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Review

Unique Molecular Characteristics of the Environmental Responses of Mucosal Macrophages

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Abstract. *Macrophages are thought to be the cells that initially respond to environmental information and transmit this information to other immune cells. We hypothesize that there is a "network system" consisting of various tissue macrophages; the macrophages respond to stimulation and transmit secondary information to neighboring cells, which is important for the maintenance of homeostasis. Macrophages exist in all animal organs as tissue macrophages, and their cellular characteristics may change as an adaption to tissue-specific environments. It is believed that mucosal macrophages are particularly important in the macrophage network system because mucosa exist where there is regular exposure to foreign substances. However, the molecular mechanism by which intestinal mucosal macrophages respond to the external environment is not yet clear. In this review the biological characteristics of mucosal macrophages are introduced and how they recognize and eradicate various foreign substances is discussed.*

Maintaining Homeostasis with the Macrophage Network

Macrophages play a central role in the immunological defense mechanism against invasive pathogens; they are the first cells to recognize foreign substances and to remove them by phagocytosis. Thereafter, they transmit this primary information to neighboring cells by secreting cytokines, by cell adhesion and by migration and antigen presentation. Kohchi *et al.* presented a concept called the "macrophage network" hypothesis in which there is an information signal-transfer system from local activated macrophages to other macrophages by cell-to-cell contact (1). Tissue macrophages are variously named depending on their location, for example, microglias in the brain, Kupffer cells in the liver and alveolar macrophages in the lung. Macrophages were originally monocytes that differentiated from bone marrow cells, migrated to various tissues of the body *via* the circulatory system (2) and differentiated into various tissue-specific macrophages (3). Tissue macrophages retain the essential functions of macrophages, expressing different characteristics depending on their tissue location. For example, Kupffer cells and alveolar macrophages have similar abilities for antigen presentation and secretion of immunological mediators. However, Kupffer cells have substantially higher phagocytic activity than alveolar macrophages, while the macrophages produce significantly greater quantities of reactive oxygen species and nitric oxide (NO) than Kupffer cells (4). Thus, it is thought that macrophages play a central role in homeostasis maintenance based on the macrophage network hypothesis (the signal transduction system utilizing macrophage-to-macrophage interactions).

The Mechanism Used by Macrophages for the Recognition and Elimination of Foreign Substances

Macrophages can eliminate foreign substances by phagocytosis, recognizing and targetting a wider range of foreign substances than other phagocytes, such as neutrophils. Moreover, macrophages can recognize foreign bodies, such as microorganisms, and also cells derived from the body itself, such as wasted erythrocytes and cancer cells (5, 6). Therefore, the target of macrophages may be all foreign substances, which are recognized by various types of

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receptors; these differ from specific antigen receptors, such as T-cell receptors and B-cell antigen receptors. Especially important are the pattern recognition receptors (PRR) that are involved in the discrimination of foreign substances (7).

In mammals, the molecules of PRR include Toll-like receptors (TLRs), which are homologs of insect TLR. TLR was originally discovered as a differentiation factor in *Drosophila* and was later found to be involved in the self-defense response (immunity) (8, 9). Currently, eleven human homologs of TLRs (1 to 11) have been identified (10-12). Each TLR recognizes a specific structure of a microorganism, such as the flagellum and/or cell-wall structures, and then evokes innate immunity responses. TLR4 was first discovered as the LPS receptor in mammals (13). Generally, TLRs have leucine-rich repeats in the extracellular region, and a highly homologs cytoplasmic Toll/interleukin-1 receptor (TIR) domain, similar to that of the interleukin-1 receptor family. After ligand binding, a signal cascade of TLRs involves adaptor molecules with TIR domains, such as MyD88, TIRAP, TRIF and TRAM. In particular, MyD88 appears to be a control adaptor molecule, which is associated with NF-κB and MAPK (p38 and JNK) activation. However, TLR3 and TLR4 have another signal transduction cascade through activation of IRF3 and NF-κB that does not involve MyD88 (10). Macrophages secrete pro-inflammatory cytokines, such as TNF, which activate neighboring cells which then eliminate foreign substances.

Expression of Recognition Receptors of Foreign Substance in Intestinal Mucosa

Defense from foreign substances is important for maintaining homeostasis. The gastrointestinal mucosa has the largest internal surface in the body in contact with the external environment: in order to enable efficient absorption of nutrients and water, the surface area is equivalent to 1.5 tennis courts (14). However, besides food, the intestine also contains large numbers of microorganisms and microbial products (15). It is known that many bacteria in the intestinal flora have an effect on the digestion and absorption of food, as well as on immunity (16-19). In order to avoid unnecessary inflammation, there is no inflammatory response in intestine with concomitant bacteria or their components (20). When pathogenic bacteria invade the intestine, a defense response is induced by immune cells, such as macrophages in the lumen. If the defense mechanism of the intestine fails, septicemia or endotoxin shock occurs (21-23). Many researchers have investigated the humoral and cell-mediated immunity of the acquired immune systems, but little research has been conducted on mucosal immunity, and this type of immune system is substantially different. The foreign substance recognition receptor group, including TLRs, has only recently been discovered, leading to a rapid increase in understanding of the mechanism of mucosal immunity. It is now clear that the tolerance of normal intestinal mucosa is inversely correlated with surface receptor expression on intestinal epithelial cell (IEC), which limits front-line recognition of foreign substances (24, 25), and is positively correlated with an increased expression of a downstream signaling suppressor, Tollip (26), and the existence of external regulators which suppress TLR-mediated signaling pathways (20).

Characteristics of Intestinal Macrophages

Most macrophages are found in the intestine, where they are believed to play a role in the elimination of undesirable foreign substances and pathogens that could otherwise enter the body from the intestine (27). We hypothesized that intestinal macrophages have the most important position in the macrophage network. However, there are only a few reports on the mechanism of foreign substance recognition and elimination by intestinal macrophages. Previous papers have reported that intestinal macrophages can phagocytose foreign substances but are not then activated (28). In addition, it has been found that intestinal macrophages could phagocytose latex particles (29), but could not produce either tumor necrosis factor (TNF) or NO after stimulation with various macrophage-activating materials, such as LPS, TPA and PWM (28, 30, 31). Their hypo-responsiveness to foreign substances has been attributed to lower expression or defects in the recognition receptors on the intestinal macrophages (32).

Expression of LPS Receptors in Intestinal Macrophages

Immunohistochemical and flow cytometric studies have shown that intestinal macrophages do not express CD14 (LPS receptor) (28, 32-36), CD89 (Fcα receptor) (28, 32, 35), CD16 (28, 36), CD32, CD64 (Fcγ receptor), or CD25 (IL-2 receptor) (28). In particular, detection of CD14, a general surface marker of macrophages, has been widely recognized as a specific feature of intestinal macrophages. In CD14 knockout mice, the mice were more resistant to LPS-induced lethal shock (37) and produced significantly smaller amounts of cytokines in response to LPS (38). CD14 exists as a glycosyl phosphatidylinositol (GPI)-anchored membrane protein on macrophages. As it lacks a transmembrane domain, it is incapable of transmitting a signal through the membrane. Therefore, it was postulated that an accessory molecule performs signal transduction, creating a functional LPS-receptor together with CD14. TLR4 and MD-2 are already known to be accessory

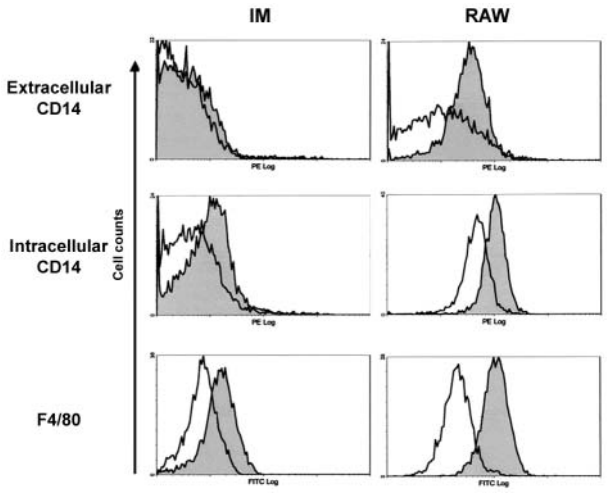

Figure 1. *Intracellular expression of CD14 in intestinal macrophages. CD14 protein on the cell surface and in the cell was compared in mouse intestinal macrophages and RAW264 cells (mouse monocyte cell line) by flow cytometry.*

molecules for CD14. It was confirmed, by a mapping analysis of LPS using LPS-hyporesponsive C3H/HeJ mice (39) and TLR4 knock-out mice (40), that TLR4 is the major LPS receptor. Also, MD-2 was identified as the assembly molecule with TLR4 (41). Although direct binding of LPS to TLR4 has not been proven, MD-2 is thought to be an essential molecule for LPS and TLR4 binding because of its ability to directly bind with LPS (42-44). Also, because CD14 and TLR4/MD-2 molecules recognize different portions of LPS, these molecules appear to transfer intracellular signals synergistically (45, 46). There have been several reports on the regulatory mechanism of CD14 expression by intestinal macrophage: (i) it is possible to constitute CD14 intestinal macrophage-like cells using co-culture with IECs (47, 48); (ii) Smythie *et al.* reported that a factor released from the intestinal stroma suppressed the expression of CD14 on monocytes (28); (iii) Austin *et al.* studied an *in vitro* model of epithelial injury and loss, and reported that intestinal macrophages that migrated from the lamina propria expressed CD14 and responded to LPS (49). These phenomena demonstrate that intestinal macrophages suppress CD14 expression, although the molecular mechanism is still not clear.

For CD14 mRNA, there are reports comparing the expression or non expression of CD14 mRNA of intestinal macrophages with that of peripheral blood macrophages. It is believed that the reason that expression of CD14 in the colon was higher than in the small intestine (50), was the difficulty in detecting these small genes with the experimental techniques used (the Northern-blotting method and RT-PCR). In our experiment, macrophages were isolated from rat colons and then analyzed by real-time PCR as this technique is able to detect this small gene. The results confirmed that intestinal macrophages express more CD14 mRNA than alveolar macrophages, but less than peritoneal macrophages (51). Like intestinal macrophages, alveolar macrophages reside in the mucosa and have a direct interface with the external environment. Their CD14 expression remains at the lowest level when they are not stimulated (52). At lower concentrations of LPS (<0.1 ng/ml), alveolar macrophages behaved much like intestinal macrophages as they did not produce either TNF or NO after LPS stimulation. However, at high concentrations of LPS (>10 ng/ml), one to ten times higher TNF and NO production levels were observed than had occurred in peritoneal macrophage cell lines and monocyte cell lines (53-55). These results indicate that there is a correlation between CD14 expression and LPS responsiveness because of the increase of CD14 mRNA after LPS stimulation. Thus, intestinal macrophages are also thought to express sufficient CD14 mRNA as a response to LPS. In the m-IC_{C12} intestinal epithelial cell line, the expressed TLR4/MD-2 proteins are localized on the Golgi apparatus and not on extracellular membranes (56, 57). This fact suggests that it is possible that CD14 and TLR4/MD-2 proteins exist intracellularly in intestinal macrophages. We also confirmed the intracellular expression of CD14 protein in intestinal macrophages by flow cytometry after increasing membrane permeability and found both CD14 and the TLR4/MD-2 protein complex expressed in the cells (Figure 1). These results suggest that intestinal macrophages synthesize LPS-associated receptor proteins in the intracellular region, but these proteins are not transported onto the membrane surface of the intestinal macrophages. In the above mentioned report on m-IC_{C12} cells, it was demonstrated that intracellular TLR4/MD-2 was able to transduce LPS signals, and that gp96 of chaperone is related to the transportation of TLR4/MD-2 onto the cell membranes. After an epithelial injury, the signal transduction system of intracellular CD14 or TLR4/MD-2 is also activated in intestinal macrophages, as in the m-IC_{C12} cells. This is apparent since they express membrane CD14 and LPS responsiveness after migration out of the lamina propria.

Macrophages that recognize and eliminate foreign substances play an important role in the maintenance of homeostasis. Intestinal macrophages are not activated by foreign substances, and it is still unclear how they exchange information with other cells. It is well known that low expression of CD14 by intestinal macrophages is associated with hypo-responsiveness to foreign substances. Our previous studies also indicate that, intestinal macrophages do not express CD14 on membrane surfaces, and in macrophages from various

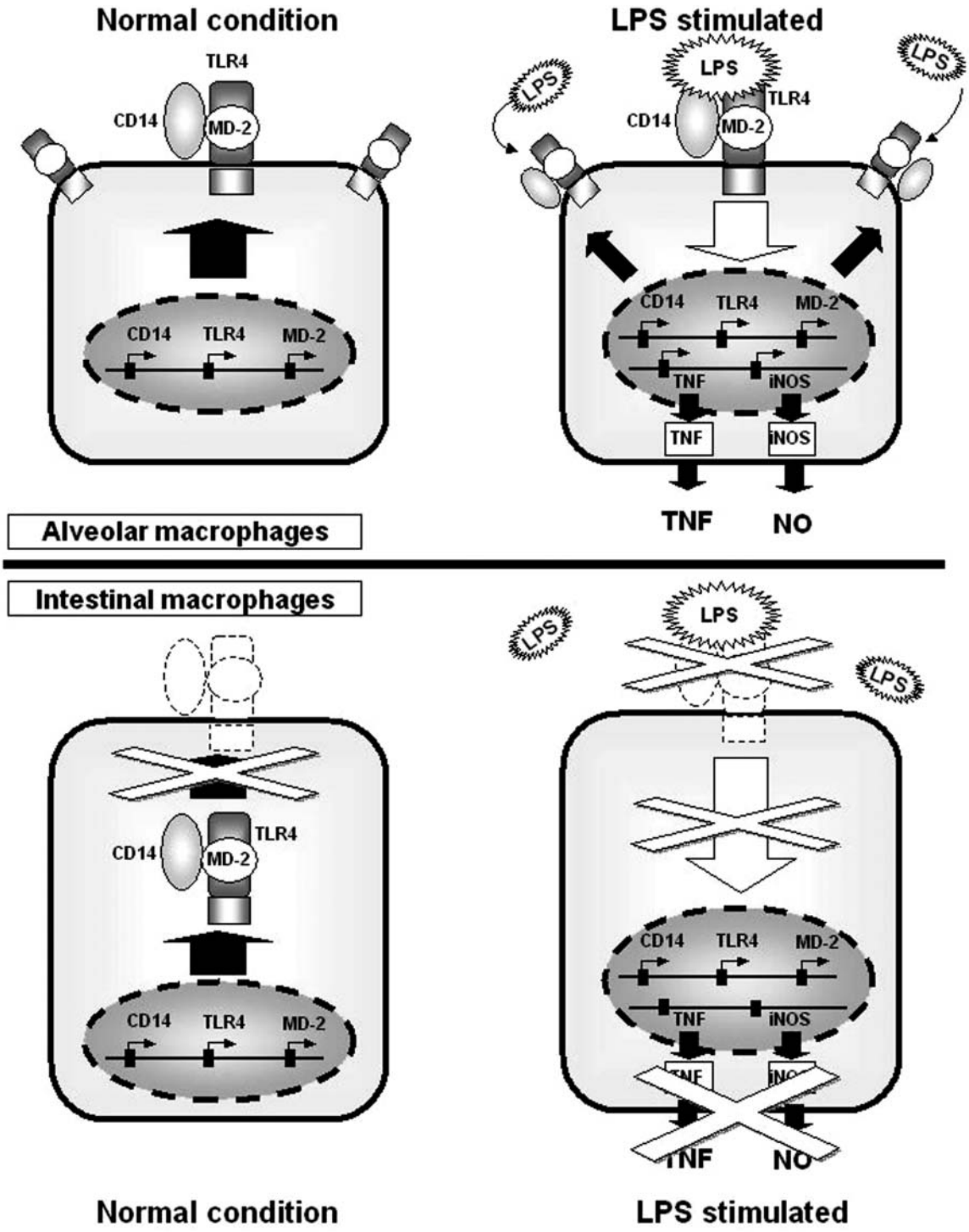

Figure 2. *The expression of the LPS receptors in alveolar and intestinal macrophages. Although alveolar macrophages express little CD14 in a normal state, expression was increased by stimulation and was related to an increased production of TNF and NO.*

tissue there are no differences in the different mRNA expressions of signal transduction associated with molecules except for CD14 (51). However, so far there have been no reports on the mechanism of inhibition of CD14 protein expression in intestinal macrophages. Our recent studies strongly suggest that CD14 expression of intestinal macrophages is inhibited at the level of membrane transport. Moreover, this is believed to be a

completely different inhibitory mechanism than that employed by alveolar macrophages, which also exist in the mucosa (Figure 2). These phenomena require further investigation.

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