

Review

## Utilization of Macrophages in Anticancer Therapy: The Macrophage Network Theory

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**Abstract.** *Appropriate and rational modulation of innate immunity may enhance the therapeutic efficacy of emerging immune therapies for treating cancer. One of the crucial cells of innate immunity is the macrophage. The purpose of this article was to review those issues that suggest ways of exploiting macrophage local functions in immune therapy, and to discuss the suitability of low molecular-weight lipopolysaccharides as potent modulators of macrophage functions for immune therapy of cancer.*

### Development of immune therapy

At present, therapeutic interventions for various types of cancer are divided into four categories, namely: (1) surgical operation, (2) chemotherapy, (3) radiation therapy, and (4) immune therapy. Immune therapy, especially focusing on the acquired properties of the immune system, is the newest antitumor therapy.

It is well established *in vivo* that tumor cells are foreign targets of the immune system. During the last decade, several tumor-associated antigens (TAA) such as MAGE for melanoma, HER-2/neu for breast cancer and CEA for colorectal cancer have been identified(1-3). Currently, a promising hypothesis suggests that certain tumor cells can

be eliminated with immune therapies that use antigens to generate cytotoxic T lymphocytes (CTL)(4, 5). The following immune therapies are being developed to target a number of cancers (Figure 1). (i) Lymphokine-activated killer cells (LAK), CTL, or tumor infiltrating lymphocytes (TIL) are passively administered to the patient's body after activation *in vitro* by stimulation with IL-2 and/or TAA (6). (ii) Dendritic cells (DCs) belong to the myeloid lineage and possess the strongest antigen presentation activity to helper T cells. They have been incorporated in therapies with the purpose of generating CTL more efficiently (7, 8). (iii) Vaccine therapy using killed tumor cells or TAA as a vaccine has also been tested for generating CTL endogenously in a patient's body (9, 10). Each technique augments a specific tumor-killing activity through proper activation of adaptive immunity. Other mechanisms are often combined to kill tumor cells and/or to maximize the antitumor effects of a host immune system. For example, (iv) biological response modifiers (BRMs) are used to activate the patient's immune system, which might be crucial as an adjuvant(11). (v) The drug delivery system, such as a missile therapy in which a chemotherapeutic drug is conjugated or encapsulated with a suitable antibody against TAAs, might be crucial for the reduction of side-effects(12).

These immune therapies are now combined with gene-engineering techniques which make it possible to express TAAs or major histocompatibility antigen classI (MHC classI) or accessory molecules in patient's tumor cells, or to express cytokines by possible effector cells *in vivo* and/or *ex vivo* (13-15). These advances in immune therapy are significant and suggest that future successes in establishing new therapeutic options will complement chemotherapy or radiation therapy in cancer.

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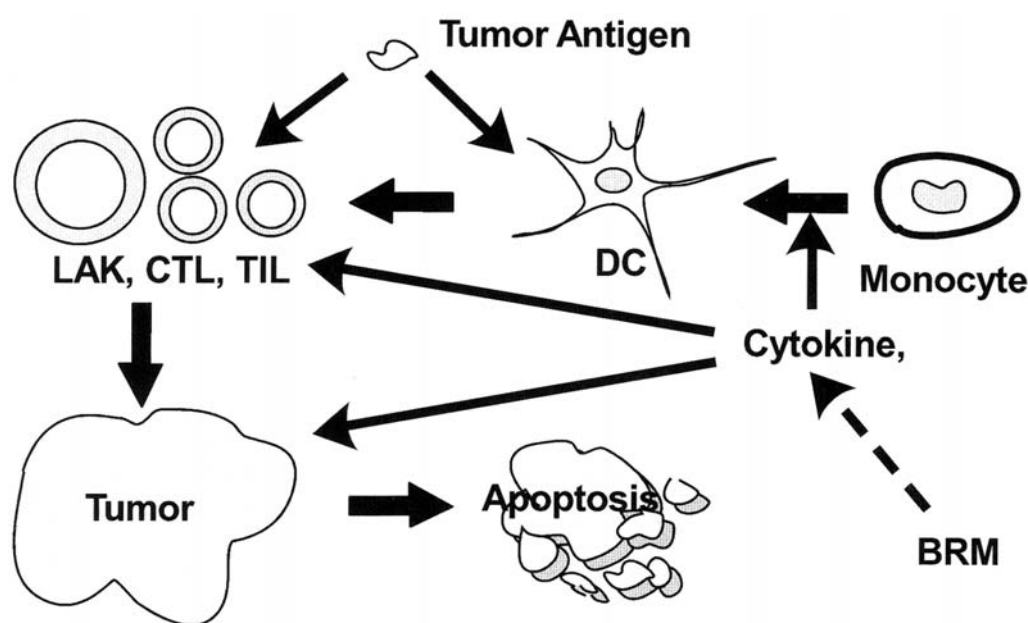


Figure 1. Scheme for immune therapy based on acquired-immunity principles. (1) LAK, CTL, or TIL are passively administered to the patient's body after activation *in vitro* by stimulation with cytokine and/or TAA. (2) DC cells possess strong antigen presentation activity. (3) Vaccine therapy using killed tumor cells or TAA as a vaccine for generating CTL. (4) BRMs and cytokine are also used to activate the patient's immune system.

However, in clinical settings, the efficacy of the immune therapies described above are still limited. There may be a number of factors that contribute to the discrepancy in the efficacy of immune therapy between clinical settings and model systems (including *in vivo* and *in vitro* systems). A major factor appears to be that, after escaping the host immune surveillance, the antigenicity of tumor cells is not necessarily high. It has been shown that most tumor cells lack MHC class I molecules, which is essential for inducing CTL(16). This might be a reason why experimental results focusing on adaptive immunity are not particularly effective in clinical settings.

### Macrophages in immune therapy

The molecular basis of innate immunity is being elucidated by recent progress in immunology. The significance of innate immunity, either by itself and/or in combination with adaptive immunity, has encouraged the re-evaluation of innate immunity (17).

Macrophages are classified into the major cell types involved in the innate immune system. Until recently, manipulation of macrophages has not been considered important for immune therapies.

Macrophages have pleiotropic immune regulatory functions. The main functions are: (i) phagocytosis of foreign molecules or damaged cells, (ii) activation of the

acquired immune system by antigen presentation, and (iii) secretion of multiple cytokines to act as regulators and effectors in immune responses (18, 19). Thus, macrophages are involved in almost all aspects of immune therapy. Also, macrophages are found in or around almost all tumors, although the role of tumor-associated macrophages is still controversial (20). Consequently, if it were possible to activate appropriate functions of the macrophages at a local tumor site, it would provide a new strategy for immunotherapy that is based not only on adaptive immunity but also on another modality.

To fully and appropriately utilize the functions of macrophages locally at tumor sites, it is necessary to understand the tissue-specific characteristics of macrophages, and the regulatory mechanisms involved in the recognition and elimination of the tumor cells.

### Tissue specificity of macrophages

Macrophages exist in a variety of tissues as tissue-specific macrophages(18). They are called microglia in brain, alveolar macrophages in lung, intestinal macrophages in intestine, Kupffer cells in liver, and so on (Figure 2). Of the tissue macrophages, it is the alveolar macrophages and intestinal macrophages that are exposed directly to the external environment. However, the response of each type of macrophage to lipopolysaccharide (LPS) can be quite

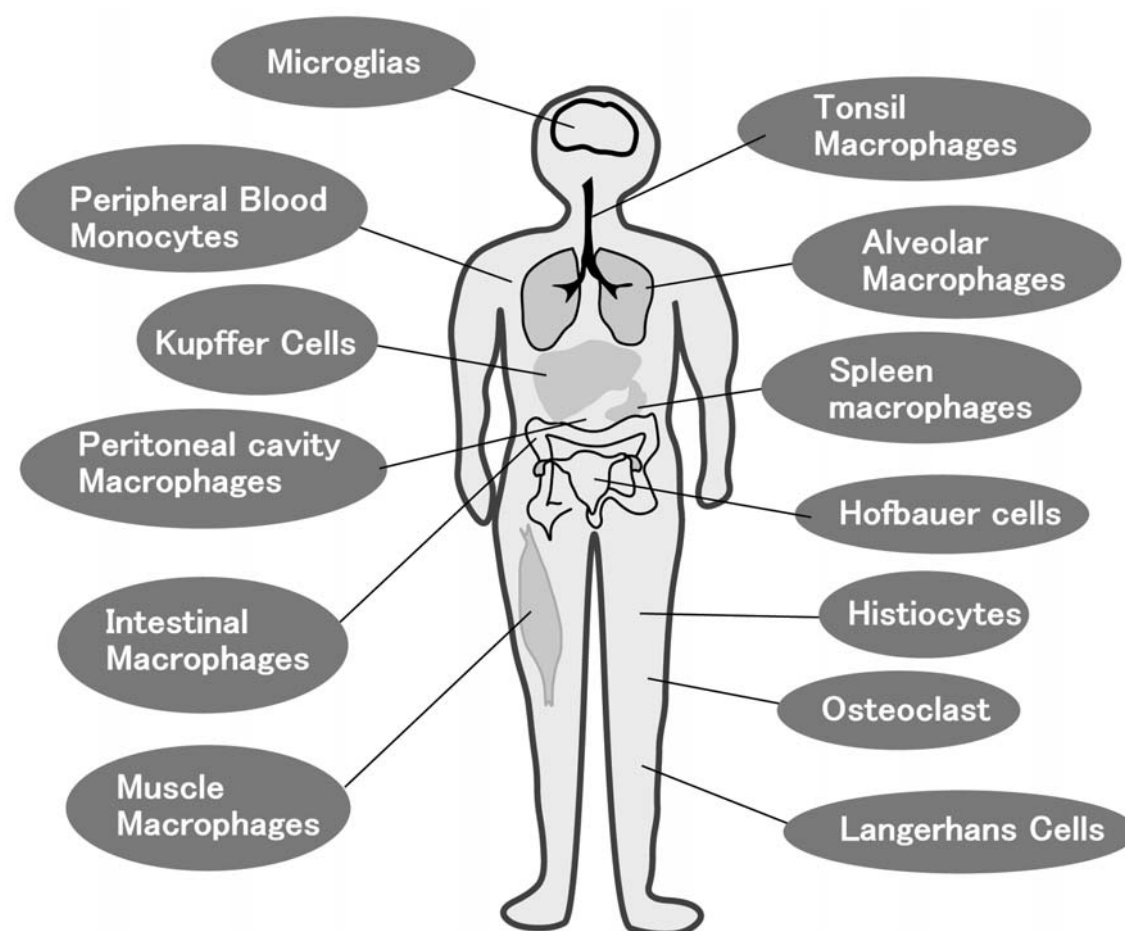


Figure 2. Distribution of macrophages. Tissue-specific macrophages exist in a variety of tissues.

different. We have recently examined the production of tumor necrosis factor (TNF) and nitric oxide (NO) by rat intestinal macrophages, alveolar macrophages and peritoneal macrophages in response to LPS. Production of TNF and NO was not observed in intestinal macrophages even with a high concentration of LPS (100  $\mu\text{g/ml}$ ). On the other hand, alveolar macrophages responded to LPS and produced both molecules. However, the amount of NO produced by alveolar macrophages is lower than the amount produced by peritoneal macrophages (manuscript in preparation). These results clearly show that responses to external stimuli, as exemplified by LPS, are not identical for each type of macrophage. Thus, the tissue specificity of macrophages must be considered when they are utilized for the purpose of regressing tumors.

Recently, there has been remarkable progress in the study of the recognition of foreign substances, namely the pathogen-associated molecular pattern (PAMP) receptors (21). It has been shown that LPS provokes a cellular

response by attaching to toll-like receptors (TLR) 4 (one of the PAMP receptors), which is followed by clustering of several cytoplasmic adaptor molecules and, finally, by induction of activation of transcription factors NF- $\kappa\text{B}$  and/or IRF3(22). Inductions of TNF and iNOS, which is an enzyme catalyzing the oxidative deamination of L-arginine to produce NO, depends on NF- $\kappa\text{B}$  activation(23).

We examined the expression of the mRNAs of several receptors and adaptor molecules that are involved with LPS responses in intestinal macrophages, alveolar macrophages and peritoneal macrophages by real-time PCR to determine the regulatory mechanism in which TNF and NO are produced in response to LPS. The level of expression of the mRNAs corresponding to each molecule is different for each type of tissue macrophage. This differential expression of mRNAs is relevant to the signal transduction of LPS, and may be partly responsible for the different LPS-susceptibilities for each type of tissue macrophage. However, the expression profile of these mRNAs was not

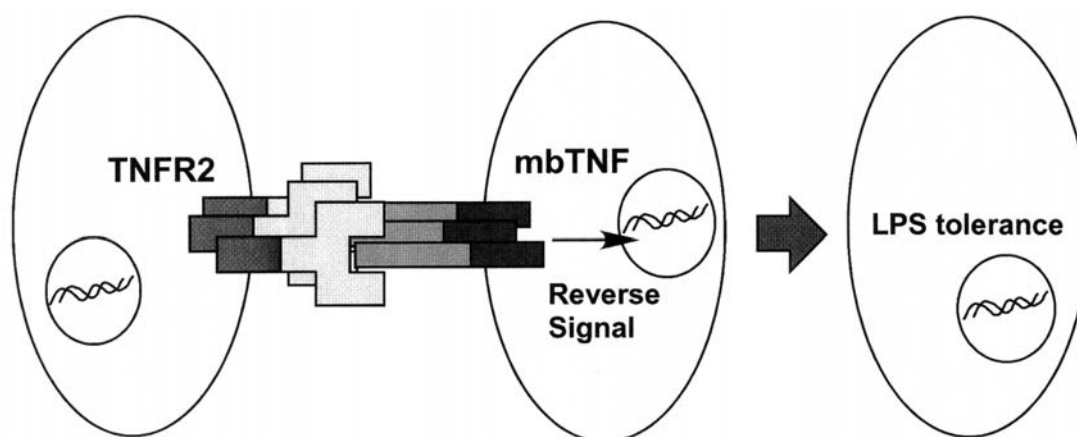


Figure 3. Induction of an LPS-tolerance state in macrophages using a reverse-signal mechanism. The state of LPS tolerance or priming in macrophages can be modified by cell-to-cell contact. The crosslink of membrane-bound TNF reverse signal with TNFR2 leads to an LPS-tolerance state in THP-1 cells.

necessarily coordinated with the expression of the effector molecules such as TNF and NO, for each type of macrophage. In some instances, the mRNA expression levels of TLR4, CD14, Myd88 and TRAF6 (which are all considered to be molecules essential to signal transduction from LPS) in intestinal macrophages are comparable or higher than the expression by alveolar macrophages. This suggests that other regulatory mechanisms may be involved in the production of TNF and NO, and that the mechanisms might also be regulated differently for each type of tissue macrophage (manuscript in preparation). These results clearly show that tissue macrophages have different characteristics depending on the tissue where they exist.

### Acquired functional diversity of macrophages

As described above, the responses of macrophages to LPS are different depending on tissue type. In addition, macrophages exposed to LPS show reduced responses to a second stimulation with LPS, which is termed "LPS tolerance" (24). On the other hand, IFN- $\gamma$  pretreatment appears to augment TNF production by macrophages in response to LPS, although IFN- $\gamma$  by itself is not capable of stimulating TNF production. This phenomenon is termed "priming" (25).

Interestingly, LPS tolerance or priming could be modified by cell-to-cell contact. We tested this phenomenon using THP-1 cells, a human acute-monocytic-leukemia cell line. THP-1 cells grow in suspension. After co-culture with cells adherently grown, THP-1 cells were re-collected and stimulated with LPS. When, THP-1 cells were co-cultured with NIH 3T3 cells, the production of TNF in response to LPS was repressed. On the other hand, when THP-1 cells were co-cultured with COS-1 cells, the production of TNF

in response to LPS was enhanced. These results suggest that macrophages could be induced into opposite states, LPS tolerance or priming, by cell-to-cell contact. This phenomenon is of interest, because this functional diversity is acquired by contiguous substances, *i.e.* self and non-self.

Although the molecular mechanism for LPS tolerance and priming are not fully understood, down- and up-regulation of receptors and/or adaptor molecules for LPS signal transduction appeared to be involved (26-29).

On the other hand, a "reverse signal" mechanism may be involved in the induction of LPS tolerance by cell-to-cell contact. We first mentioned the reverse signal, by which TNFR could act as a ligand to transmit some signals through membrane-bound TNF (30-34). THP-1 cells co-cultured with NIH3T3 cells up-regulate TNFR2 expression, but not THP-1 cells co-cultured with COS-1 cells. Between the two TNF receptors, TNFR2 is known to be the principle receptor to bind membrane-bound TNF (35). Our preliminary experiment suggested that crosslinking of membrane-bound TNF with TNFR2 led to the LPS tolerance state in THP-1 cells (manuscript in preparation). Thus, there is a possibility that TNFR2 might be involved in the reductive expression of TNF through a reverse-signal mechanism (Figure 3).

Both LPS tolerance and priming are phenomena reflecting elaborate regulation of immune and inflammatory processes *in vivo*. An understanding of the mechanism would facilitate the use of macrophage functions in immune therapy.

### Suitability of lipopolysaccharide as a potent modulator of macrophage function

One of the strongest activators for macrophages is LPS (36, 37), although there are exceptional cases as observed for



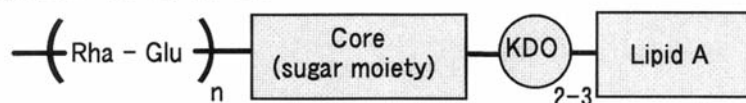
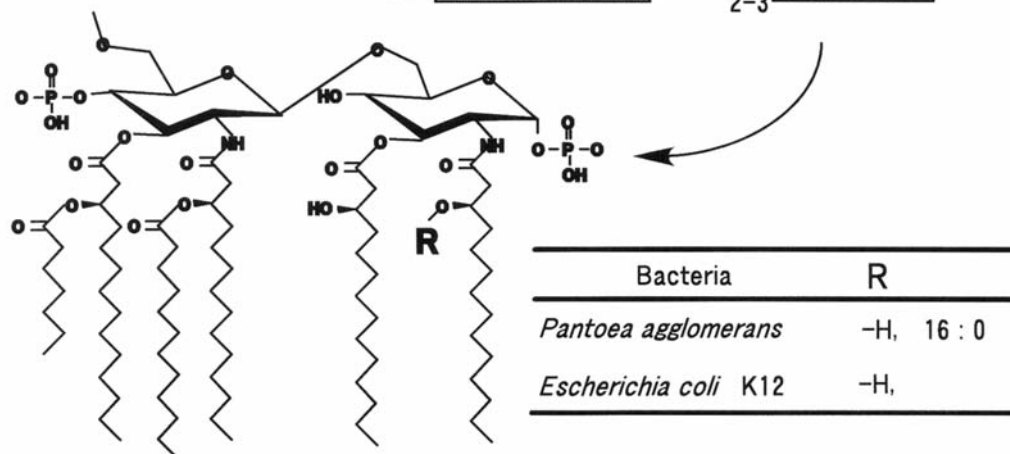
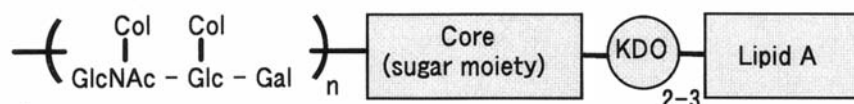
*Pantoea agglomerans* LPS: LPSP*Escherichia coli* (0111) LPS: LPSe

Figure 4. Structure of LPSP and LPSe. LPSP is distinguished from LPSe by (1) the number of fatty acid chains in the lipid A moiety, (2) component of sugar moiety, and (3) the average molecular weight.

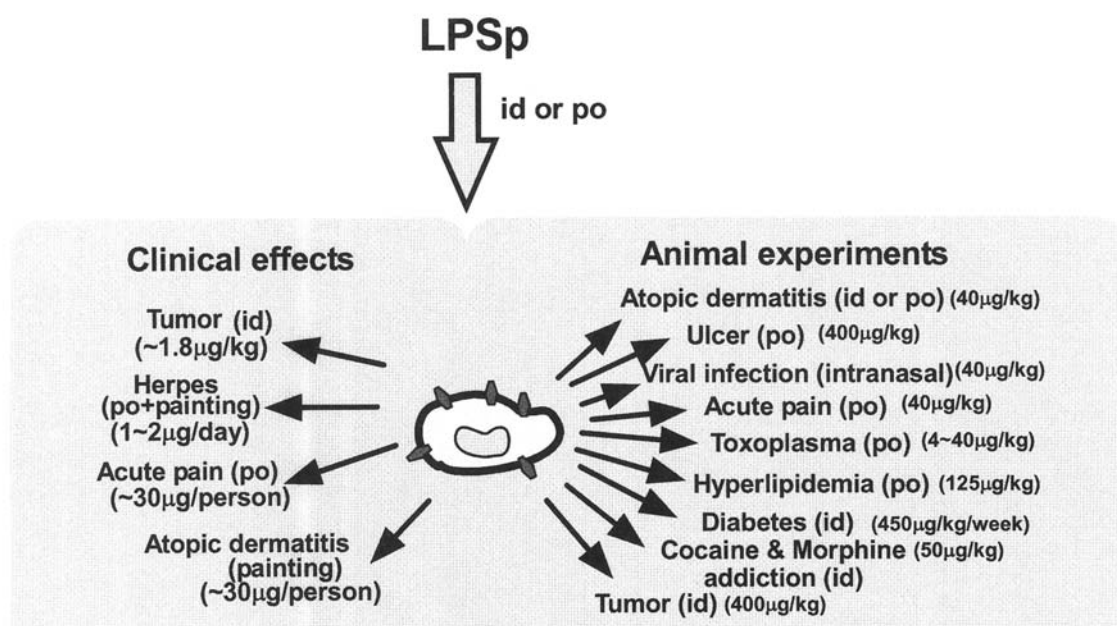


Figure 5. Therapeutic effects of LPSP. LPSP appeared to have therapeutic effects on various diseases in the clinical settings as well as in animal models, when administered orally or intradermally.

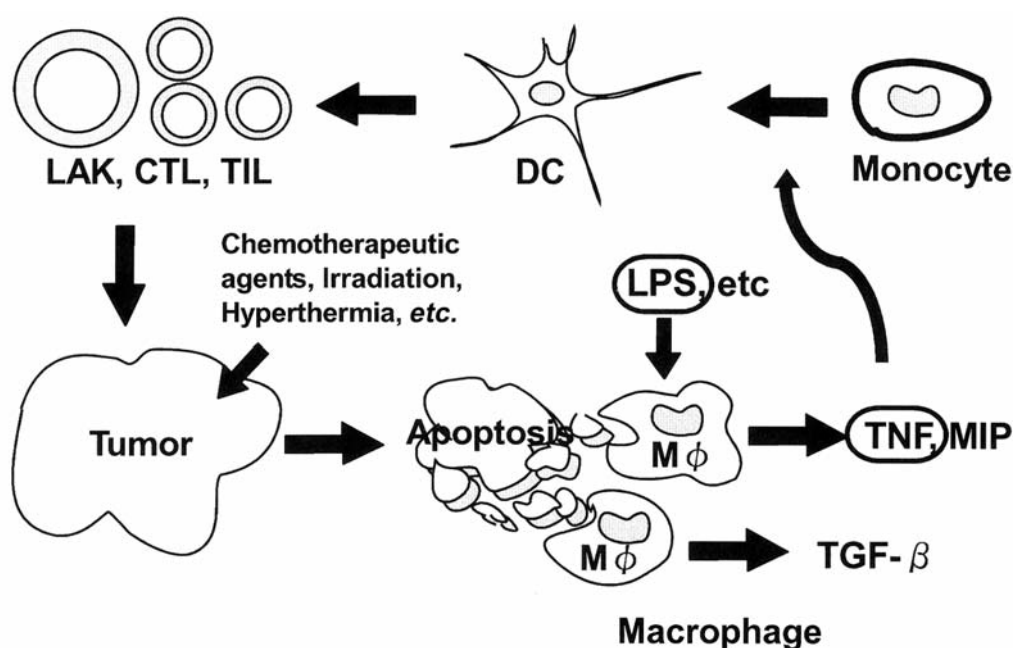


Figure 6. Importance of macrophages in immune therapy. Macrophages stimulated with LPS during phagocytosis of apoptotic bodies induced by chemotherapy or radiation therapy, secrete cytokines such as TNF or MIP. TNF promotes differentiation of monocytes to dendritic cells which have strong antigen presentation ability. As a result, the acquired immune system can be efficiently invoked specifically around tumor lesions.

intestinal macrophages (38). We discovered that LPS derived from *Pantoea agglomerans* (LPSp) has an interesting feature related to macrophage activation when compared to standard LPS obtained from *E.coli* (LPSe) (39). *P. agglomerans* is a Gram-negative symbiotic bacteria isolated from wheat, fruit and other plants (39-41). LPSp is distinguished from LPSe in the following ways: (i) number of fatty acid chains in lipid A moiety: seven in LPSp vs. six in LPSe, (ii) component of sugar moiety; combination with rhamnose and glucose is the unit in LPSp vs. combination with N-acetylglucosamine, glucose, galactose and 2 colitoses is the unit in LPSe; (iii) the average molecular weight of LPSp is about 5kDa, which is smaller than that of LPSe, which is about 20kDa (Figure 4) (42). Each of these physical differences in LPSp would be reflected in the biological characteristics of LPSp and would explain how it functions differently than LPSe.

Although LPS is known to be highly toxic when it is in the blood stream, it was not toxic when it was administered orally, percutaneously, or intradermally. We have tested the therapeutic effects of LPSp administered orally or intradermally on various diseases in clinical settings as well as in animal models. It has been shown that LPSp appeared to have therapeutic effects on various diseases including cancer (Figure 5) (39, 43-57). Depending on the usage, LPS

can be a useful potent drug because it activates macrophages that regulate the immune system. Of the various types of LPS, it appears that LPSp, with its small molecular weight, might have an advantage in the activation of mucosal macrophages through oral or intradermal administration.

In our preliminary testing using an *in vitro* model, our results show a unique therapeutic potential for LPSp compared to that of LPSe. An alveolar macrophage cell line, NR8383, was treated either by LPSp or LPSe, followed by co-culture with A549, a human lung adenocarcinoma cell line, or SLC, a rat lung carcinoma cell line. After a 4-h treatment with LPSp or LPSe, NR8383 was co-cultured with A549 or SLC for 24h, and apoptosis of lung carcinoma cells was measured by the secretion of lactate dehydrogenase. Cytotoxic activity to A549 or SLC was significantly augmented by treatment with LPSp compared to that with LPSe.

### Usefulness of macrophage activation in immune therapy

Activation of macrophages may affect not only tumor cells but also host cells *in vivo*. Luca *et al.* (58) demonstrated that signals generated by apoptotic cells are integrated with signals from LPS to promote macrophage responses that are

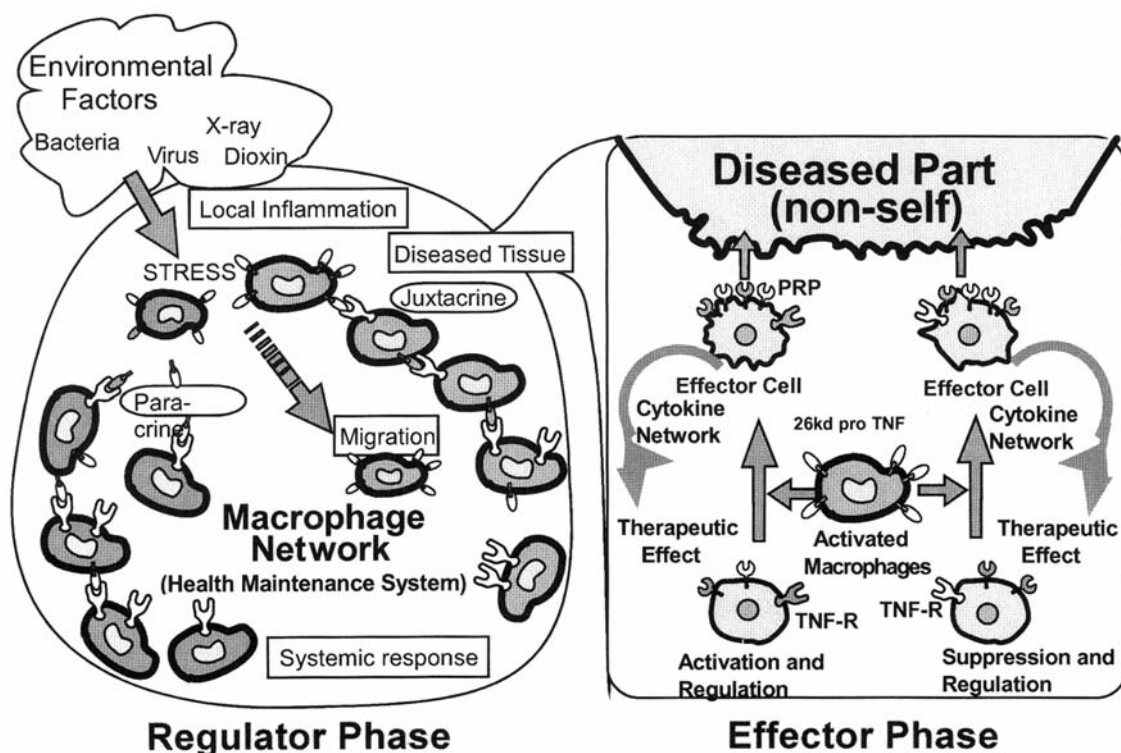


Figure 7. Macrophage network. Initially, tissue macrophages respond to external environmental stimuli and can transmit this information throughout the body by paracrine, juxtacrine, through production of cytokines, and/or by macrophage migration. The formation of the macrophage network functions to maintain homeostasis in the body. Macrophages also function for host defenses in diseased tissues both directly and indirectly.

qualitatively different from responses to stimuli given individually. Additionally, Ohno *et al.* (59) demonstrated that the number of macrophages that migrated into a tumor mass was a good prognosis factor in gastric cancer or in uterine cancer(60). Taking this data into consideration, we hypothesize that macrophage activation would benefit immune therapy (see Figure 6). Both chemotherapy and radiation therapy partially induce apoptosis in tumor cells, and the destroyed tumor cells (apoptotic bodies) are phagocytosed by macrophages. Usually, macrophages that are involved in phagocytosing apoptotic bodies secrete TGF- $\beta$  and this causes the inflammatory process to proceed to down-regulation(61). However, macrophages stimulated with pathogen-associated molecules, such as LPS, during phagocytosis, secrete cytokines such as TNF or macrophage-inflammatory protein (MIP); these chemicals are not secreted when only phagocytosis has occurred (58). Among the cytokines, TNF has been reported to promote differentiation of monocytes to DCs (62). The DCs show the strongest antigen-presenting function. Thus, these cells are now the focus of attention in finding ways to establish

immunotherapy in conjunction with adaptive immunity. For example, if the macrophages around and/or in tumor lesions are simultaneously manipulated to secrete TNF through PAMP receptors by LPSp, the acquired immune system could be efficiently invoked specifically around tumor lesions. Previously, we had reported that a combination of cyclophosphamide (CY), a chemotherapeutic drug, along with LPSp induced an inflammatory state around tumor lesions, and effectively induced CTL (63). Although, CY is not known to bind PAMP receptors, the drug can induce TNF specifically around tumor lesions (64). Also, CY could dramatically augment the antitumor effects of endogenous TNF-inducing therapy, which is one of the immune therapies (65). Our results can be adequately explained by this hypothesis. Thus, in developing an immune therapy for cancer, local macrophages could play a pivotal role. In particular, the TNF secreted from activated macrophages appears to be very important. However, a method for local application of activated macrophages is still missing. For this, a suitable drug delivery system will be required.

## Macrophage network

The innate immune system has an important role in the maintenance of homeostasis in the body. One aspect of homeostasis is adapting to the external environment. Macrophages, especially mucosal macrophages, initially respond to the external environmental stimuli and transmit this information throughout the body, by paracrine, juxtacrine, through production of cytokines, and/or migration of the macrophages (Figure 7) (18). This action of macrophages seems to be pivotal in the maintenance of homeostasis. We can tentatively call the global regulation in the body by the tissue macrophages a "macrophage network." Understanding the molecules and mechanisms involved in this network would help to explain the basic regulatory mechanisms that maintain homeostasis. Macrophages are also well-known to function for host defense in diseased tissues directly and indirectly(66, 67). Thus, the utilization of macrophage regulator and effector functions as targets of immune therapy would open new strategies against cancer.

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