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Specific messenger RNA expression for signal transduction molecules by lipopolysaccharide in intestinal macrophages

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Summary

Intestinal macrophages are known to display profound inflammatory anergy in response to lipopolysacchraide (LPS). To study the mechanisms of unresponsiveness of intestinal macrophages to LPS, we compared the mRNA expression of molecules associated with signal transduction of intestinal macrophages with those of other tissue macrophages. Also cellular localization of CD14 protein was examined. Intestinal, alveolar and peritoneal macrophages were isolated from rats or mice. The expression of mRNA was assessed by real-time PCR, and cellular localization of CD14 protein was examined by flow cytometry. Cellular responses to LPS were examined by production of TNF and NO. The expression of CD14 mRNA in intestinal macrophages was lower than for peritoneal macrophages but higher than for alveolar macrophages. The mRNA expression of other molecules corresponding to intracellular signal transduction in intestinal macrophages was similar with alveolar and peritoneal macrophages. Despite the presence of CD14 mRNA, proteins of CD14 were not detected on cell surfaces of intestinal macrophages, and induction of TNF or NO responding to LPS were not detected. Flow cytometric analysis demonstrated that CD14 protein was not expressed on the cell surface but was expressed inside intestinal macrophages. The unresponsiveness of intestinal macrophages after LPS exposure is considered to be largely attributed to the lack of CD14 protein on their cell surfaces. However, CD14 protein was expressed inside of the cells, suggesting that post-transcriptional regulation rather than transcriptional suppression may play a dominant role in determining the phenotype of the intestinal macrophages.

Keywords: intestinal macrophages, TNF-α, LPS, signal transduction molecules

Introduction

Macrophages are derived from monocytes, which are originally produced from pluripotent stem cells in the bone marrow. The monocytes then migrate into different tissues of the body through the circulatory system and differentiate into tissue-specific macrophages [1]. While macrophages have common functions in that they recognize and exclude foreign substances, tissue macrophages also develop unique characteristics depending on the tissue environment where the macrophages eventually reside. The intestinal macrophages make up 80percent of the tissue macrophages in the body and reside in the gastrointestinal mucosa [2], which is the largest surface in the body that comes in contact with the external environment [3].

Because they are directly exposed to the external environment, intestinal macrophages have characteristics that differ from those of other tissue macrophages. Intestinal macrophages are localized in the subepithelial region of the lamina propria, and they recognize and eliminate microorganisms and microbial products that breach the epithelium. Consequently, though intestinal macrophages are the greatest barrier of defense against infection in the body, their response to foreign substances such as Gram-negative bacteria, needs to be so strictly controlled so that unnecessary inflammation does not occur. It is known that intestinal macrophages do respond differently than other tissue macrophages. For example, exposure to lipopolysaccharide (LPS), which is a cell-wall component of Gram-negative bacteria, is known as one of the strongest stimuli that can induce macrophages to

produce tumour necrosis factor (TNF) which causes inflammation. However, intestinal macrophages produce little TNF after LPS stimulation, which is probably why the proinflammatory reaction of intestinal macrophages is characterized by profound anergy [4–6].

A prerequisite for a proinflammatory response by macrophages after exposure to LPS requires the existence of molecules that can bind LPS on the cellular surface. This then needs to be followed by the provocation of intracellular signals to activate the transcription of proinflammatory cytokines, such as TNF. CD14 is the most prominent molecule known to initiate cellular responses to LPS and that binds LPS [7]. However, intestinal macrophages were known not to express CD14 on the cell surface [8–11]. This is thought to be associated with their hyporesponsiveness to LPS [12].

Bacterial components are first recognized by the innate immune system. This system then provokes intracellular signals that eradicate the bacterial components. LPS is the most prominent bacterial component and is one of the strongest activators of the innate immune system [13]. The innate immune system is an ancient system that has been preserved throughout the animal kingdom, and it acts as the principle mechanism in preventing infection by means of phagocytosis and secretion of bactericidal factors [14]. Also, in higher vertebrates it plays an essential role in the induction of the acquired immune system by antigen presentation and secretion of cytokines.

It is known that Toll-like receptors are involved in this process, and it is believed that understanding the molecular interactions of Toll-like receptor (TLR) families would explain how cells (mainly macrophages of the innate immune system) recognize and eradicate various foreign substances. At present, 11 TLRs are known to exist in mammals, and each type of TLR recognizes a different bacterial component [15–17]. Interestingly, intracellular signals provoked by different TLRs partly share common processes that transmit cellular signals and produce effector molecules such as TNF. MyD88 was found to be one of the most commonly used adapter molecules in cells, and it activates IRAK and TRAF6 followed by the activation of transcription factors NF-κB and IRF3, and proinflammatory cytokines are produced [18]. Of the TLRs [19,20], TLR4 is mainly associated with MD-2 [21] and works downstream of CD14 in delivering LPS signals into the cell [22]. Thus, it is thought that activation of the innate immune system is largely regulated by expression of TLR-associated signal transduction molecules, and that their inhibitory factors act cooperatively.

Although CD14 mRNA expression by intestinal macrophages is nearly the same as for monocytes, the level of protein expression by intestinal macrophages is dramatically lower than for monocytes [23]. These observations indicate that it is possible for intestinal macrophages to regulate the expression of CD14 by other processes, such as at a post-transcriptional level, rather than at the transcriptional level. This means that anergy of intestinal macrophages for TNF production in response to LPS may not be inherent but is reversible under certain specific environmental conditions. Accordingly, we confirmed that intestinal macrophages could produce TNF in response to LPS under specific conditions, such as contact with membrane-bound IgA [24]. Thus, it is reasonable to hypothesize that the molecules assuring intra cellular signalling followed by production of TNF in response to LPS also exists in intestinal macrophages. This would allow these cells flexibly in regulating their response to external stimuli (such as LPS). However, until now, there has not been a molecular basis that supports this hypothesis for intestinal macrophages except that MD-2 mRNA expression was reported to be impaired specifically by colonic macrophages [25]. Therefore, we believed that by examining and comparing the expression of TLR and their associated signal transduction molecules in intestinal macrophages with those of other tissue macrophages, we could understand the unique regulatory mechanisms underlying the responsiveness to foreign substances by intestinal macrophages.

This study investigated how tissue-specific characters of macrophages and expression of TLR and their associated signal transduction differed between intestinal macrophages and other tissue macrophages.

Materials and methods

Isolation of tissue macrophages

Colon tissue was isolated from Spraque-Dawley (SD) rats (5 weeks old; Japan SLC, Inc.) or C3H/HeN mice (11 weeks old; Japan SLC, Shizuoka, Japan). Resected tissues were cut, and incubated at 37 °C, for 30 min in PBS containing 1 mM ditiothreitol (Invitrogen Japan, Tokyo, Japan) and 1 mM EDTA (Wako Pure Chemical Industries, Osaka, Japan) to remove mucus and epithelial cells [26]. Then tissue was minced and incubated for 60 min in RPMI 1640 (Sigma) containing 400 u/ml collagenase (Wako Pure Chemical Industries), 2·0 μ/ml dispase (Roche), 200 μ/ml deoxyribonuclease (Sigma-Aldrich, St. Louis, MO, USA), 100 μg/ml ampicillin (Meiji Seika, Tokyo, Japan), 50 μg/ml gentamicin (Sigma), and 10% heat-inactivated fetal calf serum (FCS; HyClone Laboratories, Logan, UT, USA). The digest was filtered with a stainless mesh, and then centrifuged through Percoll (Sigma), and a resultant preparation of mononuclear cells was collected from the Percoll-medium. Rat cells were suspended in RPMI 1640 containing 10% FCS, 100 μg/ml ampicillin, and 50 μg/ml gentamicin, and incubated overnight at 37 °C in 5% CO_2. Non-adherent cells were removed with warm PBS, and the adherent cells were used for experiments. Final purity of macrophages (67·3%) was confirmed by immunohistochemical analysis. Mouse cells were purified from the mononuclear cell population by eltriation [9]. Final purity of macrophages (68·7%) was confirmed by flow cytometry. RAW264 cells, mouse leukaemic monocyte cell

line used as the control cells, were maintained in RPMI1640 supplemented with 10% FCS at 37 °C in 5% CO_2.

Alveolar macrophages were isolated as described previously [27]. Alveolar macrophages recovered with saline from SD rats, were used after overnight incubation at 37 °C in 5% CO_2. Final purity of macrophages (67·8%) was confirmed by immunohistochemical analysis.

To collect peritoneal macrophages, 2 ml of 4·05% thioglycolate (Nissui Seiyaku, Tokyo, Japan) medium was injected peritoneally into SD rats. After 4 days, cells were recovered with intraperitoneal injection of PBS and incubated overnight. Adherent cells were used for experiments. Final purity of rat macrophages (84·3%) was confirmed by immunohistochemical analysis.

Phagocytosis assay

Phagocytic capacity was assessed as follows: 1×10^4 cells in 200 μl RPMI 1640 containing 10% FCS, 100 μg/ml ampicillin, and 50 μg/ml gentamicin were added in 96-well plates. Fluorescent latex beads (2 μm in diameter; Polysciences, Warrington, PA, USA) were added at a ratio of 1:10 (cell:beads), and incubated for 24 h. Subsequently, nonphagocytosed beads were removed with PBS. Cells were harvested with 0·25% trypsin/1 mM EDTA, and fixed with 5% formalin on glass slides. Phagocytic cells were counted using a fluorescence microscope.

Immunohistochemistry

The corresponding antibodies (Ab) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for the identification of CD14, CD33 and CD68. Each type of tissue macrophage was fixed with cold acetone on a glass slide for 10 min, and dried at room temperature for 60 min. The cells on the slide were then incubated for 60 min at room temperature with 1·5% normal serum in PBS to block nonspecific binding, and incubated for 60 min at room temperature with 1/100 diluted primary Ab or irrelevant isotype-matched Ab in PBS. Subsequently, the slides were washed three times in PBS. The cells on the slides were then incubated for 60 min at room temperature with 1/2500 diluted biotinylated secondary Ab in PBS, followed by incubation with ABC complex (Vectastain ABC kit; Vector Laboratories, Peterborough, UK) for 45 min. Finally the cells were counterstained with haematoxylin.

Flow cytometry

Mouse intestinal macrophages and mouse peritoneal macrophages (5×10^5 cells) were washed twice with 1 ml of 3% BSA/PBS. For the staining of surface antigens, cells were incubated with the PE-conjugated mAb CD14 (BD PharMingen, San Diego, CA, USA), or FITC-conjugated mAb F4/80 (Serotec USA, Washington, DC, USA) for 15 min at room temperature. For the staining of intracellular CD14, cells were treated with Intra Prep™ Permeabilization Reagent (Sigma) and incubated with the PE-conjugated mAb CD14 for 15 min at room temperature. Control Abs included PE- and FITC-labelled irrelevant rat mAb of the same isotype used in the same concentration as the other Abs. After washing, cells were fixed in 0·5% paraformaldehyde/PBS and analysed by flow cytometry (EPICS Altra, Beckman Coulter, Tokyo, Japan).

Expression of mRNA in each type of macrophage

Total RNA was isolated from intestinal macrophages, alveolar macrophages, and peritoneal macrophages using Trizol reagent (Invitrogen Japan) following the manufacturer's instructions. Total RNA was reverse transcribed with Super Script II (Gibco) at 42 °C. Real-time PCR was performed with DyNAmo Master Mix (Finnzymes, Espoo, Finland) that allow an automated quantification of the amplified products in real time with the DNA Engine Opticon™ System (MJ Research, Tokyo, Japan). Primer sequences are presented in Table 1. The thermal cycling conditions comprised 10 min at 95 °C and then 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s, followed by a standard melting curve analysis. Annealing temperatures were optimized for the primer pairs

Table 1. Primer sequences used for amplification in real-time PCR.

Target	Forward primer	Reverse primer
β-actin	GAGACCTTCAACACCCCAGC	ACAGAGTACTTGCGCTCAGG
CD14	GGAAACCTAGGCCAGAGGGA	AGCAAAGCCAAAGTTCCTGA
TLR4	GAGGACTGGGTGAGAAACGA	GAAACTGCCATGTCTGAGCA
MD2	CCTCCGATGCAATTATTTCC	CTTCGGCAATTCTATGGAGT
MyD88	GAGATCCGCGAGTTTGAGAC	CTGTTTCTGCTGGTTGCGTA
TIRAP	CCAAGAAGCCTCGAGACAAG	TGTGGCTGTCTGTGAACCAT
IRAK4	TCACGAATGACTTCGACGAG	CCAAGCTTCTTCACCGCTAC
TRAF6	CAGCGCTGTGCAAACTACAT	GCAGTTCTGGCTTAGCATCC
TRIF	ACTGCCCAGTCTGTCAGGAG	CTAGTCACACTTCCGCGACA
TNF-α	GTCGTAGCAAACCACCAAGC	TGTGGGTGAGGAGCACATAG
iNOS	GTGGTGACAAGCACATTTGG	GTCATGAGCAAAGGCACAGA
INF-γ	ACCCGTCACAGATGGAGAAG	AGTCTCATTCCACCCAGTGC

Annealing temperatures were 60°C.

used. The number of copies in each real-time PCR reaction was normalized to that of β-actin.

TNF induction

Intestinal macrophages, alveolar macrophages and peritoneal macrophages (1×10^5 cells/well) were cultured in 96-well plates and treated with LPS (100 ng/ml) for 3 h. Supernatants were harvested for measurement of TNF. TNF activity was measured by L-929 cytotoxicity assay as described previously [28].

NO induction

Intestinal macrophages, alveolar macrophages, and peritoneal macrophages (1×10^5 cells/well) were cultured in 96-well plates and treated with LPS (100 ng/ml). After 24 h, a portion of supernatant from each well was removed and was tested for NO production. NO production was measured by Griess reaction [29]. 100 µl of supernatant was mixed with an equal volume of Griess reagent (1% sulphanilamine, 0·1% naphthylethylenediamine dihydrochloride, 2·5% phosphoric acid) in duplicate 96-well plates at room temperature for 10 min in the dark. Chromophore absorbance at 550 nm was measured. Sodium nitrite was used as the standard.

Statistical analysis

Data are expressed as means (± S.D). Statistical analyses were preformed using Student's *t*-test. Differences were considered as significant at *P*-value of < 0·05.

Results

Characterization of intestinal macrophages

Before testing signal transduction by LPS in intestinal macrophages, we first measured the fundamental characteristics of both intestinal macrophages and of other tissue macrophages (alveolar macrophages and peritoneal macrophages). Phagocytosis, the most essential function of macrophages, was measured using the incorporation rate of latex beads. The results demonstrated that none of the tissue macrophages were significantly different with respect to phagocytosis (intestinal macrophages; 71·2 ± 3·5%, alveolar macrophages; 79·5 ± 8·4%, and peritoneal macrophages; 70·7 ± 14·5%) (Fig. 1).

Macrophage-specific surface markers were also tested by immunohistochemical analysis. As shown in Fig. 2, intestinal macrophages expressed CD33 and CD68, but not CD14. Alveolar macrophages expressed CD14 weakly, and CD33 and CD68 strongly. These results are similar to the expression pattern that was reported previously [6,8,12].

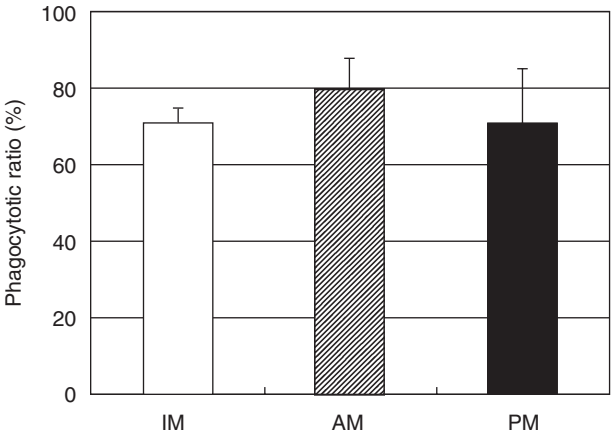

Fig. 1. Phagocytic activity of macrophages. Latex beads and cells were mixed together and incubated for 24 h. The ratio of beads to cells was 10–1. The percentage of cells that phagocytosed latex beads was counted under a fluorescence microscope. IM, intestinal macrophages; AM, alveolar macrophages; PM, peritoneal macrophages.

Expression of mRNA of the molecules associated with the LPS-signal transduction under nonstimulated conditions

Induction of an inflammatory reaction following recognition of LPS is a complicated process accompanied by activation of various molecules. While CD14 and TLR4 are known to be involved in the major signalling pathway producing TNF, little information on mRNA expression had been obtained until now. CD14 is a major receptor of LPS [7]. CD14-LPS complex is further associated with TLR4/MD-2 complex, and a signal is transduced into the cells. The transmitted signal activates MyD88, TIRAP, IRAK and TRAF6, and induces NF-kB by phosphorylation of IKK [30]. At the same time, the TRIF pathway, which is not mediated by MyD88, activates IRF3 and induces IFN-β. In this paper, we investigated the level of mRNA expression of these receptors, adaptors, and signalling molecules in intestinal macrophages by real-time PCR.

The mRNA expression of CD14 in mucosal macrophages (intestinal and alveolar) was lower than in peritoneal macrophages, and it was higher in intestinal macrophages than in alveolar macrophages. Also, it is significant that expression of TLR4 in intestinal macrophages was lower than in alveolar and peritoneal macrophages, and that mRNA expression of MD-2 in intestinal macrophages was lower than in peritoneal macrophages or alveolar macrophages (Fig. 3a). By contrast, the expression of molecules involved in intracellular signal transduction (MyD88, TIRAP, IRAK, TRAF6 and TRIF) in intestinal macrophages was comparable to that of alveolar and peritoneal macrophages. The expression of mRNAs by intestinal macrophages was even slightly higher than for alveolar macrophages, though not

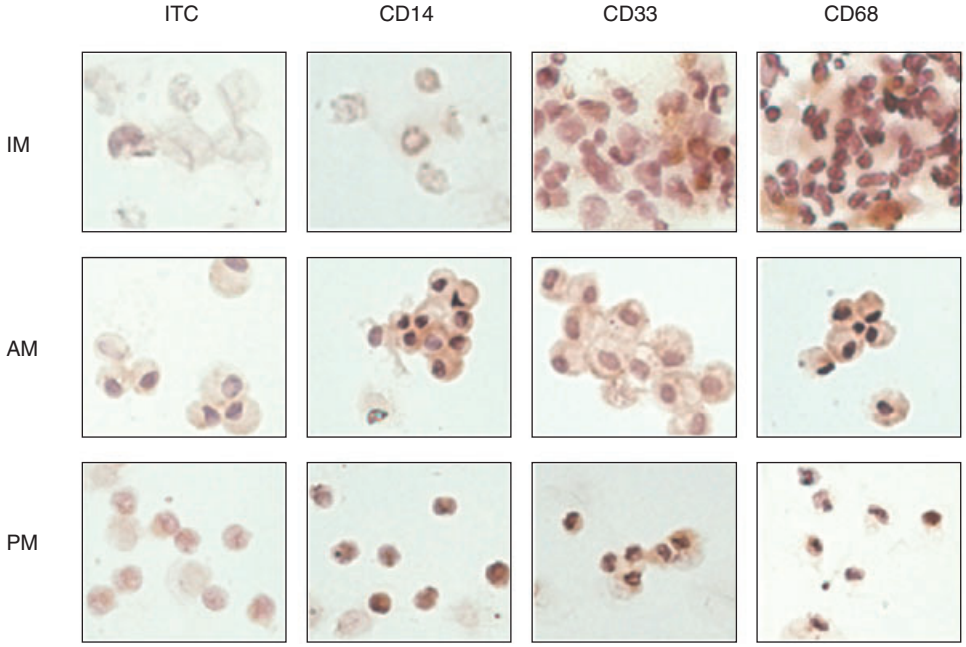

Fig. 2. Immunohistochemical analysis of intestinal macrophages (IM), alveolar macrophages (AM) and peritoneal macrophages (PM). Cells were fixed on glass slides. Cells were stained with antibodies for CD14, CD33, CD68 and the isotype control (ITC). Representative images are shown (magnification × 400).

significantly (Fig. 3b). These results suggest that the specific unresponsiveness of intestinal macrophages to LPS is not caused by transcriptional repression of the molecules for LPS receptors and intracellular signalling molecules, since their mRNAs are expressed at almost comparable levels with other macrophages, even though the mRNA expression of TLR4 and MD-2 were slightly lower than other macrophages.

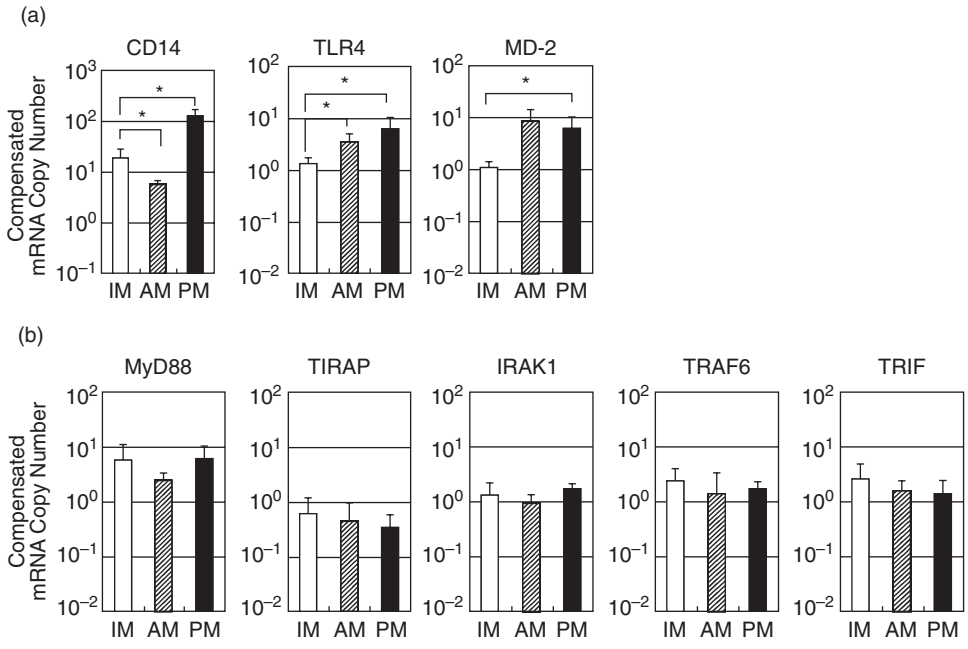

Fig. 3. Expression of mRNA of the molecules associated with LPS signal transduction under nonstimulated condition. Expression of the molecules was analysed by real-time PCR. Results are expressed as relative copy number in relation to that of the housekeeping gene, β-actin. (a) Expression of LPS receptors. (b) Expression of the intracellular signal transduction molecules. $^*P < 0{\cdot}05$. IM, intestinal macrophages; AM, alveolar macrophages; PM, peritoneal macrophages.

Expression of mRNA of the molecules associated with the LPS-signal transduction after LPS stimulation

Although there is little information concerning mRNA expression of the molecules relevant to signal transduction in response to LPS by tissue macrophages, these molecules appear to be ready to act during an invasion of Gram-negative bacteria. Also mRNA expression of these molecules, especially CD14 and TLR4 [31], are reported to be modulated in response to LPS. These results suggest that it might be a common feature of tissue macrophages that the molecules involved in signal transduction after LPS exposure are already present, but are only partly inducible with LPS stimulation. For this reason, we investigated whether intestinal macrophages have different features than other tissue macrophages regarding mRNA expression of the molecules relevant to signal transduction in response to LPS. We analysed mRNA expression of receptor, adaptor and signal transduction molecules in intestinal macrophages and compared the values with those of alveolar and peritoneal macrophages.

There was no significant induction of CD14 mRNA expression in intestinal macrophages. By contrast, there was 10 times more enhancement in alveolar and peritoneal macrophages after LPS stimulation. With respect to the mRNA expression of TLR4 after LPS stimulation, there were no significant differences between any of the tested macrophages. The mRNA expression of MD-2 was reported to be increased in human monocytes 3 h after LPS stimulation [32]. However, in our experiments, mRNA expression of MD-2 had not changed significantly in intestinal and alveolar macrophages, and was only observed to decrease in peritoneal macrophages (Fig. 4a). Also, the mRNA expressions of the molecules for intracellular signal transduction (MyD88, TIRAP, IRAK, TRAF6 and TRIF) were not remarkably induced after LPS stimulation in any of the macrophages (Fig. 4b). These results demonstrate that there is no significant influence on the expression of mRNA of LPS receptors and intracellular signal transduction molecules in intestinal macrophages after exposure to LPS.

Production of effector molecules with LPS

Next, we investigated TNF and NO production responding to LPS stimulation. Both TNF and NO were not induced after LPS stimulation by intestinal macrophages at the concentration of LPS where alveolar and peritoneal macrophages secrete detectable levels of both molecules (Fig. 5a). To address whether the absence of induction of both molecules in intestinal macrophages is dependant on transcriptional regulation, we further examined mRNA expression of TNF and iNOS, NO-producing enzymes.

Without LPS stimulation, TNF mRNA was almost non-existent for each type of macrophage (Fig. 5b). After LPS stimulation, high expression was induced in alveolar and peritoneal macrophages, but a change of expression was not observed in intestinal macrophages.

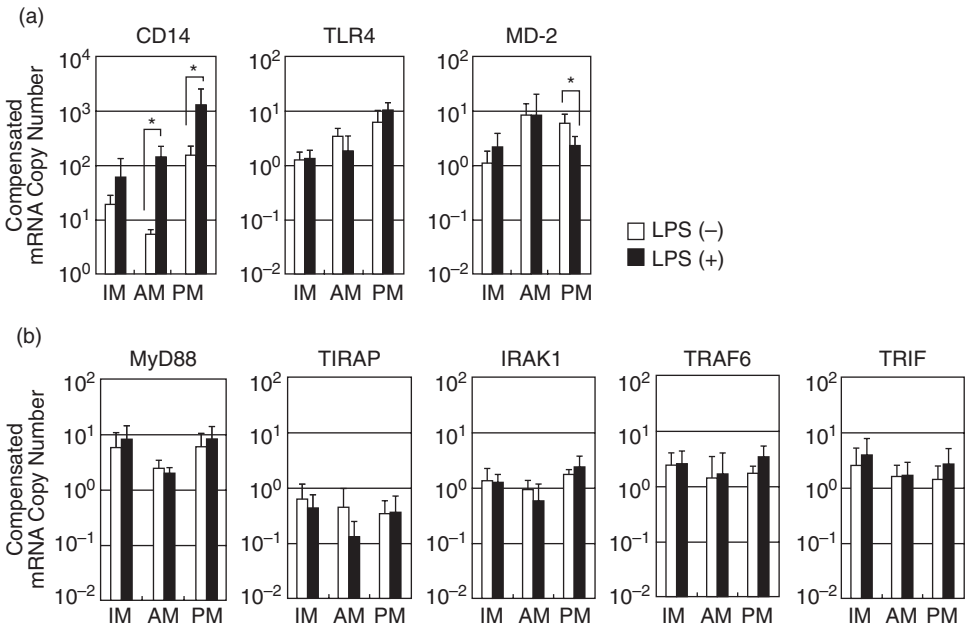

Fig. 4. Change in expression of mRNA of the molecules associated with the LPS signal transduction by LPS. Intestinal macrophages (IM), alveolar macrophages (AM) and peritoneal macrophages (PM) were cultured with (■) or without (□) LPS (100 ng/ml) for 4 h. Expression of the molecules was analysed by real-time PCR. Results are expressed as relative copy number in relation to that of the housekeeping gene, β-actin. (a) Expression of LPS receptors. (b) Expression of the intracellular signal transduction molecules. $^*P < 0{\cdot}05$.

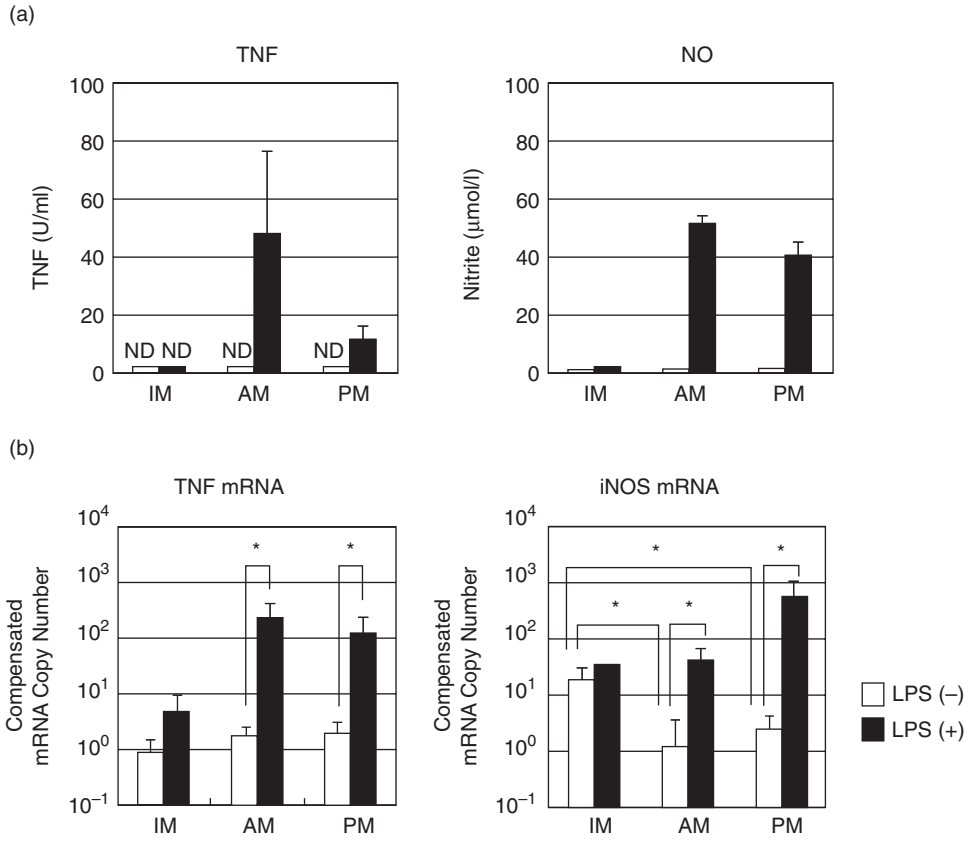

Fig. 5. Change in expression of mRNA of TNF and iNOS and the products with LPS. Intestinal macrophages (IM), alveolar macrophages (AM) and peritoneal macrophages (PM) were cultured with (■) or without (□) LPS (100 ng/ml) for 4 h. (a) Expression of TNF protein and NO. ND: not detected. (b) mRNA expression of TNF and iNOS, analysed by real-time PCR. Results are expressed as relative copy number in relation to that of the housekeeping gene, β-actin. $^*P < 0{\cdot}05$.

Expression of iNOS mRNA under nonstimulated conditions in intestinal macrophages was higher than for other macrophages. It is note worthy that, the basal level of iNOS mRNAs did not contribute to the NO production (Fig. 5a). However, induction of iNOS mRNA with LPS stimulation was not observed, which was different than the significant induction shown by other types of macrophages (Fig. 5b).

The above results show an absence of induction of TNF and NO responding to LPS stimulation in intestinal macrophages. This is ascribed to the absence of induction of corresponding mRNAs.

Intracellular expression of CD14

The absence of mRNA induction of TNF and iNOS in response to LPS means that the LPS signal is not transduced in intestinal macrophages. Because the mRNA expression of molecules concerned with LPS signal transduction in intestinal macrophages was almost the same as for alveolar and peritoneal macrophages (Fig. 5b), post transcriptional regulation of those molecules (possibly CD14 protein expression) in intestinal macrophages seemed to correlate with the absence of LPS signal transduction. To examine the regulatory step for surface expression of CD14 protein, we tested intracellular expression of CD14 by flow cytometry. In this experiment, we used mouse intestinal macrophages (obtained by elutriation) because the mouse antibodies suitable for distinguishing intestinal macrophages are available. The expression pattern was compared with mouse peritoneal macrophages. As shown in Fig. 6, CD14 proteins were detected both intracellularly and on the surface of RAW264 cells (mouse monocyte cell line). Although surface expression of CD14 was not detected, intracellular expression was observed in intestinal macrophages.

Discussion

Intestinal macrophages are the first cells that develop as part of the innate immune system, and the macrophages supply the major functions by interacting with pathogenic microbes [14]. In previous studies we showed that intestinal macrophages had different cytological characteristics compared to other tissue macrophages, probably because they are directly exposed to substances from the external environment. The

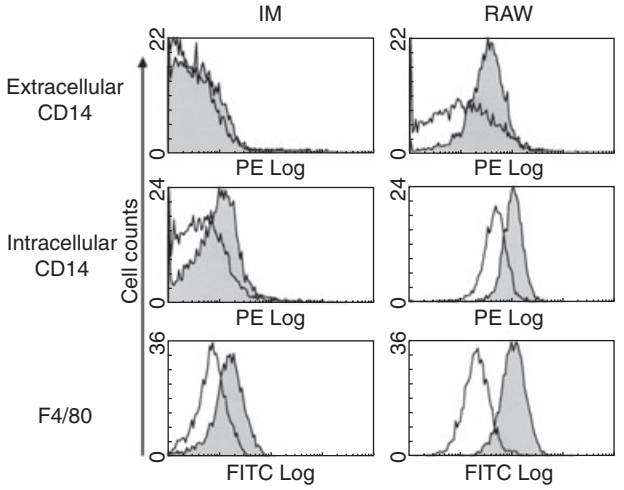

Fig. 6. Expression of CD14 in intestinal macrophages analysed by flow cytometry. Mouse intestinal macrophages (IM) and RAW264 cells (RAW) were stained with FITC-conjugated F4/80 and PE-conjugated CD14 antibodies (■). Isotype control Abs served as negative control staining (□).

biggest difference known for intestinal macrophages is that they do not show a response to many foreign substances, including LPS [4–6]. However, it has not been clear what kind of molecules were involved or how these molecules interacted to suppress the response to foreign substances.

It is known that phagocytotic activity, which is thought to be the basic and common characteristic of macrophages in general, was retained in intestinal macrophages [8,26,33]. Our results also show that the phagocytotic activity of intestinal macrophages is essentially the same as for other tissue macrophages (Fig. 1).

To elucidate the molecular mechanisms of unresponsiveness of intestinal macrophages to LPS, we qualitatively and quantitatively analysed the expressions of relevant molecules including receptors, cellular adaptors, and signal transducers. Signal transduction by LPS in intestinal macrophages was compared with two tissue macrophages known to produce TNF in response to LPS. One of the tested macrophages was peritoneal macrophages. The other was alveolar macrophages, which like intestinal macrophages, resides at an interface with the external environment. Phenotypic characteristics of each macrophage are shown in Fig. 2. Only intestinal macrophages were negative for the surface expression of CD14, which is consistent with the characteristics reported previously [8,9,12]. Differences in the expressions of mRNA that correspond to the molecules including receptors, cellular adaptors, and signal transduction molecules were not apparent enough to explain the unresponsiveness of intestinal macrophages to LPS (Fig. 3). Although several earlier reports demonstrated that intestinal macrophages failed to express CD14 mRNA [12], our results demonstrated that intestinal macrophages expressed CD14 mRNA, apparently at a higher level than alveolar macrophages but at a lower level than peritoneal macrophages. Supporting this view is the report by Ortega-Cave *et al.* [34] that the expression of both CD14 and TLR4 was heterogeneous depending on the region from which intestinal macrophages were isolated. Thus, some differences might be dependant on which intestinal region was used in each experiment.

Although there were no apparent differences in the level of expression of mRNA among the tested macrophages, there were marked differences among these cells in the expression of corresponding proteins. As shown in Fig. 3a, although the level of mRNA expression of CD14 in intestinal macrophages without stimulation with LPS was significantly higher than that in alveolar macrophages, the protein expression of CD14 on the cell surface of intestinal macrophages was barely detectable immunohistochemically (Fig. 2). Flow cytometric analysis using mouse intestinal macrophages showed that intracellular expression of CD14 protein was detected in the same way as for RAW264 cells (Fig. 6). These results suggest that CD14 transport from the intracellular region to the membrane surface had been inhibited in intestinal macrophages. Similar regulation of the protein-transport step was also reported for TLR4 [35]. TLR4 works downstream of CD14 and delivers the LPS signal. The LPS hyporesponsiveness of C3H/HeJ mice (which bear a mutation in the signalling domain of TLR4 proteins) indicates that TLR4 is indispensable for LPS signalling [19,36]. The mRNA expression of TLR4 in intestinal macrophages was not absent, but was low compared to that of other macrophages. Moreover, intestinal epithelial cells were reported not to express the TLR4 protein on their cell surface in spite of the expression of the corresponding mRNA [37]. Also, in crypt epithelial cells in intestine, TLR4 was reported to be localized on the Golgi apparatus instead of cell surfaces suggesting that transportation of TLR4 might be impaired in these cells [35]. Thus, down-modulation of cell-surface TLR4 proteins, in addition to the low expression of the corresponding mRNA might contribute to the hyporesponsiveness to LPS in intestinal macrophages. Physical association of MD-2 with TLR4 is critical for LPS responses [22,38]. The results of an experiment using mice lacking MD-2 indicated that MD-2 is an indispensable molecule for LPS responses [39]. Shirai *et al.* [25] proved that mRNA expression of MD-2 in colonic macrophages was low, and suggested that LPS hyporesponsiveness was possibly due to the low expression of MD-2. Our data also showed that mRNA expression of MD-2 in intestinal macrophages was lower than in alveolar and peritoneal macrophages (Fig. 3a). By contrast to the results for LPS receptors, i.e. CD14 protein and TLR4 and MD-2 mRNA, apparent difference in mRNA expression for intracellular signalling molecules was not observed in our experiments (Fig. 3b). Thus, although we cannot make a definitive conclusion until more complete analyses for protein expression and activation of proteins have been completed, it appears that the low protein of cell-surface LPS-receptor molecules (including CD14, TLR4 and MD-2)

might be related to the hyporesponsiveness to LPS in intestinal macrophages.

It was believed that the unresponsiveness of intestinal macrophages, the lack of both TNF and NO production in response to LPS, was thought to be physiologically appropriate in that this suppresses unnecessary inflammation. This is necessary because intestinal macrophages are the first phagocytotic cells located in the subepithelial lamina propria that come in contact with foreign substances such as bacteria, which have breached the epithelium. As shown in Fig. 5b, the level of expression of iNOS mRNA in intestinal macrophages without LPS stimulation was significantly higher than that in alveolar and peritoneal macrophages. The level without LPS stimulation was even comparable with that in alveolar macrophages after LPS stimulation. However, NO production by intestinal macrophages was hardly detectable, but was dramatically higher in alveolar macrophages (Fig. 5a).

Though it has been reported that intestinal macrophages do not produce TNF, we found that intestinal macrophages had the inherent ability to produce TNF after exposure to specific stimuli, such as sarcophaga lectin [24]. Thus, unresponsiveness of intestinal macrophages should not be considered as an inherent characteristic but as a plastic one that can be cancelled by certain stimuli.

In Crohn's disease, TNF is produced in the intestinal lumen, and the presence of the TNF is correlated with the progression of the disease. This study indicates that it is possible that the intestinal macrophages might be responsible for producing the TNF. Thus, the hypothesis that it is recruited monocytes, not intestinal macrophages, that produce the TNF-inflamed lesions of Crohn's disease may not be true.

Tissue macrophages are derived from blood monocytes. Accordingly, it has been assumed that the tissue-specific characteristics of intestinal macrophages (such as their unresponsiveness to LPS exposure) were induced and regulated within the environmental conditions of intestinal lamina propria. Smythies *et al.* [40] provided evidence that the unresponsiveness was partly due to TGF-β secreted by intestinal stroma cells. Also, we proved that membrane-bound IgA could revive the responsiveness to LPS, and resulted in production of TNF after LPS stimulation. All these phenomena indicate that intestinal macrophage have diverse regulatory systems. This makes it possible for them to behave as the first innate immune cells that interact with microorganisms and microbial products of the external environment.

Because both intestinal macrophages and alveolar macrophages reside at a boundary with the external environment, one would expect that they would have similar characteristics in terms of their response to foreign substances. However, our study indicates that the LPS response of intestinal macrophages is regulated differently than for alveolar macrophages. In the case of Crohn's disease, it would be very useful to know whether a shift in localization of CD14 or TLR4 in intestinal macrophages to the cell surface occurs because of exposure to particular stimuli such as sarcophagi lectin or membrane-bound IgA.

In conclusion, we investigated the unique LPS-response characteristics of intestinal macrophages and compared these responses with those of alveolar macrophages and peritoneal macrophages. Although the intestinal lumen is normally exposed to large quantities of bacteria, inflammatory responses are hardly detectable under normal conditions. In this regard, Yoshino *et al.* [41] reported that LPS administered orally could affect the systemic immune system and inflammatory responses, suggesting that LPS can be absorbed through intestinal lamina propria. In a similar way, we reported that the small molecular weight of LPS extracted from *Pantoea agglomerans* showed protective and/or curative effects to a variety of diseases including infectious diseases and auto-immune diseases [42,43]. It is still unclear whether these effects are caused by LPS directly through lamina propria or are mediated by the immune system resident in the intestine. Further analyses of the mechanisms of how intestinal macrophages respond to LPS are still needed in order to clarify the regulatory mechanisms in intestinal macrophages. It is anticipated that such knowledge will allow the development of novel therapeutic approaches and medications for a variety of currently intractable diseases.

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