Brief Definitive Report

Upregulation of Mouse CD14 Expression in Kupffer Cells by Lipopolysaccharide

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Summary

Western blot analysis showed that a monoclonal antibody against recombinant mouse CD14 (mCD14), designated rmC5-3, specifically reacted with mouse macrophage cell line J774, but not myeloma cell line NS1. Fluorographic and immunocytochemical analysis demonstrated specific binding of rmC5-3 with mouse resident macrophages, inflammatory monocytes and neutrophils, and macrophage cell lines. Immunohistochemical staining using rmC5-3 showed that CD14-positive Kupffer cells (KC) were small in number in the liver in nonstimulated mice. The number of stained KC, which were rich in the midzonal and periportal regions, gradually increased with time after intraperitoneal injection of lipopolysaccharide (LPS), peaked 6 h after injection, and returned to normal by 20 h after injection. Staining intensity over time was proportional to the number of KC. A slight increase in mCD14 expression was observed in peritoneal macrophages 2 h after LPS administration in vivo using flow cytometric analysis. mCD14 mRNA became detectable at 1 h after the intraperitoneal injection of LPS (20 μg/mice), and the level dramatically increased with time, peaking at 3 h, and sharply dropped at 6 h. The resident peritoneal macrophages demonstrated a constitutively high mCD14 mRNA expression, which slightly increased 2 h after LPS (100 ng/ml) stimulation in vitro. The level of mCD14 expression in macrophages did not increase after intraperitoneal injection of LPS (20 μg/mice).

Kupffer cells (KC) are one of the members of monocytic lineage and are located in the sinusoids of the liver, the organ containing the largest pool of mononuclear phagocytes (1). Portal blood, which carries nutrients together with a number of stimuli, perfuses the sinusoids. KC share many functions with macrophages. Stimuli to KC as well as macrophages trigger signals for the production of a variety of bioactive substances such as TNF-α, IL-1α and -β, IFN-α and -β, prostaglandins, leukotrienes, platelet activating factors, and nitric oxide (2–10), all of which act locally and systemically to regulate cell functions. LPS from gram-negative bacteria is the most important stimulant that could consistently be maintained in the portal blood. KC, therefore, can be expected to have a special function for handling LPS entering the liver via the hepatic portal circulation in the forefront of the liver.

Macrophages have a central role in mediation of the biological effects of LPS. First, LPS-stimulated monocytic cells produce monokines such as TNF-α and IL-1. Second, they can eliminate and detoxify LPS from the blood. Several binding sites for LPS on the cell surface of macrophages have been reported. LPS can also interact with the macrophage membrane after binding to plasma proteins. A 60-kD acute-phase protein called LPS-binding protein (LBP) has been shown to bind to the lipid A moiety of LPS (11). LPS–LBP complexes are a ligand for a 55-kD phosphatidylinositol-linked protein CD14 on macrophages. LPS–LBP complexes can stimulate production of TNF-α by macrophages at concentrations far below those required for stimulation by LPS alone (12, 13). KC have also been shown to have CD14, although features of expression of CD14 on KC have not been investigated because of the limited animal probes available for CD14 (14).

We previously cloned the mouse CD14 (mCD14) cDNA and gene (15, 16). In this communication, we raised a mAb against mCD14 and observed expression features of mCD14 in KC compared with macrophages.

Materials and Methods

Animals. Lou rats and BALB/c mice were bred and maintained in our animal facilities in conventional and specific pathogen-free (SPF) conditions. 7–8-wk-old male BALB/c mice were used for in vivo experiments. Nude mice were purchased from Shizuoka Animal Center (Hamamatsu, Japan).

Cell Lines. Murine macrophage cell lines J774 and aHINS-B3 (15), and murine myeloma cell line NS1 were used.

Preparation of Antigen. A mCD14 cDNA clone, designated MS7X (17) and encoding the entire mature mCD14 sequence, was

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used for the construction of the cDNA to be inserted into the expression vector. This plasmid was cleaved with NcoI and BamHI, and the cDNA fragment was isolated, and inserted into the NcoI and BamHI sites of pET-11d (18). *Escherichia coli* K12 strain HB101 was used as the host for initial cloning of the resulting plasmids and for maintaining the plasmids. Plasmids expressing mCD14 were propagated in an *E. coli* strain BL21(DE3), derivative of BL21 cells [F^- ompT r_B^- m_B^-]. Cultures were grown at 37°C for 4 h in M9ZY medium supplemented with ampicillin (100 μg/ml). Induction was commenced by 1 mM isopropyl-β-D-thiogalatopyranoside (IPTG) when the medium containing the cells reached an OD_{660} of ~0.6. The temperature was maintained at 37°C for 2 h until induction was terminated by rapidly cooling the cells to 4°C by the addition of crushed ice.

Bacterial cells were harvested by centrifugation, and disrupted with sonicators. Sonicates were spun at 12,000 rpm for 5 min and the supernatants and pellets were recovered for analysis. Aliquots were lysed in sample buffer (15% glycerol/4.5% SDS/1 mM 2-ME/93.5 mM Tris-HCl/0.25% bromophenol blue, pH 6.8) and heated for 2–3 min at 100°C; the proteins separated on polyacrylamide gels either were stained with Coomassie brilliant blue or were electrophoretically transferred to nitrocellulose sheets and allowed to react with rabbit anti-mCD14 peptide antisera (anti-pmCD14) followed by anti-rabbit IgG conjugated with peroxidase (19). The immunoblots were washed, and allowed to react with horseradish peroxidase–conjugated goat anti-rabbit IgG (H and L chain–specific; Cappel Laboratories, Cochranville, PA) followed by substrate. The major band with an apparent molecular mass of 50 kD was efficiently induced. Analysis of the samples obtained from a larger scale demonstrated that recombinant mCD14 (rmCD14) was sequestered into inclusion bodies in *E. coli*.

The inclusion bodies were solubilized with 6 M urea in lysis buffer (50 mM Tris [pH 8.0]/1 mM EDTA/100 mM NaCl) for 1 h at room temperature. After centrifugation, the supernatant was added with SDS loading buffer supplemented with 2-ME, and subjected to SDS-PAGE. The 50-kD band was excised from the gel stained with Coomassie blue and electrically eluted. The eluted samples were precipitated with trichloracetic acid, solubilized with acetone, and dried. After solubilization of the dried samples with Tris buffer containing 0.02% SDS and 0.1% 2-ME, the sample was dialyzed against decreasing concentration of urea solution (4–0.5 M) and finally against PBS.

Production of Monoclonal Anti-rmCD14 Antibody. Lou rats received multiple subcutaneous injections of rmCD14 emulsified with Freund's complete adjuvant in footpads, both thighs, and the back and nape of the neck. 2 wk later, each rat was intraperitoneally injected with rmCD14 in PBS. These procedures were repeated. Spleen cells taken from the rats 3 d after the final sensitization were hybridized with mouse myeloma line SP2/O-Ag14 by using polyethylene glycol (PEG 4000; Boehringer Mannheim, Tokyo, Japan) as described previously (20). Hybridomas were selected in the medium containing hypoxanthine, aminopterin, and thymidine. Ag-specific clones were screened by ELISA and cloned at least twice by limiting dilution. Stable clones were expanded in vitro in medium supplemented with 5% bryclone (Dainippon Seiyaku Co., Tokyo, Japan). Antibodies thought to be specific for the rmCD14 on the basis of initial screening were examined by ELISA. One mAb designated rmC5-3 was selected for the present experiments. The mAb was isotyped as IgG1 by ELISA. Ascites were collected from peritoneal cavities of nude mice given 10^7 cloned cells after treatment with pristane (Wako, Tokyo, Japan) 1 wk previously.

Western Blot Analysis. Purified rmCD14 were prepared as above. J774 and NS1 cells (1.5×10^6) were lysed with lysis buffer containing 1% NP-40, 5 mM PMSF, and 10 μg/ml leupeptin. The samples were electrophoresed on 10% polyacrylamide minislab gels using the buffer system as described previously (19). Protein was transferred to the nitrocellulose membrane using the semidry transblot system (Nihon Eido Co., Tokyo, Japan). Blots were blocked with 3% bovine serum albumin solution in PBS for 2 h at room temperature and were sequentially incubated with rmC5-3 (1:200) at 4°C overnight. After three washes with PBS, the membrane was treated with ^{125}I-labeled affinity purified antibody to rat IgG (Amersham International, Little Chalfort, UK) (1:500) for 2 h at room temperature. After further washing, the nitrocellulose membranes were exposed to X-omat film (Eastman Kodak Co., Rochester, NY).

Immunohistochemistry. Immunostaining was carried out on acetone-fixed smears prepared from resident and peptone-induced peritoneal exudate cells and Carnoy-fixed liver sections using an indirect immunoperoxidase staining technique. Briefly, binding of rmC5-3 to cells and sections was detected using horseradish peroxidase–conjugated rabbit anti-rat IgG (H and L chain–specific), followed by substrate. Smears and sections incubated in the absence of primary antibody or with an irrelevant primary antibody were included as negative controls.

Fluorographic Analysis. Mouse macrophage cell lines J774 and aHINS-B3, mouse resident peritoneal cells and peritoneal cells after stimulation with LPS (20 μg/mice) were stained with rmC5-3 followed by fluoresceinated rabbit anti-rat Ig, and the analysis was performed using FACS IV® (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Preparation of Cells and Tissues. Resident and inflammatory exudate peritoneal cells were obtained from nontreated mice and mice that received intraperitoneal injection with 10% proteose peptone (Difco Laboratories, Detroit, MI) 24 h previously, respectively. The liver was taken from nontreated mice and mice intraperitoneally injected with LPS (Sigma Chemical Co., St. Louis, MO) (20–600 μg/mice) 1–20 h previously, respectively. Resident peritoneal macrophages were cultured in DMEM supplemented with 10% FCS on plastic petri dishes for 1 h, followed by washing with DMEM. The peritoneal macrophages were stimulated with LPS (100 ng/ml) for 1–24 h. LPS-induced peritoneal cells were obtained from mice after intraperitoneal injection with LPS (20 μg/mice) 1–24 h previously, and the macrophage fraction was prepared as above.

Northern Blot Analysis. Northern blot hybridization was performed as previously described (19). Briefly, total RNA prepared from tissues and cells was electrophoresed through a 1.5% agarose–6% (vol/vol) formaldehyde gel and blotted onto a nylon membrane. The membranes were exposed to UV for 7 min and then prehybridized and hybridized with $3–5 \times 10^6$ cpm/ml of ^{32}P-labeled RNA probe prepared from mCD14 cDNA MS7X (16).

Relative expressions of mCD14 message measured using BAS1000 bioimaging analyzer (Fuji Film, Tokyo, Japan) were determined after normalization to levels of β-actin mRNA.

Results

Western Blot Analysis of Reactivity of mAb rmC5-3. To examine reactivity of rmC5-3 with cells, rmC5-3 was tested for its ability to react with purified rmCD14, and lysates prepared from J774 and NS1 cells by Western blotting. rmC5-3 reacted with purified rmCD14, and detected a band in lysates from J774 cells but not from NS1 cells (Fig. 1). Immunocytochemical and flow cytometric analysis using rmC5-3 demonstrated specific binding of rmC5-3 with mouse resi-

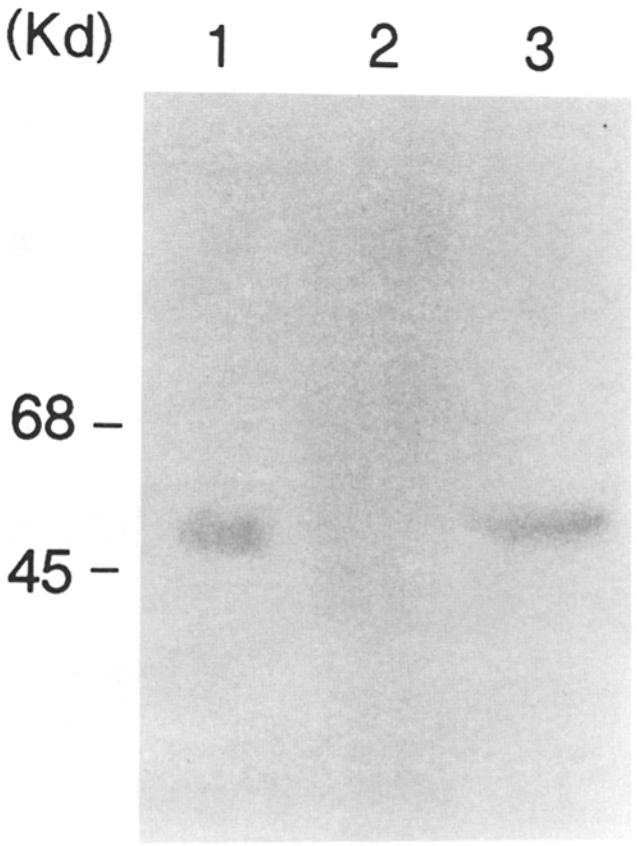

Figure 1. Western blot analysis of reactivity of rmC5-3. Samples were electrophoresed on 10% polyacrylamide gels. Purified recombinant mCD14 (lane *1*); lysates from NS1 cells (lane *2*); lysates from J774 cells (lane *3*).

dent macrophages, inflammatory macrophages and neutrophils, and mouse macrophage cell lines J774 and aHINS-B3 (data not shown).

Immunohistochemical Analysis of the Liver and Flow Cytometric Analysis of Peritoneal Macrophages Using rmC5-3. Immunohistochemical staining using rmC5-3 was performed for the liver from untreated SPF BALB/c male mice. A few cells morphologically thought to have characteristics of KC were stained with rmC5-3 (Fig. 2 *A*). The numbers of stained KC, which were rich in midzonal and periportal regions, gradually increased with time after intraperitoneal injection of LPS (20–600 μg/mice), peaking 6 h after injection and returned to normal by 20 h after injection (Fig. 2, *B* and *C*). Staining intensity over time was proportional to the number of KC. mCD14-positive KC were slightly larger in number in nonstimulated conventional mice than in nonstimulated SPF mice, and gradually increased after LPS stimulation as for in SPF mice (not shown).

Resident peritoneal cells and cells collected from 2 to 6 h after intraperitoneal injection of LPS (20 μg/mice) were stained with rmC5-3 and subjected to flow cytometric analysis. The peritoneal cells contained mainly two size populations, and the larger population, thought to consist of mainly macrophages, was analyzed. The cells from the 2-h-old site showed slightly higher expression of mCD14 than resident cells,

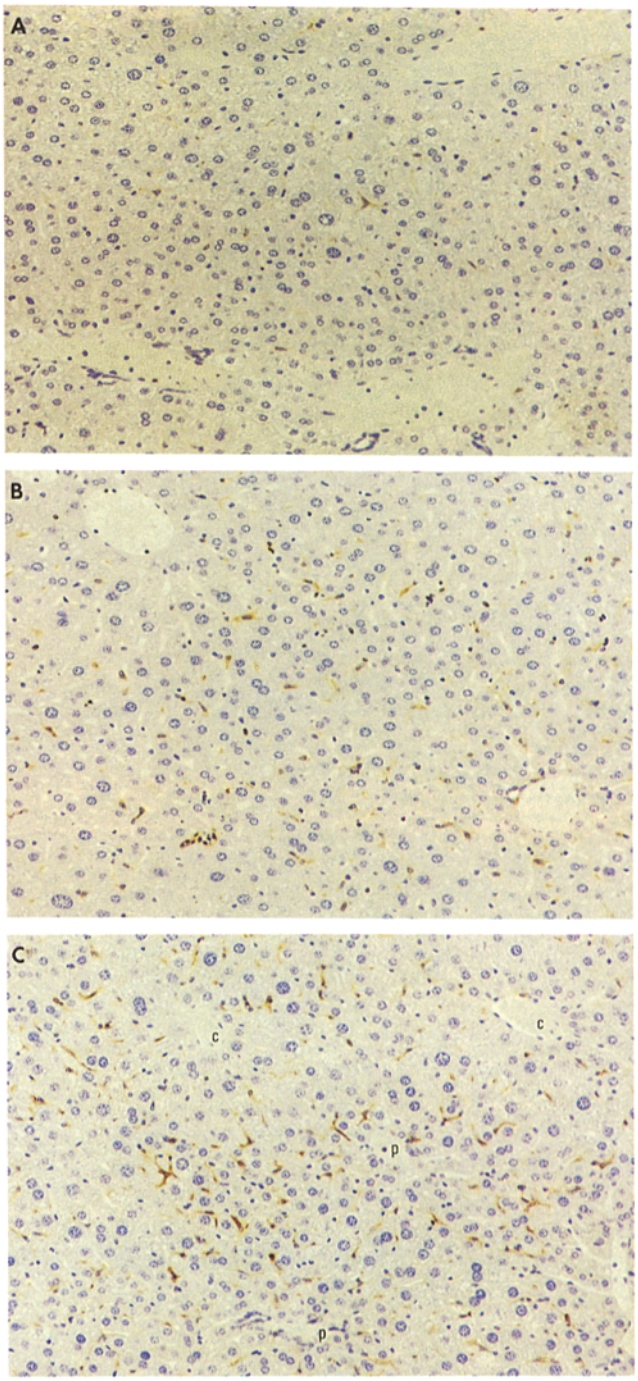

Figure 2. Effect of LPS stimulation on mCD14 expression in KC. (*A*). Immunohistochemical staining of the normal liver. (*B*). Immunohistochemical staining of the liver 3 h after LPS stimulation. (*C*). Immunohistochemical staining of the liver 6 h after LPS stimulation, indicating central vein (*c*); portal vein (*p*). Original magnifications are ×400.

whereas those from the 6-h-old site showed no enhanced expression (data not shown).

Northern Blot Analysis of mCD14 mRNA in the Liver and Macrophages. mCD14 mRNA in the normal liver is below the limits of detection using Northern blot analysis. To confirm

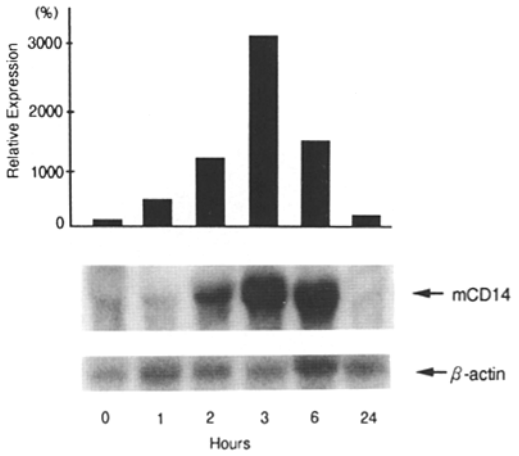

Figure 3. Effect of LPS on mCD14 mRNA expression in the liver. Total RNA (20 μg/lane) from the liver was electrophoresed in a 1.5% formaldehyde gel, blotted onto nylon membrane, and hybridized with a ^{32}P-labeled antisense mCD14 RNA probe. Lanes contained RNA from the liver after the various stimulation times by LPS (20 μg/mice) in vivo.

the observation of increased mCD14-positive KC detected by immunohistochemical analysis, it is necessary to determine whether the increase was accompanied by an increase in the mRNA level and compare the time course of expression of protein and mRNA levels. Northern blot analysis revealed that mCD14 mRNA became detectable at 1 h after the intraperitoneal injection of LPS (20 μg/mice), and that the level increased with time, peaked at 3 h, and sharply declined at 6 h (Fig. 3). An early rise of mRNA expression would explain enhanced mCD14 synthesis at 6 h. Increase in the expression of mCD14 mRNA by LPS was found in a dose-dependent fashion (not shown).

CD14 has been shown to increase or decrease after stimulation with LPS in human monocytes (21–23). The effect of LPS on CD14 mRNA expression in macrophages was tested in vitro and in vivo. We compared mCD14 mRNA expression in peritoneal macrophage fraction before and after stimulation with LPS (100 ng/ml) in vitro for 1–24 h. The resident macrophage fraction demonstrated constitutive mCD14 mRNA expression. The results revealed that the levels of mCD14 mRNA increased to about 1.7 times of the level of resident macrophages 3 h after LPS stimulation (Fig. 4 *A*). The resident macrophage fraction prepared from nonstimulated peritoneal cells after a 1-h incubation on petri dishes contained about 40% of macrophages. Percentages of macrophages in the macrophage fraction prepared from peritoneal cells after intraperitoneal injection of LPS (20 μg/mice) 1–24 h LPS earlier followed by incubation on petri dishes for 1 h was comparable with that in the resident cell preparation. The levels of mCD14 mRNA in macrophage fraction did not increase after LPS injection (Fig. 4 *B*).

Discussion

Of all macrophage functions, one of the most important is probably for KC to react to microorganisms and stimulating substances carried by the portal blood. In particular, it is important for KC to develop a specialized strategy to detect and detoxify LPS because LPS are the stimulators most frequently encountered by KC. Here, we demonstrated that the number of mCD14-positive KC cells was very small, but the number of positive KC and the intensity of staining of mCD14 greatly increased, peaking at 6 h after LPS administration using peroxidase antiperoxidase technique. Furthermore, we revealed that the level of expression of mCD14 mRNA in the liver markedly increased, peaking 3 h after LPS stimulation. The level of upregulation increased by more than a factor 30. Hepatocytes showed no mCD14 expression. mCD14 expression in macrophages in the liver should be comparable with that in peritoneal macrophages. In addition, the increase in the expression of mCD14 mRNA by LPS in macrophages was relatively small. Enhanced levels of mCD14 mRNA expression in the liver, therefore, are most likely to reflect that in KC. In contrast, peritoneal macrophages constitutively express mCD14, and showed low levels of increase in mCD14 2 and 6 h after intraperitoneal injection of LPS (20 μg/mice). Similarly, peritoneal macrophages showed constitutively high levels of mCD14 mRNA expression, which showed low levels of enhancement 2–3 h after LPS stimulation in vitro. Taken collectively, KC show unique features in the mCD14 expres-

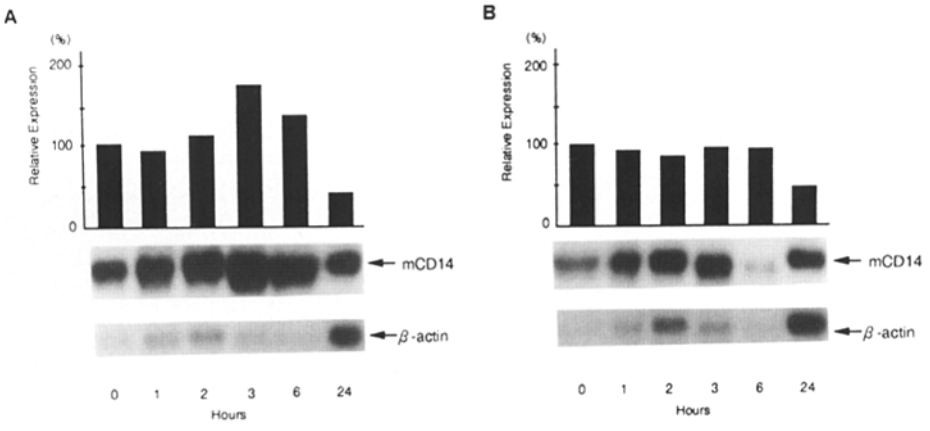

Figure 4. Effect of LPS on mCD14 mRNA expression in macrophages. (*A*) mCD14 mRNA expression in macrophages stimulated with LPS (100 ng/ml) in vitro. Lanes contained RNA from macrophages after the various stimulation times. (*B*) mCD14 mRNA expression in macrophages stimulated with LPS (20 μg/mice) in vivo. Lanes contained RNA from macrophages after the various stimulation times.

sion in nonstimulated and stimulated conditions when compared with peritoneal macrophages.

It has been reported that human CD14 is strongly expressed in KC in the liver specimen surgically prepared from normal humans (14). In contrast, immunohistochemical analysis revealed that the majority of KC in nonstimulated SPF mice did not express detectable levels of mCD14. The number of mCD14-positive KC remained slightly larger in nonstimulated conventional mice than in nonstimulated SPF mice. Therefore, the discrepancy may reflect the technical differences used in these studies, or the differences between human and mouse. In any event, the number of mCD14-positive KC is gradually increased after intraperitoneal LPS stimulation, peaking at 6 h irrespective of breeding condition.

The increase was found in the midzonal and periportal area. KC have been reported to show functional heterogeneity (24). Periportal KC showed a higher phagocytic and lysosomal enzyme activities as compared with midzonal and perivenous KC, suggesting slight differences in the localization between KC capable of expressing mCD14 by LPS stimulation and KC with a high endocytic activity. It has been shown that liver parenchymal cells show the metabolic zonation (25). Heterogeneity of KC may possibly be paralleled with such zonation.

The mechanism of difference of mCD14 expression between KC and peritoneal macrophages remains unclear. Profound respiratory burst defect (26) has been shown for KC which derive from monocytes whose capacity to mount a respiratory burst is a general characteristic. The respiratory burst defect of KC is suggested to be due to a deactivation mechanism (27). On the other hand, expression of mCD14 in KC is an upregulation of function. Therefore, other reasons should be considered for the presence of such a phenotype of macrophages. Peritoneal macrophages contained the cells that could be induced to express mCD14 by LPS, although their content should be low because the enhancement of mCD14 mRNA expression was small. If such cells represent a distinct macrophage lineage, KC may arise from them.

Enhanced expression of CD14 by LPS (10^{-2}–10 ng/ml) in vitro has been reported using whole blood human monocytes. The upregulation was suggested not to accompany protein synthesis because it was not affected by cycloheximide (21). The enhancement peaked 1–3 h after LPS administration. Thus, features and mechanisms of CD14 expression in monocytes are different from our present results. The difference may be attributed to that of species and source of macrophages (monocytes) and techniques used in these studies. Other reports show that high doses of LPS (100 ng/ml) downregulate the expression of CD14 in human monocytes (22, 23). Wright (22) demonstrated that the downregulation occurred 18 h after LPS stimulation. The data could be comparable with the present results that LPS (100 ng/ml) downregulates the expression of mCD14 mRNA at 24 h in vitro. Bazil and Strominger (23) showed the downregulation of CD14 expression 1–3 h after LPS stimulation. However, it is difficult to compare the results with our data because the decrease of CD14 is assigned to shedding and no data is available for the production of CD14. IFN-γ downregulates the expression of CD14 in mature monocytic cell lines and blood monocytes (28, 29). Since LPS induces IFN-γ in vivo, the effect of IFN-γ could affect the expression of mCD14 by LPS in vivo.

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