 6.7), 1 mM NaCl, and 0.01% Triton X-100), and chips were washed using 0.1 M -morpholino)ethanesulfonic acid (MES) buffer (MES (pH 6.7), 1 mM NaCl, and 0.01% Triton X-100), and chips were washed using 0.1 M MES buffer at 45°C. Data were collected by laser scanning, and pixel values were analyzed with GeneChip 3.0 software (Affymetrix). Expression values of transcripts as scoring as present (according to an internal program algorithm) were normalized, according to the total signal intensity on the chip, and compared. In agreement with the indications of the manufacturer and other users (13), transcripts with a ratio of normalized expression levels between 2.0 or 0.5 were regarded as modulated in the single experiment under evaluation. We considered a gene as CCL5-responsive if it satisfied these criteria in at least three of four independent experiments with cell preparations from different donors and was not acting in the opposite manner in the fourth experiment.

**Quantitation of gene expression by real-time PCR analysis**

Total RNA was prepared using TRIlol reagent (Life Technologies), and treated with DNase I using the DNA-free kit (Ambion, Austin, TX), and reverse transcribed for 1 h at 42°C using an oligo(dT)12-18 primer (Life Technologies) and the SuperScript II RT kit (Life Technologies). A 0.1 μM of CD54 primer reagents mix (Applied Biosystems, Foster City, CA) containing 1 × SYBRGreen PCR buffer, 3 mM MgCl2, 100 μM dATP, dCTP, and dGTP, 200 μM dUTP; 0.025 μM AmpliTaq Gold DNA polymerase; 0.01 μM AmpErase UNG, and 2 pmol/μl gene-specific forward and reverse primers designated using the Primer Express software (Applied Biosystems, Foster City, CA) and listed in Table I. The reaction conditions were as follows: 2 min at 50°C (one cycle), 10 min at 95°C (one cycle), and 15 s at 95°C and 1 min at 60°C (40 cycles). Gene-specific PCR products were continuously measured by means of an ABI PRISM 5700 detection system (PerkinElmer, Norwalk, CT). Samples were normalized using the housekeeping gene β-actin. Five replicates for each experimental point were performed, and differences were assessed with the two-tailed Student’s t test. Results are expressed as the relative fold increase of the stimulated over the control group, which was used as a calibrator.

**Cytokine measurement by ELISA**

After purification, monocytes were resuspended at 2.5 × 10^6/ml in RPMI 1640 medium supplemented with 2% FCS, plated on hydrophobic petriperm dishes, and incubated at 37°C in 5% CO2. After 6-h cell stimulation, cell supernatants were collected, and cytokine secretion levels were measured using ELISA. The data were analyzed using the GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA).

**Table I. Quantitative real-time PCR analysis of CCL5/RANTES effect on target genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>CCL5 30 ng/ml</th>
<th>CCL5 100 ng/ml</th>
<th>CCL5 300 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2/MCP-1</td>
<td>5'-CATTGTGCCAGGACGATCTG-3'</td>
<td>9.3</td>
<td>19.8</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>5'-CCTCCAGAAGGTGGTTGCTT-3'</td>
<td>3.6</td>
<td>4.6</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>5'-GCCAAGAAGGCTGCGCAG-3'</td>
<td>4.2</td>
<td>3.5</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>Pre-B colony-enhancing factor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD166</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-GGATGCTGCCCCAACAAGAAG-3'</td>
<td>7.2</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>C5a receptor</td>
<td>3.2</td>
<td>5.2</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>EMP-1/BB</td>
<td>6.9</td>
<td>15.9</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>MMP-1</td>
<td>3.5</td>
<td>3.7</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>uPA-R</td>
<td>2.8</td>
<td>1.9</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>Plasminogen activator inhibitor-2</td>
<td>90.5</td>
<td>103.5</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>CYP1B</td>
<td>1.7</td>
<td>3.7</td>
<td>7.3</td>
</tr>
<tr>
<td>Mitogen-activated protein</td>
<td>5'-ACACCCCTCTTGGCATGATG-3'</td>
<td>12.4</td>
<td>8.2</td>
<td>3.5</td>
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<tr>
<td>Mitogen-activated protein</td>
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<td>1.2</td>
<td>3.9</td>
<td>3.0</td>
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<tr>
<td>Mitogen-activated protein</td>
<td>5'-GTGCCACATCTGACTAT-3'</td>
<td>0.7</td>
<td>2.6</td>
<td>3.9</td>
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<tr>
<td>Mitogen-activated protein</td>
<td>5'-AGTGGGCGGCGCGAATGTCATG-3'</td>
<td>1.8</td>
<td>3.7</td>
<td>2.4</td>
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<tr>
<td>Mitogen-activated protein</td>
<td>5'-ATCCACCCACGGAGGAAGTTA-3'</td>
<td>1.7</td>
<td>2.1</td>
<td>1.5</td>
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<tr>
<td>Mitogen-activated protein</td>
<td>5'-TCCGGGCGGGAAGCTGCTTGG-3'</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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<tr>
<td>Mitogen-activated protein</td>
<td>5'-AGGAAGAAGGCGGTGTAAG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Monocytes were stimulated for 2 h with CCL5/RANTES at the indicated concentrations. Total RNA was extracted, quantified, DNase-treated and reverse transcribed. Samples were normalized according to actin expression level. Target genes were amplified with indicated forward and reverse primers. Results, expressed as the ratio of stimulated cells over control cells, represent the mean of two independent experiments with two different donors."
supernatants were collected, and cells were lysed by three cycles of freezing and thawing. Cytokine concentrations were measured in triplicate for each sample using sandwich ELISAs based on a rabbit antiserum and a mAb (SD3-F7) for monocyte chemotactic protein CCL2/MCP-1 (14) and a biotinylated anti-IL-1β mAb (Endogen, Woburn, MA) for IL-1β (15).

Results

Gene expression profile induced by CCL5

The gene expression profile activated by the prototypic inflammatory chemokine CCL5 in human monocytes was analyzed using oligonucleotide array technology. Fig. 1 shows one experiment in which monocytes purified from healthy donors were stimulated under nonadherent conditions with 300 ng/ml CCL5 for 2 h. By way of comparison, monocytes were also stimulated for 2 h with 100 ng/ml LPS, a prototypic activation signal for mononuclear phagocytes. In agreement with previous reports (16, 17), LPS modulated a significant number of genes (~4%), up-regulating 105 and concomitantly down-regulating 136 transcripts in the experiment shown (Fig. 1A). As shown in Fig. 1B, monocyte exposure to CCL5 also resulted in the regulation of transcription of a restricted subset of genes (~2%), with up-regulation of 114 genes and mild inhibition (0.4- to 0.5-fold) of nine genes in this particular experiment.

The effect of CCL5 on gene expression was then examined at 2 and 8 h in four other independent donors in the same experimental system. In resting cells, 1495 ± 154 genes scored as expressed. Although a considerable donor-to-donor variation was observed (see individual data at the website http://www.marionegri.it/profile1), we identified 42 genes whose expression levels reproducibly increased after monocyte exposure to CCL5, while none of the expressed genes was found to be consistently down-regulated. Fig. 2 shows the time course of CCL5-responsive genes, clustered according to gene function. A first set of stimulated transcripts was represented by cytokines and their receptors, including IL-1β (9.84 ± 2.92- and 6.34 ± 1.70-fold at 2 and 8 h, respectively), CCL2 (6.85 ± 4.03- and 7.42 ± 9.90-fold at 2 and 8 h, respectively), CCL3/macrophage inflammatory protein-1α (MIP-1α); 2.45 ± 1.07- and 1.62 ± 0.75-fold at 2 and 8 h, respectively), CCL4/MIP-1β (3.29 ± 1.60- and 2.23 ± 0.56-fold at 2 and 8 h, respectively), and CXC ligand (CXCL) 8/IL-8 (5.40 ± 4.87- and 2.37 ± 1.97-fold at 2 and 8 h, respectively). Among receptors, the CC chemokine receptor CCR1 scored as induced (4.71 ± 1.93- and 2.69 ± 1.41-fold at 2 and 8 h, respectively). A second set of chemokine-inducible transcripts is related to interaction with and digestion of extracellular matrix components and includes the urokinase-type plasminogen activator receptor (uPA-R; 2.07 ± 0.87- and 1.47 ± 0.51-fold at 2 and 8 h, respectively), matrix metalloprotease-9 (MMP-9; 1.58 ± 1.17- and 3.40 ± 2.20-fold at 2 and 8 h, respectively), and MMP-19 (2.37 ± 1.31- and 1.81 ± 0.61-fold at 2 and 8 h, respectively). Moreover, CCL5 induced the appearance of variable region spliced transcripts of the hyaluronate receptor CD44 (3.61 ± 1.99- and 1.96 ± 1.03-fold at 2 and 8 h, respectively). Other functional sets included intracellular enzymes (e.g., cytochrome P450 isoform CYP1B1), cytoskeleton components (e.g., β2-tubulin), and molecules involved in cell signaling (e.g., mitogen-activated protein kinase phosphatases) and regulation of gene expression (e.g., cAMP response element binding protein-2).

To confirm array data and to investigate effect of CCL5 concentration on gene induction, a panel of CCL5-responsive genes was tested using different methodologies. Real-time PCR experiments confirmed the induction of all 16 molecules tested, including at least one molecule for each functional group (Table 1). In most cases gene induction was detectable at the lowest concentration tested (30 ng/ml) and was optimal at higher concentrations. Interestingly, despite the fact that different monocyte donors were used for array and real-time PCR, the rank order of gene induction was similar, and a significant correlation (r = 0.876; n = 16) was observed in the two sets of measurements. Induction of IL-1β, CCL2, and uPA-R transcripts was also confirmed by Northern blot analysis (not shown).

Transcript vs protein induction

Having observed CCL5-mediated transcript stimulation, it was important to assess whether protein production was, in fact,
stimulated. As shown in Fig. 3A and concordant with mRNA data, monocyte exposure to 300 ng/ml CCL5 for 6 h resulted in CCL2 secretion, although LPS was a more potent CCL2 inducer (0.14 ± 0.11, 0.12 ± 0.07, 0.11 ± 0.08, and 1.23 ± 0.97, 1.23 ± 0.97, and 2.87 ± 1.90 ng/ml for control, CCL5-treated, and LPS-treated cells, respectively; n = 5). Similarly, CCL5 augmented the percentage of cell adhesion to hyaluronate recognized by CD44 and the expression of uPA-R (data not shown). On the contrary, as shown in Fig. 3B, CCL5-induced IL-1β mRNA was not followed by protein secretion after 6 h of stimulation, different from what observed with LPS treatment (0.07 ± 0.03, 0.05 ± 0.02, and 1.64 ± 1.18 ng/ml for control, CCL5-treated, and LPS-treated cells, respectively; n = 5). To test whether CCL5 could induce intracellular accumulation of IL-1β, in a second series of experiments (three donors; not shown) we analyzed the cell lysate, detecting high concentrations of IL-1β in response to LPS (3.521 ± 0.592 ng/ml), but only minor concentrations with CCL5 (0.043 ± 0.004 ng/ml). Again, CCL5 did not induce IL-1β release, unlike LPS.

Comparison of the transcriptional programs activated by CCL5 and LPS
As shown in Fig. 4, of 105 genes stimulated by monocyte treatment with 100 ng/ml LPS for 2 h (quadrants A + B + C), only 21 were also induced by monocyte treatment with 300 ng/ml CCL5 for 2 h (quadrant C), including IL-1β (4.51- and 4.53-fold induction for LPS and CCL5, respectively) and CCL2 (3.73- and 4.36-fold induction for LPS and CCL5, respectively). LPS stimulated the expression of a series of cytokines and chemokines that were unaffected by CCL5, including TNF-α (2.60- and 0.69-fold induction for LPS and CCL5, respectively), IL-6 (4.87- and 1.07-fold induction for LPS and CCL5, respectively), and CCL23/myeloid progenitor inhibitory factor-1; 6.34- and 1.60-fold induction for LPS and CCL5, respectively). Similarly, cyclooxigenase-2 was stimulated by LPS, but not by CCL5 (5.22- and 1.21-fold induction, respectively). In this particular experiment, 72 genes were stimulated by CCL5 but were unaffected by LPS (Fig. 4, quadrant F), including MMP-19 (0.88- and 2.41-fold induction for LPS and CCL5, respectively), lectin-like oxidized low density lipoprotein receptor (oligoclonal) 1 (CLP1), and fibrinogen alpha chain (FGB).
receptor (1.42- and 2.40-fold induction for LPS and CCL5, respectively), and the decay-accelerating factor receptor (1.16- and 2.49-fold induction for LPS and CCL5, respectively), while 21 genes induced by CCL5 were oppositely regulated by LPS (Fig. 4, quadrant I), including CCR1 (0.35- and 2.43-fold of induction for LPS and CCL5, respectively; in agreement with data reported in Ref. 18), and plectin (0.40- and 2.16-fold of induction for LPS and CCL5, respectively). As expected, LPS was able to down-regulate a consistent set of genes that resulted unaffected by CCL5 treatment (114 in the experiment shown; Fig. 4, quadrant H), including CCR5 (0.33- and 0.76-fold induction for LPS and CCL5, respectively; in agreement with data reported in Ref. 18), and the M-CSF receptor (0.40- and 1.97-fold induction for LPS and CCL5, respectively; in agreement with data reported in Ref. 16).

Discussion

The results presented in this study show that the prototypic inflammatory chemokine CCL5 activates a defined transcriptional profile in fresh human monocytes consisting of stimulation of at least 42 genes of 5600 examined. Consistent gene suppression was not observed after exposure to CCL5, whereas down-regulated genes were detected when LPS was used in the same conditions. Although chemokine-inducible genes were heterogeneous and belonged to different classes, some of them could be grouped into functional clusters.

First, induction of selected components, agonists or receptors, of the cytokine and chemokine systems was observed. Chemokines have been recently shown to modulate the expression of Th1/Th2-polarizing cytokines (11, 19–21). In contrast, the capacity of chemotactic agents to induce proinflammatory cytokines in mononuclear phagocytes has been the object of inconsistent findings (8–10, 22). In our experiments CCL5 consistently stimulated IL-1α transcripts, but not other cytokines, such as TNF-α and IL-6, that were strongly induced by LPS as expected. However, IL-1β production was not observed after CCL5 stimulation despite transcript induction. It has previously been reported that nonchemokine chemotactic agents require a second signal (cell adhesion or primary proinflammatory mediators) for triggering IL-1β production (22). Hence, chemotactic agents prime mononuclear phagocytes for IL-1β production by inducing transcript expression, and tissue-derived second signal may induce protein production. CCL5 also stimulated the expression of a selected set of chemokines with overlapping, but distinct, spectra of action (1, 2), including CCL2, CCL3, CCL4, and CXCL8, whose transcript induction (strongest compared with other chemokines) was associated with protein induction. In addition to selected agonists, CCL5 also stimulated expression of chemotactic receptors, including the C5a receptor and CCR1 (a receptor for CCL3, CCL5, and CCL7/MCP-3). Therefore, chemokine-induced chemokine production may set in motion an amplification and diversification cascade for leukocyte recruitment.

A second set of genes was related to interaction with extracellular matrix components and included spliced CD44, MMP-9, MMP-19, and uPA-R. CD44 variable region splice variants interact with hyaluronate and represent an important mechanism for
tissue invasion by leukocytes and metastatic tumor cells (23). The uPA-R is localized at the leading front of migrating cells and allows localized and polarized digestion of extracellular matrix components (24). MMP-19 and MMP-9 hydrolyze basement membrane and extracellular extracellular matrix components, such as fibronectin, laminin, and type IV collagen (25), and have been shown to mediate extravasation and tissue invasion (26). Therefore, the prototypic CC chemokine CCL5 activates a transcriptional program related to cell migration and tissue penetration.

The CCL5-induced genes included the aryl hydrocarbon receptor and the cytochrome P450 isoform CYP1B1. The epoxide hydride cytochrome P450 isoform CYP1B1 is the predominant cytochrome P450 isoform expressed in all mononuclear phagocytes (27). It metabolizes, among other substrates, estradiol to carcinogenic metabolites and is involved in the generation of all-trans-retinoic acid, a regulator of cell differentiation. CYP1B1 is constitutively expressed in various cell types, and it is inducible by dioxin through aryl hydrocarbon receptor transcriptional activity and is constitutively expressed in all mononuclear phagocytes and is inducible by retinoic acid, a regulator of cell differentiation. CYP1B1 is constitutively expressed in various cell types, and it is inducible by dioxin through aryl hydrocarbon receptor transcriptional activity (28) and TNF-α, which induces CYP1B1 expression while inhibiting other cytochrome P450 isoforms (29). Chemokine-mediated modulation of cytochrome P450 CYP1B1, either directly or indirectly through aryl hydrocarbon receptor transcriptional activity, could be one mechanism by which chemokines participate in carcinogenesis (1) and in the regulation of precursor cell differentiation (30).

The miscellaneous transcripts stimulated by CCL5 included the lectin-like oxidized low density lipoprotein receptor, which plays a major role in atherosclerosis (31). CCR2 and CCL2 gene-targeted mice are protected from the development of atherosclerotic lesions (32), and it remains to be elucidated whether the chemokine-regulated transcripts described in this study play a role in the pathogenesis of atherosclerosis and cardiovascular diseases.

The results presented in this study show that the prototypic CC chemokine CCL5 activates a restricted transcriptional profile in human mononuclear phagocytes. The chemokine-activated transcriptional profile overlaps with, but is clearly distinct from, that induced by LPS, which is used as a prototypic primary proinflammatory signal. In particular, a set of chemokine-inducible transcripts is related to leukocyte recruitment and could be relevant to prevent monocytes for extravasation and interaction with extracellular matrix components, and chemokine-induced chemokine production may set in motion a novel amplification loop of leukocyte recruitment.

Acknowledgments
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