FEBS Letters 425 (1998) 490–494 FEBS 20066

A novel lipopolysaccharide inducible C-C chemokine receptor related gene in murine macrophages

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Received 16 January 1998; revised version received 4 March 1998

Abstract To identify genes induced in activated macrophages, we screened a cDNA library prepared from the lipopolysaccharide (LPS)-treated cell line, RAW264, using the suppression subtractive hybridization technique. One of the clones isolated was dramatically induced by LPS in macrophages. The predicted protein sequence of this gene contains the domain unique to seven transmembrane receptors, and shows similarity with mouse C-C chemokine receptor 5 (CCR5). Therefore, we designated it LPS inducible C-C chemokine receptor related gene (L-CCR). Northern blot analysis revealed that L-CCR was specifically expressed in differentiated macrophages after LPS stimulation. These results show that L-CCR is a novel C-C chemokine receptor related gene induced by LPS in macrophages and may play an important role in inflammatory responses.

Key words: Lipopolysaccharide; Macrophage; Chemokine receptor; Gene expression regulation; Subtraction technique; Inflammation

1. Introduction

Macrophages play a central role in host defense against infection of microbial organisms and invasion of tumor cells by performing phagocytosis, antigen presentation, tumor cell killing and so on [1,2]. Macrophages have been shown to be activated by several inflammatory mediators. These include lipopolysaccharide (LPS), an endotoxin derived from Gram-negative bacteria [3], and cytokines such as IFN-γ [4,5], granulocyte/macrophage colony stimulating factor [6], tumor necrosis factor-α (TNF-α), and interleukin 1α/β (IL-1α/β) [7]. Among these, LPS is the most potent inducer of the inflammatory response for macrophages [8]. Indeed, macrophages, once activated by LPS, secrete several cytokines, such as macrophage inflammatory protein-1α/β (MIP-1α/β) [9], IL-6 [10], TNF-α [11], and IL-1α/β [12], and exhibit enhanced expression of cell surface antigens such as major histocompatibility complex II [13,14] and B7-1/2 [15]. These molecules are all important for the immune and inflammatory response and some of them are autocrine macrophage activators. In addition, several LPS inducible transcription factors, such as ATF-3 and IRF-1, were identified from the macrophage cell line RAW264.7 [16,17].

To isolate novel genes that are induced in activated macrophages and responsible for the inflammatory processes, we prepared a cDNA library from cultures of the LPS stimulated mouse macrophage cell line, RAW264, and screened the library by the suppression subtractive hybridization technique [18]. One of the cDNA fragments isolated was used for further study. The isolation and analysis of the full sequence of this gene revealed that this is a novel molecule which belongs to the C-C chemokine receptor (CCR) family and we designated this molecule LPS inducible chemokine receptor related gene (L-CCR). Northern blot analysis of L-CCR demonstrated that L-CCR was specifically expressed in the LPS activated macrophage cell line RAW264. L-CCR was dramatically induced in normal mouse macrophages when stimulated with LPS. These data indicate that L-CCR may be a chemokine receptor important for the immune response against microbial infections.

2. Materials and methods

2.1. Subtractive hybridization and cloning of L-CCR

Subtraction was performed essentially according to the manufacturer's instruction of the PCR-select subtraction kit (Clontech). Briefly, 1×10^8 RAW264 cells were stimulated with 100 ng/ml of LPS for 4 h. Cytoplasmic RNA was obtained from RAW264 cells by guanidine isothiocyanate-cesium chloride gradient centrifugation [19]. Poly(A)$^+$ RNA was purified using Oligotex-dt30 latex beads (Takara). Comple-

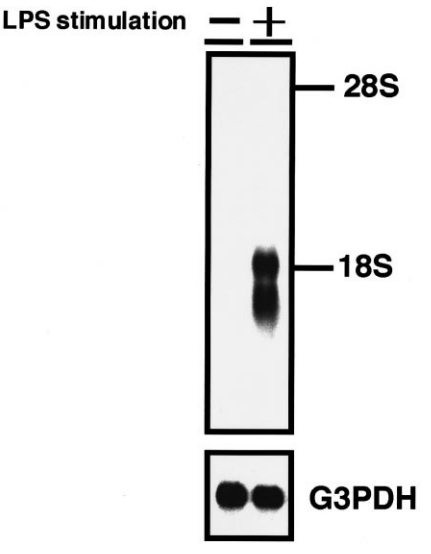

Fig. 1. Induction of L-CCR expression by LPS. Northern blot analysis of an LPS induced gene in RAW264 macrophage cell line. Poly(A)$^+$ RNA (2 mg) from RAW264 cells before (−) and after (+) LPS stimulation was separated on 1% formaldehyde agarose gel, transferred to nylon membrane, and hybridized with radiolabeled PCR fragment of L-CCR (obtained by subtraction). G3PDH is the internal control.

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PII S0014-5793(98)00299-3

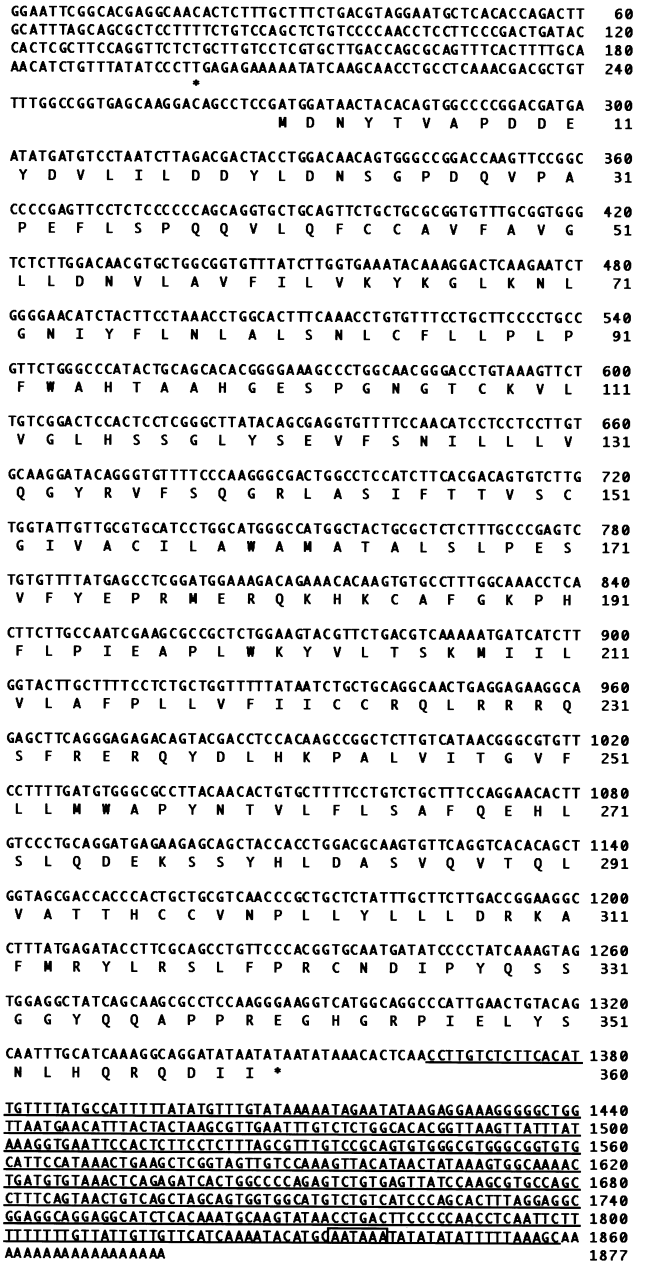

Fig. 2. L-CCR cDNA sequence and deduced amino acid sequence (DDBJ/GenBank/EMBL accession number AB009384). cDNA sequence of L-CCR is shown. The short isoform lacks the sequences underlined. Polyadenylation site is in a box. Stop codons are asterisked below.

mentary DNAs were synthesized from 2 μg of poly(A)$^+$ RNA from RAW264 stimulated with LPS for tester and 2 μg of poly(A)$^+$ RNA from unstimulated RAW264 cells for driver. Following *Rsa*I digestion of cDNAs, adaptors 1 and 2R were ligated to the tester. Tester cDNA was hybridized with excess driver cDNA. After hybridization, differential cDNAs were selectively amplified by suppression PCR. Nested PCR products were ligated into pGEM-T vector (Promega). A subtracted plasmid library was constructed and independent clones were amplified by colony PCR. Differential screening against 500 clones was performed according to the manufacturer's instruction (Clontech). Positive clones were sequenced by autosequencer (ABI). Sequence comparisons against DDBJ databases as well as protein homology prediction were obtained from the DDBJ blast server (http://www.ddbj.nig.ac.jp/E-mail/homology-j.html) [20]. To obtain the full sequence of different clones, a cDNA library was constructed from poly(A)$^+$ RNA of RAW264 stimulated with 100 ng/ml of LPS for 4 h, using the ZAP-cDNA Gigapack III gold cloning kit (Stratagene) according to the manufacturer's directions.

The sequences of the obtained clones were determined for both strands.

2.2. Northern blot analysis

B cell leukemia (BCL-1), myeloma cells (MOPC 315), and thymoma cells (EL-4) were cultured in RPMI 1640 containing 10% FCS, 100 μg/ml of streptomycin, 10 U/ml of penicillin G. NK cells (5E3) were cultured in RPMI 1640 in the presence of 500 U/ml of IL-2 (Genzyme). Monocytic leukemia cells (M1) were cultured in EMEM containing 10% FCS. RAW264 (obtained from Riken cell bank) and fibroblast cells (NIH 3T3) were cultured in DMEM containing 10% FCS, 100 μg/ml streptomycin (Meiji), 10 U/ml of penicillin G (Meiji).

In the case of peritoneal macrophages, C57BL/6 mice were intraperitoneally injected with 2 ml of 4% thioglycolate. Three days after injection, peritoneal exudate cells were elicited by washing the peritoneal cavity with 5 ml of ice-cold PBS. Cells were cultured for 2 h and washed. Adherent cell monolayers were used as peritoneal macrophages.

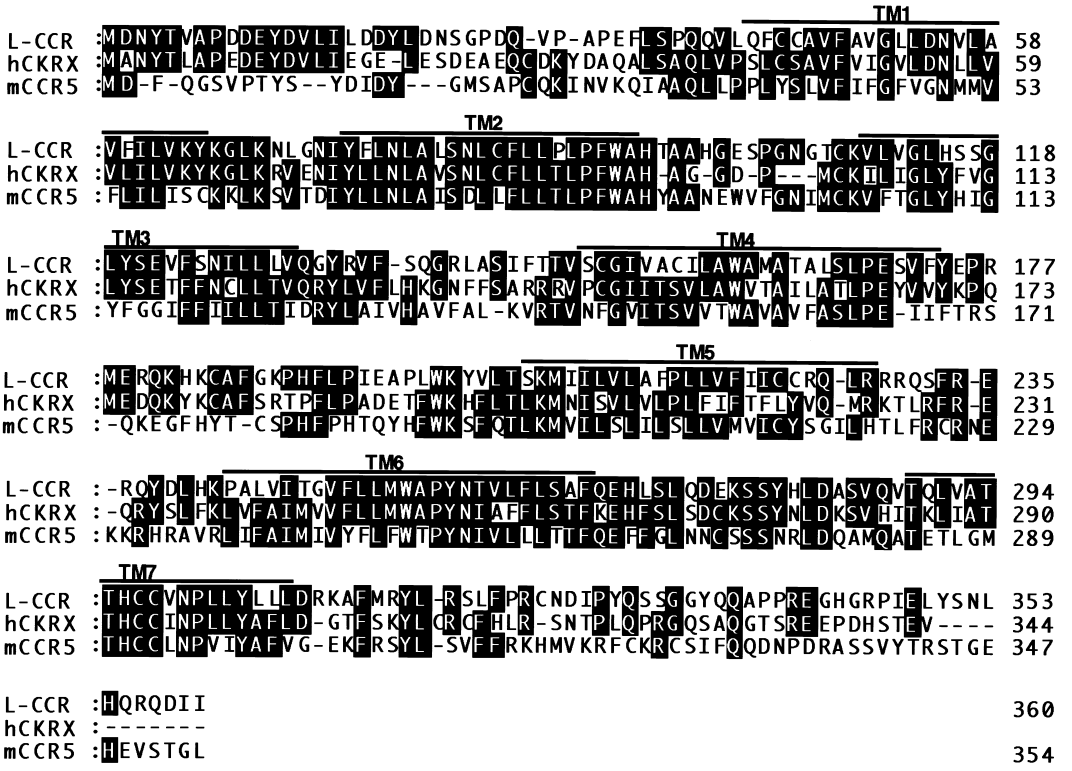

Fig. 3. Alignment of L-CCR protein sequence against human CKRX and mouse CCR5. Putative transmembrane domains are overlined (TM 1–7) and identical amino acid residues are highlighted with solid boxes.

Ten million cells were stimulated with 100 ng/ml of LPS, 250 U/ml of IFN-γ (Genzyme), 1000 U/ml of TNF-α (Genzyme), 400 U/ml of IL-1β (Genzyme), 1000 U/ml of IL-2, 100 U/ml of IL-4 (Genzyme), or 1000 U/ml of IL-6 (Genzyme) for 4 h.

For time course experiments, ten million cells were harvested after treatment with 100 ng/ml of LPS for 0–24 h.

Total RNA was extracted using TRIzol reagent (Gibco), electrophoresed, transferred to Hybond-N^+ (Amersham) nylon membrane, and hybridized with the cDNA probe of L-CCR. For L-CCR probe preparation, full length cDNA was cut with *Apa*I and *Eco*RV. The fragment from nucleotides 551 to 1243 was used as a probe.

3. Results and discussion

To identify LPS inducible genes in macrophages, we utilized the suppression subtractive hybridization technique based on PCR amplification [18]. We stimulated the mouse macrophage cell line RAW264 with LPS for 4 h, extracted mRNA, and synthesized double strand cDNA. cDNA from LPS stimulated macrophages was digested with *Rsa*I, ligated to adaptor, and amplified by PCR after hybridization with driver cDNA from non-stimulated macrophages. The resulting PCR products were subcloned and 120 clones were sequenced. As expected, many cDNA fragments were known genes induced by LPS stimulation. These include MIP1-α/β, IFN-γ, granulocyte colony stimulating factor (G-CSF), TNF-α, IL-1β, MIP-2, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and interferon inducible protein 10 (IP-10). Besides the known genes, six clones contained an unknown but identical sequence. This gene was not expressed in non-stimulated macrophage cell line RAW264, but dramatically induced by LPS stimulation (Fig. 1). Northern blot analysis revealed that this gene has long and short forms of transcript, about 1.9 kbp and 1.4 kbp (Fig. 1). To obtain the full length cDNA of this gene, we prepared a cDNA library in the λZAPII vector from RAW264 cells activated by LPS for 4 h, and screened with the probe of the PCR fragment. Three cDNA clones were isolated and analyzed. The length of two cDNA clones obtained was 1877 bp and matched that of the longer band detected by Northern blot analysis, indicating that this clone contains the full length cDNA (Fig. 1). This cDNA clone has an open reading frame of 1080 bp encoding a protein of 360 amino acids (Fig. 2) (the nucleotide sequence data reported in

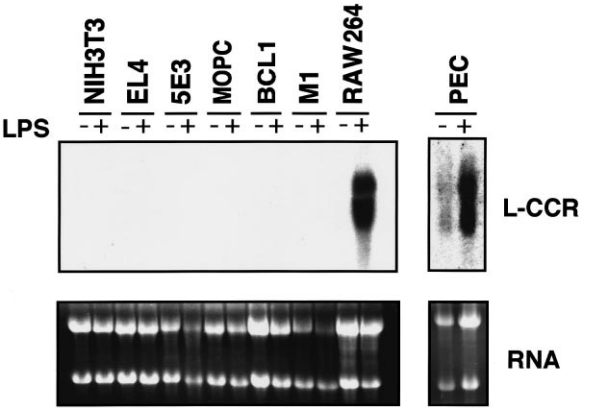

Fig. 4. Expression of L-CCR mRNA in several hematopoietic cell lines and mouse peritoneal macrophages. Indicated cell lines and mouse peritoneal macrophages (PEC) were stimulated with 100 ng/ml of LPS for 4 h. Total RNA (25 μg/lane; 20 μg/lane for PEC) from stimulated (+) or non-stimulated (−) cells was separated on 1% formaldehyde agarose gel, transferred to nylon membrane, and hybridized with radiolabeled L-CCR cDNA fragment. The lower column shows ethidium bromide stained ribosomal RNA of the upper column.

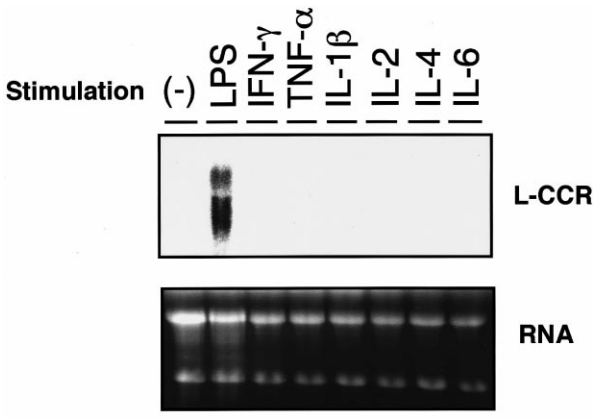

Fig. 5. Induction of L-CCR with several cytokines. RAW264 cells were stimulated with the indicated reagents for 4 h. Total RNA (25 μg/lane) was separated on 1% formaldehyde agarose gel, transferred to nylon membrane, and hybridized with radiolabeled L-CCR cDNA fragment. The lower column shows ethidium bromide stained ribosomal RNA of the upper column.

this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with accession number AB009384). It has the putative initiating methionine with the consensus Kozak sequence and the preceding in-frame stop codon [21]. In the 3′ untranslated region, a typical polyadenylation signal, AATAAA, was found upstream of the poly(A) tail. An additional clone also contained the same coding region and poly(A) tail, but 513 bp of nucleotides in the 3′ untranslated region were absent (Fig. 2). The length of this clone matched the shorter band detected by Northern blot analysis. This shorter cDNA may be a product of alternative polyadenylation signal usage of the L-CCR, using an atypical polyadenylation signal, AATATA, existing upstream of the poly(A) tail.

The deduced amino acid sequence of this gene shares significant sequence identity with the known C-C chemokine receptors, containing the characteristic domain of seven transmembrane receptors (Fig. 3). It is most similar to mouse CCR5 (DDBJ/GenBank accession number AF019772) with 35.8% identity and shares 34.9% identity with mouse CCR1 (DDBJ/GenBank/EMBL accession number U29678) at the amino acid level. Therefore, we named this gene LPS inducible C-C chemokine receptor related gene (L-CCR). In addition, homology search using the DDBJ blast system revealed that a recently deposited novel human chemokine receptor, designated human chemokine receptor x (CKRX) (DDBJ/GenBank accession number AF014958), showed 51.1% homology with mouse L-CCR at the amino acid level (Fig. 3). This implied that the L-CCR may be a mouse homologue of the human CKRX. Thus, we identified and molecularly cloned a novel mouse CCR family member, which is induced by LPS in macrophages.

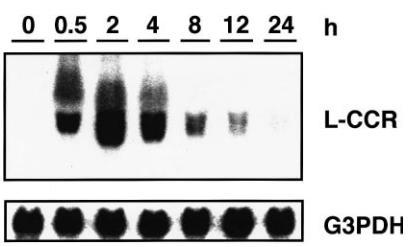

Fig. 6. Time course of L-CCR mRNA in RAW264 cells. RAW264 cells were treated with 100 ng/ml of LPS for 0–24 h as noted. Northern blot analysis was performed as described in the legend of Fig. 5. G3PHD is the internal control.

To examine the expression profile of L-CCR, we performed Northern blot analysis of mRNA in several types of cell lines before and after LPS stimulation. Abundant expression of mRNA of the expected size was observed in LPS activated RAW264. In cell lines such as M1 (myeloid leukemia cells), BCL-1 (B cell leukemia cells), MOPC 315 (myeloma cells), EL-4 (T-cells), 5E3 (NK cells), and NIH 3T3 (fibroblast cells), expression of L-CCR mRNA was not detected before and after LPS stimulation (Fig. 4). Weak expression was observed in thioglycolate elicited peritoneal macrophages, and its expression was dramatically increased by LPS stimulation (Fig. 4). These results demonstrate that L-CCR is not expressed in lymphoid cells but is induced by LPS in differentiated monocytes/macrophages. Several recent findings have demonstrated that chemokine receptor expression is regulated by LPS, for example, CCR2, CCR1, and CCR5 are expressed in resident monocytes/macrophages and down-regulated by LPS [22,23]. In addition, the combination of LPS and TNF-α inhibited the expression of the IL-8 receptor in neutrophils [22]. So far, there have been no reports about upregulation of the chemokine receptor in response to LPS. Therefore, L-CCR may be the first chemokine receptor which is upregulated by LPS.

Besides LPS, several inflammatory cytokines such as IFN-γ, TNF-α, and IL-1 have been shown to affect macrophage activities. In addition, IL-2 has been shown to upregulate the expression of CCR2 or CCR5 in macrophages [22,23]. Therefore, we next analyzed the expression of L-CCR in response to these cytokines in RAW264. As shown in Fig. 5, expression of L-CCR mRNA was not induced by stimulation with IFN-γ, TNF-α, IL-1β, IL-2, IL-4, or IL-6. These data demonstrate that L-CCR is specifically induced by LPS stimulation in the RAW264 macrophage cell line.

In order to analyze the time course of accumulation of L-CCR mRNA, RAW264 cells were harvested after treatment with LPS for 0–24 h. As indicated in Fig. 6, L-CCR was induced by 30 min, reaching a peak at 2 h.

In conclusion, we identified a novel CCR related gene, L-CCR, by the suppression subtractive hybridization method. Contrary to other CCR families, L-CCR is not expressed in non-stimulated cells, but is dramatically induced by LPS stimulation in macrophages, demonstrating that L-CCR is the LPS inducible gene in macrophages. In addition, time course analysis revealed that the induction of L-CCR was quite rapid and L-CCR could be classified as an immediate early gene. Therefore, L-CCR may play an important role in controlling bacterial infection. Further study, such as identification of its ligands and generation of L-CCR deficient mice, will delineate the precise role of L-CCR in vivo.

Acknowledgements: We thank T. Yoshimoto for providing several cell lines, Kiyoshi Takeda for preparation of the manuscript, T. Kawai for preparation of the figures, H. Sanjo for technical support, K. Hoshino for helpful advice and T. Aoki for secretarial assistance. This work was supported by CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation (JST) and grants from the Ministry of Education of Japan.

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