REVIEW

Toll-like receptor signaling in anti-cancer immunity

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Abstract : It is important to augment the anti-cancer host response in cancer treatment. Recent studies suggested that the signaling via Toll-like receptors (TLRs) which are newly identified receptor molecules recognizing many pathogens, are involved in the induction of anti-cancer immunity. Seya *et al.* demonstrated that maturation of dendritic cells (DCs) and cytokine induction by the cell wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG-CWS) are induced via both TLR2 and TLR4. Akira *et al.* discovered a new molecule of TLR family, TLR9, recognizing unmethylated bacterial CpG-DNA, whose clinical use is expected for cancer therapy as a potent inducer of a helper T cell 1 (Th1)-type T-cell response. TLR9-deficient mice did not show any responses to CpG-DNA, including Th 1 cytokine production and maturation of DCs. We have obtained two molecules, a lipoteichoic acid-related molecule isolated from streptococcal agent OK-432, and a plant-derived 55-kDa protein that can induce Th1 response and elicit a strong anti-cancer effect *in vivo* and *in vitro*. Our basic experiments demonstrate that TLR4 signaling is intimately involved in anti-cancer immunity induced by these immunopotentiators. Our clinical examination in oral cancer patients also suggests the requirement of both TLR4 and MD-2 in the OK-432-induced anti-cancer host response. Establishment and clinical use of the methodology for human cancer therapy by utilizing TLR signaling is greatly expected.
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INTRODUCTION

Toll-like receptors (TLRs) that are expressed mainly on macrophages and dendritic cells (DCs), are recently identified receptor molecules that recognize many types of pathogens in addition to host-derived proteins. Macrophages and DCs are not only primarily involved in innate immunity, but are also essential for establishment of adaptive immunity as antigen-presenting cells (APCs). Thus, TLR signaling promotes activation of an innate immune response, and then triggers antigen-specific adaptive immunity (1-4). In the immunotherapy against malignant diseases, it was suggested that the induction of tumor antigen-specific cytotoxic T lymphocytes (CTLs) is most important for eliminating tumor cells, and most immuno-oncologists have discussed how to induce adaptive immunity against cancer. However, the precedent activation of the innate immune system is essential for the subsequent induction of antigen-specific immunity. TLR-mediated activation of innate immunity should be important for the establishment of an effective anti-cancer immune response. Recently, several studies proposed the significance of TLR signaling in the induction of anti-cancer immunity. In this review,

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we present the recent progress of these studies including our investigation, focused on the involvement of TLR signaling in anti-cancer immunity.

TLRs, and their ligands

The toll gene controls dorsoventral pattern formation during the early embryonic development of *Drosophila melanogaster* (5). Interestingly, toll participates in anti-microbial immune responses upon infection in adult *Drosophila* (6). Recently, several mammalian homologues of the *Drosophila* Toll receptor protein (Toll-like receptors : TLRs) were identified. TLRs are transmembrane proteins and represent a newly recognized family of vertebrate pattern recognition receptors in the innate immune system. A prerequisite for the development of an effective host defense is the recognition of pathogens. TLRs are involved in this first step (2-4).

Eleven members of the TLR family (TLR1 to TLR10 and RP105)(7-12), co-factors (CD14, MD-1 and MD-2)(13-15) and their ligands have been reported (12, 16-46) as shown in Table 1. The well-characterized TLRs are TLR2, TLR4 and TLR9. TLR4 recognizes Gram-negative bacteria-derived lipopolysaccharide (LPS)(32-34) as well as Gram-positive bacteria-derived lipoteichoic acid (LTA)(17, 35). It was also reported that TLR 4 mediates LPS-mimetic signal transduction by an anti-cancer agent Taxol, a plant-derived diterpene, in mice but not in humans (36). In addition, TLR4 recognizes host-derived proteins, heat shock protein (HSP) 60 (38, 39) and fibronectin fragment (40). In the antigen recognition by TLR4, MD-2 plays an essential role. MD-2 is physically associated with TLR4 on the cell surface and the TLR4/MD-2 complex confers responsiveness to bacterial components (15). TLR 2 recognizes peptidoglycan (PGN) (17, 18), lipoprotein (19-24) and lipoarabinomannan (LAM)(25, 26) derived from Gram-positive bacteria, mycobacteria or mycoplasma. Several studies reported that Gram-positive bacteria-derived LTA is recognized by TLR2 (27-29). TLR9 recognizes bacterial unmethylated CpG DNA and is the receptor that distinguishes bacterial DNA from self-DNA (46).

Subsequent to pathogen-associated molecular pattern engagement, TLRs initiate the signaling via sequential recruitment of myeloid differentiation protein (MyD) 88, IL-1R-associated kinase (IRAK)

Table 1. TLRs and their ligands

TLRs	cofactors	ligands	origin	refs.
TLR1 (associated with TLR2)		Soluble factors	*Neisseria meningitides*	16
TLR2	CD14	PGN	Gram(+), *Mycobacteria*,	17,18
		Lipoprotein	Gram(+), *Mycoplasma* *Mycobacteria, Spirochetes*	19-24
		LAM	*Mycobacteria, Spirochetes*	25,26
		LTA	Gram(+)	27-29
		Zymosan	Yeast	20,30
TLR3		dsRNA	Virus	31
TLR4	CD14	LPS	Gram(-)(*E.coli*)	32-34
	MD-2	LTA	Gram(+)	17,35
		Taxol	Plant	36
		F protein	RSV	37
		HSP60	Host	38,39
		Fibronectin EDA	Host	40
TLR5		Flagellin	Bacteria with flagella	41
TLR6 (associated with TLR2)		MALP-2	*Mycoplasma*	17,42
		Modulin	*Staphylococcus*	43
TLR7		Imidazoquinoline	(Synthetic compound)	44,45
TLR8		Imidazoquinoline	(Synthetic compound)	45
TLR9		Unmethylated CpG-DNA	Bacteria	46
TLR10				
RP105	MD-1	LPS	Gram(-)	12

PGN:peptidoglycan, LAM : lipoarabinomannan, LPS : lipopolysaccharide, LTA : lipoteichoic acid, ds : double-stranded, RSV : respiratory syncytical virus, HSP : heat shock protein, EDA : extra domain A, MALP-2 : mycoplasmal macrophage-activating lipopeptide-2 kDa

and TNFR-associated factor (TRAF)6, which in turn activate downstream mediators such as nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs) (47, 48). In addition, experiments using MyD88-deficient (MyD88-/-) mice revealed that TLR4 mediates the signaling in an MyD88-independent fashion in addition to an MyD88-dependent fashion (49). Recently, it was reported that newly identified molecule, Toll-interleukin 1 receptor (TIR) domain-containing adapter protein (TIRAP), associates with TLR4, and manages MyD88-independent signal transduction (2, 50). On the other hand, Kawai *et al.* reported that a transcription factor, interferon (IFN)-regulatory factor (IRF) 3, translocated into the nucleus in response to LPS in MyD88-/-mice (51). It was strongly suggested that IRF3 activation contributes to the MyD88-independent pathway. However, it remains uncertain whether TIRAP is involved in IRF3 activation (Fig. 1). Furthermore, most recent studies have demonstrated findings strongly suggesting that TIRAP mediates the signaling via TLR1, TLR2 and TLR6 in addition to TLR4, and is involved not only in the MyD88-independent but also in the MyD88-dependent signaling pathway (52, 53). The downstream molecular events of TLRs are significant to determine what type(s) of host response(s) are induced against different kinds of, and different doses of pathogens. Progress of this clarification is strongly expected.

TLR-mediated signaling stimulates the maturation

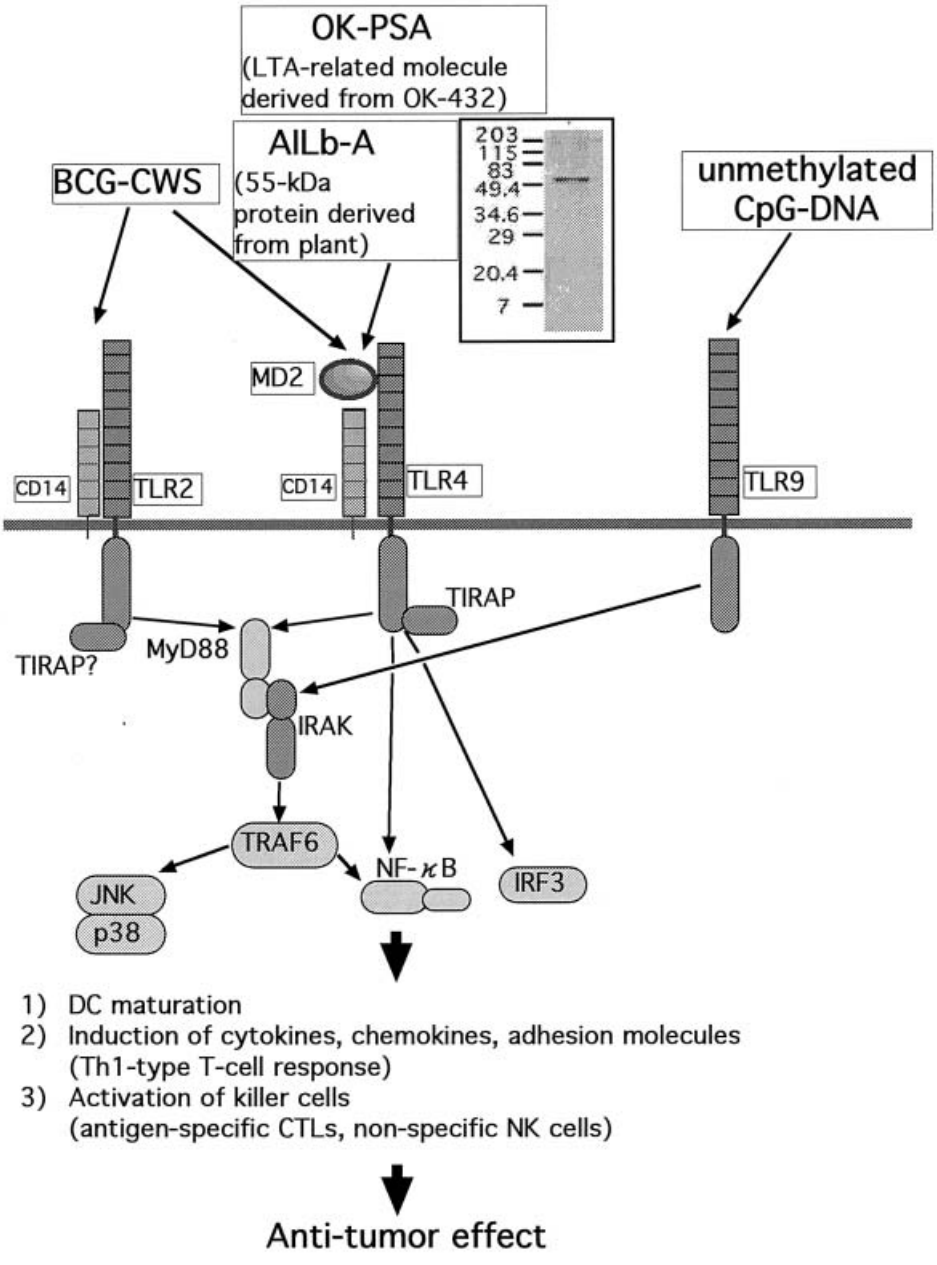


Fig. 1. Ligands with anti-cancer effects and downstream signals of TLRs

of DCs, which migrate to the regional lymph nodes, where they stimulate T cells by presentation of antigen-major histocompatibility (MHC) complex in addition to costimulatory molecules such as CD80 and CD86. TLR signaling acts to trigger adaptive immunity by enhancing expression of MHC molecules in addition to these costimulatory molecules. Furthermore, TLR signaling frequently enhances the production of IL-12, a major helper-T cell 1 (Th1)-inducing cytokine, on APCs (2-4, 54). Thus, it was strongly suggested that the DCs matured by TLR stimulation may induce T-cell differentiation toward Th1 by presenting antigens to the T cells while promoting a Th 1-leading situation in the local environment. Therefore, it is possible that the ligands of TLRs are able to be effective immunoadjuvants for cancer therapy. In the next sections, we review the recent progress focused on the TLR ligands as applications for cancer therapy.

Involvement of TLR2, TLR4, and TLR9 in anti-cancer immunity induced by the bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) or unmethylated CpG-DNA

1) BCG-CWS-induced anti-cancer host response via TLR2 and TLR4

Heat-killed mycobacterial cells suspended in mineral oil are potent immunoadjuvants to induce both cell-mediated and humoral immunity, and the CWS fraction of the cells of mycobacteria is the active immunoadjuvant component (55-58). It was reported that BCG-CWS enhances the cytotoxic activity of T cells and macrophages against cancer cells, and elicits an anti-tumor effect in mice and in rats bearing transplantable and authochthonous tumors (59, 60). Further, clinical trials with BCG-CWS were performed in patients with several types of malignancies, and it was demonstrated that BCG-CWS was effective in prolongation of survival of patients especially those with gastric cancer and lung cancer (61-67).

Recently, Seya and his co-investigators reported findings strongly suggesting that BCG-CWS augments the anti-cancer host response through TLR2 and TLR4 (Fig. 1). It was reported that BCG-CWS activates and matures DCs (58). Tsuji *et al.* reported that BCG-CWS induces tumor necrosis factor (TNF)-α secretion from DCs via both TLR2 and TLR4, and that the secreted TNF-α induces the maturation of DCs (68). The distribution profile of TLR2 and TLR4 matches the response profile of cells for BCG-CWS, and further investigation by Tsuji *et al.* suggested that the PGN portion of BCG-CWS is an active center for cytokine induction and DC maturation via Toll signaling (58). It was also reported that TLR2 mediates mycobacteria-induced proinflammatory signaling in macrophages (25). These findings strongly suggest that the signaling via TLRs is closely involved in BCG-CWS-induced anti-tumor immunity. However, Azuma and Seya suggested that TLRs are not merely the receptor for establishment of the BCG-CWS-induced anti-tumor host response, and proposed the "two receptor theory". Two types of receptors expressing on APCs, which consist of signal transducing receptor related to maturation and activation of APCs such as TLRs, and phagocytosis-related receptor to induce antigen presentation to T cells, may be essential for the establishment of antigen-specific, adaptive immunity, and BCG-CWS may augment the anti-tumor host response by activating both types of receptors (58, 69). As demonstrated previously, BCG-CWS consists of mycolic acid, arabinogalactan and PGN (57, 59, 70), and distinct portions may play significant roles in the binding and activation of the two receptors on macrophages and DCs. Identification of the phagocytosis receptor(s) is currently under investigation by Seya *et al.* (58).

2) Bacterial unmethylated CpG-DNA-induced host response via TLR9

The specific immunostimulatory effect of bacterial genomic DNA was first reported by Tokunaga *et al.*, who demonstrated that bacterial DNA activates natural killer (NK) cells and induces IFN production in addition to tumor regression in some mouse models, but vertebrate DNA does not (71-73). In 1995, Krieg *et al.* demonstrated that CpG motifs in bacterial DNA trigger direct B-cell activation (74, 75). They also reported that CpG content and methylation distinguish vertebrate and bacterial DNAs. Genomic DNA from vertebrates but not from bacteria contains very few CpG dinucleotide motifs (76). Further, CpGs are commonly methylated in vertebrates, while the CpGs are not methylated in bacteria and viruses. This suggestes the possibility that the immune system may have evolved a defense mechanism based on the recognition of unmethylated CpG-DNAs, which could be a sign of foreign DNA (76).

The immune response of synthesized oligodeoxynucleotides (ODN) with CpG motifs was examined. Many studies

demonstrated that unmethylated CpG-ODN strongly activates immunocompetent cells such as DCs, macrophages, NK cells, T cells, and B cells, and induces the Th1-like T-cell response including IFN-γ production and CTL induction, in *in vitro* and *in vivo* models (74). Roman *et al.* reported that co-injection of CpG-ODN with a protein antigen greatly enhances the T- and B-cell response to this antigen (77). Significantly, this response is strongly biased toward the generation of a Th1-dependent immunity with all its ramifications, for example a preference for IgG_2 immunoglobulin subclasses. It can be expected as an immunotherapeutic agent for cancer, allergy and infectious diseases as a potent Th1 inducer. The efficacy of CpG-DNA in preventing or treating tumor development or metastasis in mice has been examined in several experimental models. In CpG-DNA monotherapy, systemic or local administration of CpG-DNA protected 80% of syngeneic C57BL/6 mice from a lethal challenge of B16 melanoma. Further, SCID mice were also protected against tumor challenge by CpG-DNA, which suggests that neither B nor T cells are required (78). The potential of the CpG-DNA as an adjuvant for cancer vaccines was also examined. CpG-DNA-induced activation of DCs creates a Th1-like cytokine and chemokine environment in the secondary lymphoid organs that promotes cross-priming with strong IFN-γ-secreting CTLs and antibody responses to peptides and protein antigens derived from tumors, and elicits marked anti-tumor activity (79, 80). Despite its promising clinical use, the molecular mechanism by which CpG-DNA activates immune cells has remained unclear.

In 2000, Hemmi *et al.* discovered the receptor molecule recognizing bacterial DNA (46). The identified protein, TLR9, recognizes the unmethylated CpG motif in bacterial DNA, and mediates an innate immune response (Fig. 1). They generated TLR9-deficient (TLR9-/-) mice and examined the immune effect of CpG-ODN using those mice (46). TLR9-/- mice did not show any response to CpG-DNA, including proliferation of splenocytes, inflammatory cytokine production from macrophages and maturation of DCs. TLR9-/-mice showed resistance to the lethal effect of CpG DNA without any elevation of serum pro-inflammatory cytokine levels. The *in vivo* CpG-DNA-mediated Th1 response was also abolished in TLR9-/-mice. Thus, it was clarified that the signaling via TLR9 plays an important role in CpG-DNA-induced host response.

Early-stage clinical trials of CpG-DNA as an immunotherapeutic agent for cancers and as an anti-allergic agent are currently on going, and preliminary findings from these trials appear to be encouraging (81).

Involvement of TLR4/MD-2 signaling in anti-cancer immunity induced by an LTA-related molecule, an effective component of OK-432, or by a plant-derived 55-kDa protein

Recently, we obtained 2 molecules that can induce the Th1-dominant state and elicit an anti-cancer effect. One is an LTA-related molecule isolated from a *Streptococcus*-derived anti-cancer agent OK-432, and another is a 55-kDa protein from *Aeginetia indica L* (AIL), a parasitic plant, and we obtained findings strongly suggesting that these molecules enhance anti-cancer immunity via TLR4/MD-2 complex. In this section, we present the recent progress of our basic and translational research focused on the involvement of TLR4/MD-2 signaling in anti-cancer immunity induced by these molecules.

1) Isolation of an effective component responsible for OK-432-induced anti-cancer effect

OK-432, a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of *Streptococcus pyogenes* (group A) (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), was developed by Okamoto *et al.* in 1967 (82), and has been successfully used as an immunotherapeutic agent in many types of malignancies (83-87). We also reported that OK-432-based immunotherapy exhibits a marked therapeutic effect in patients with oral squamous cell carcinoma (88, 89). Previous studies helped to clarify the cellular mechanism of OK-432-induced anti-cancer immunity. Namely, OK-432 elicits anti-tumor effects by stimulating immunocompetent cells such as macrophages, T cells and NK cells, and by inducing multiple cytokines including IL-1, IL-2, IL-6, TNF-α, and IFN-γ (90-92). In addition, OK-432 induces IL-12 and polarizes the T-cell response to a Th1 dominant state (93). However, there has been limited progress in elucidating the molecular mechanism, i.e., in the identification of the effective molecule(s) for inducing anti-cancer immunity in whole bacterial preparation OK-432 and their molecular target(s), such as receptors and signal transducers on immunocompetent cells. Recently, we succeeded in isolating the LTA-related molecule that is

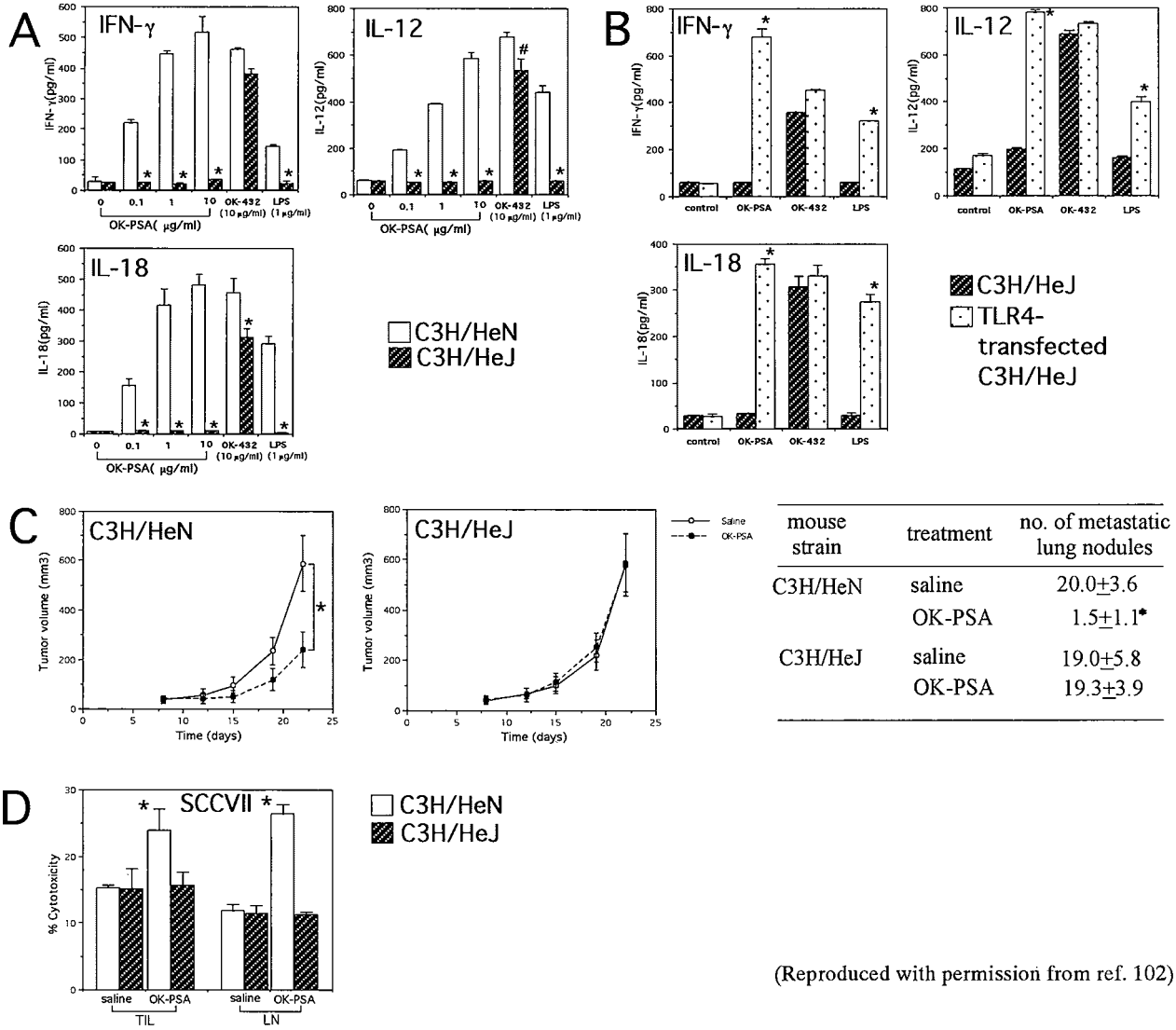
most responsible for the anti-cancer effect of OK-432. This molecule designated as OK-PSA was isolated from a butanol extract of OK-432 using a CNBr-activated Sepharose 4B affinity column bound with the monoclonal antibody TS-2, which neutralizes the IFN-γ-inducing activity of OK-432 (94, 95). We previously reported that OK-PSA is a more potent inducer of Th1 cytokines and killer cell activities on human peripheral blood mononuclear cells (PBMCs) than original OK-432, and showed a marked anti-tumor activity in tumor-bearing mice (94, 96-101)

2) OK-PSA-induced anti-cancer immunity via TLR4 signaling

We examined the role of TLR4 in the anti-tumor effect of OK-PSA using C3H/HeJ mice in which TLR4 is mutated and its function is impaired (34). C3H/HeN mice, which have the wild-type TLR4 gene, were used as control animals. In *in vitro* experiments, the spleen cells derived from C3H/HeN and C3H/HeJ mice were stimulated with OK-PSA for 48 h, then the cytokines in the supernatants were measured. Although Th1-type cytokines such as IFN-γ, IL-12 and IL-18 were significantly induced by OK-PSA stimulation in the splenocytes derived from C3H/HeN mice, these cytokines were not induced in the splenocytes from C3H/HeJ (Fig. 2A). TNF-α and IL-2 were also induced by OK-PSA in the splenocytes from C3H/HeN but not from C3H/HeJ (data not shown). Furthermore, when the expression vector including mouse TLR4 cDNA was transfected into C3H/HeJ-derived splenocytes, the splenocytes acquired the responsiveness to OK-PSA to produce Th1 cytokines (Fig. 2B). Next, we evaluated the anti-tumor effect of OK-PSA *in vivo*. C3H/HeN and C3H/HeJ mice bearing syngeneic SCCVII tumors were treated with OK-PSA. The peritumoral injection of OK-PSA resulted in significant inhibition of tumor growth and lung metastasis in SCCVII-bearing C3H/HeN mice ; however, no effect of OK-PSA was observed in C 3 H/HeJ mice (Fig. 2 C). The cytolytic activities of tumor-infiltrating lymphocytes (TIL) and draining lymph node (LN) cells derived from SCCVII-bearing mice that were administered OK-PSA were also analyzed. The cytolytic activities of TIL and LN cells against SCCVII were markedly increased by OK-PSA administration in C3H/HeN but not in C3H/HeJ (Fig. 2D). These findings strongly suggest that TLR4 signaling is involved in regulating OK-PSA-induced anti-cancer immunity (102, 103). As described above, several studies reported that Gram-positive bacteria-derived LTA is recognized by TLR2 (27-29). Recent studies by Hartung *et al.* have demonstrated that the butanol-extracted LTA in addition to synthetic LTA from *Staphylococcus aureus* induce cytokines through TLR2 but not through TLR4 (28, 29). Further, it was also reported that LTA from *Bacillus subtilis* and from *Staphylococcus aureus* induced the maturation of murine DCs via TLR4 (27). Since TS-2 mAb recognizes LTAs similar to OK-PSA, it is suggested that OK-PSA has a certain chemical structure that LTAs share in common (94, 95), while the active structure of OK-PSA may not be LTA itself. Furthermore, recent evidence in LPS recognition suggests that there are structural and functional differences among LPS molecules from different bacteria. An LPS with a conical shape (e.g. from *Escherichia coli*) induces cytokines via TLR4, while a more cylindrical LPS (e.g. from *Porphyromonas gingivalis*) induces a different set of cytokines via TLR2 (104). It is possible that OK-PSA, a ligand for TLR4, may be a member of the LTA family with a different structure.

3) OK-PSA-induced DC maturation via TLR4 signaling

To evaluate the role of TLR4 in OK-PSA-induced maturation of human DCs, we performed a neutralizing test using anti-human TLR4 mAb (HTA 125, 10 μg/ml ; provided from Drs. Miyake and Akashi, The Institute of Medical Science, The University of Tokyo). When monocyte-derived immature DCs (iDCs) from healthy donors were stimulated by OK-PSA for 48 h, the increased expression of surface markers such as MHC class II, CD80 and CD86, and enhancement of the production of IL-12 and IL-18 were observed. The expression of these markers increased by OK-PSA was almost completely inhibited by the addition of anti-human TLR4 mAb. OK-PSA-induced cytokine production was also inhibited significantly by anti-TLR4 (Table 2). We next used the monocyte-derived iDCs from patients with oral cancer. In semiquantitative RT-PCR analysis, TLR4 mRNA was strongly expressed in peripheral blood monocytes both from patient 1 and from patient 2. MD-2 mRNA was clearly detected in patient 1-derived monocytes, while it was not detected in those from patient 2 in the current RT-PCR condition (data not shown). OK-PSA stimulation increased the expression of MHC class II, CD80 and CD86 on iDCs derived from patient 1. The expression of these cell surface antigens was also increased by OK-PSA on patient 2-derived DCs. Although DCs derived both from patient 1 and



mouse strain	treatment	no. of metastatic lung nodules
C3H/HeN	saline	20.0±3.6
	OK-PSA	1.5±1.1*
C3H/HeJ	saline	19.0±5.8
	OK-PSA	19.3±3.9

(Reproduced with permission from ref. 102)

Fig. 2. OK-PSA-induced anti-cancer host response via TLR4. (A) Cytokine production by OK-PSA-stimulated splenocytes derived from C 3 H/HeN and C3H/HeJ. The splenocytes (2×10^6/ml) were stimulated with OK-PSA, OK-432 or LPS for 48 h, then cytokines in the supernatants were measured. *, $P<0.01$; #, $P<0.05$ compared with C3H/HeN mice. (B) Effect of transfection of TLR4 gene in the response of C3H/HeJ-derived splenocytes against OK-PSA. C3H/HeJ-derived splenocytes (3.5×10^6) were transiently transfected with the expression plasmid containing mouse TLR4. Twenty-four hours later, the splenocytes were treated with 1 μg/ml of OK-PSA, OK-432 or LPS for 48 h, and then cytokines in the supernatants were measured. *, $P<0.01$; #, $P<0.05$ compared with the controls. (C) Inhibition of tumor growth and lung metastasis by OK-PSA in C3H/HeN and C3H/HeJ. SCCVII-bearing C3H/HeN (n=10) and C3H/HeJ (n=10) were administered with OK-PSA (100 μg) peritumorally every other day from days 1 though 9 after the inoculation of SCCVII. *, $P<0.001$ compared with the control animals administered saline. (D) Cytotoxic activities of tumor infiltrating lymphocytes (TIL) and draining lymph node (LN) cells. SCCVII-bearing C3H/HeN and C3H/HeJ treated with OK-PSA were killed on day 23, and were analyzed for the cytotoxic activities of TIL and LN cells against SCCVII. *, $P<0.01$; #, $P<0.05$ compared with controls administered saline.

from patient 2 produced IL-12 by OK-PSA treatment, IL-12 secretion by patient 1-derived DCs was greater than that by patient 2-derived DCs. Next, these OK-PSA-treated or untreated DCs were irradiated, then cocultured with allogeneic T cells (DC : T=1 : 20) for 5 days. IFN-γ in the supernatants from the cocultivation was markedly increased when T cells were cocultured with patient 1-derived DCs treated with OK-PSA compared with patient 2-derived DCs. Futher, allo-specific CTL activity of the T cells harvested from the above culture was also examined. CTL activity was significantly increased only when the T cells were cocultured with patient 1-derived DCs activated by OK-PSA but not with DCs from patient 2 (Table 2) (Okamoto and Sato, manuscript in preparation).

4) Requirement of both TLR4 and MD-2 genes in IFN-γ induction by OK-432 administration in oral cancer patients

We evaluated the relation between the expression

Table 2. TLR 4/MD-2-mediated DC maturation by OK-PSA

TLR 4-mediated maturation of healthy donor-derived DCs by OK-PSA

	surface markers			cytokines	
	Class II	CD80	CD86	IL-12 (pg/ml)	IL-18 (pg/ml)
untreatd	545	35.8	188	20.0±1.7	4.2±0.5
OK-PSA	2217	92.2	331	3080±65	116±9.5
anti-TLR4	524	33.5	205	22.9±0.5	3.5±1.2
OK-PSA +anti-TLR4	625	33.9	210	582±22*	25.5±1.8*

Human monocyte-derived iDCs were treated with OK-PSA (10 μg/ml) for 2 days. In some experiments, anti-human TLR4 mAb (10 μg/ml) was added at 2 h before adding OK-PSA. After the completion of the cultivation, expression of surface markers on the DCs was analyzed by flow cytometry using FITC-conjugated mAb against each marker. Numerals in the table express mean fluorescence intensities. IL-12 and IL-18 in the supernatants were also measured by ELISA. *, $P<0.01$ compared with positive controls treated with OK-PSA alone.

TLR 4/MD-2-mediated maturation of oral cancer patient-derived DCs by OK-PSA

donors	treatment	surface markers			IL-12 (pg/ml)	allogeneic MLR	
		Class II	CD80	CD86		IFN-γ (pg/ml)	allo-specific CTL (% cytotoxicity)
patient 1 TLR4(+)MD-2(+)	untreatd	122	2.8	10.7	10.2±1.8	75.5±11.3	17.7±25
	OK-PSA	267	8.3	23.7	4356±254	623±52	42.5±3.3
patient 2 TLR4(+)MD-2(-)	untreatd	76.7	3.2	7.3	15.3±2.5	101±8.5	21.5±3.2
	OK-PSA	249	6.9	27.1	1872±146*	289±31*	18.2±1.5*

In patient 1, expression of both TLR4 and MD-2 genes was detected by semi-quantitative RT-PCR analysis. MD-2 mRNA expression was not detected in patient 2. Cell surface phenotype of patient-derived DCs stimulated by OK-PSA for 2 days was analyzed by flow cytometry. Numerals in the table express mean fluorescence intensities. IL-12 in the supernatants were measured by ELISA. Patient-derived DCs stimulated by OK-PSA were cocultured with allogeneic T cells derived from another oral cancer patient (DC : T=1 : 20) for 5 days, and then the supernatants of the cocultivation were analyzed for IFN-γ by ELISA. The cytotoxic activities of the T cells harvested from the above allogeneic MLR culture were assayed against antigen-specific target cells, iDCs derived from the same donor to the DCs used in MLR by a ^{51}Cr-release test. *, $P<0.01$ compared with patient l-derived DCs treated with OK-PSA.

of TLR4 and MD-2 genes and IFN-γ induction in response to OK-432 in 28 oral cancer patients. Nineteen of 20 patients (95%) who showed TLR4(+) and MD-2(+), demonstrated an increase in serum IFN-γ protein by peritumoral administration of OK-432. Serum IFN-γ protein was not detected after OK-432 administration in six of eight (75%) patients who showed TLR4(-) or MD-2(-). We detected a significant relation between increased IFN-γ protein levels in the sera of patients administered OK-432 and expression of TLR4 and MD-2 genes ($P=0.0005$ in Fisher's exact test, Table 3). Both TLR4 and MD-2 were apparently required for IFN-γ induction by OK-432 in patients with oral cancer (105). All of the patients examined in that study received therapy with OK-432 and UFT, an oral fluoropyrimidine formulation combining tegafur and uracil in a 1 : 4 ratio (Taiho Pharmaceutical Co., Tokyo, Japan) in combination with radiotherapy. Among these patients, 10 of 20 TLR4(+)MD-2(+) patients (50%) became histopathologically tumor-free after the therapy, and without surgical resection. In contrast, all eight patients who were TLR 4(-) or MD-2 (-) became tumor-free only after having their tumors surgically resected after the therapy (105). We also clarified the requirement of TLR4 signaling for OK-432-induced anti-cancer immunity in a mouse model using TLR4-/- mice (105).

These basic and clinical findings described above suggest that the target receptor molecule of original OK-432 in addition to its component OK-PSA is TLR4/MD-2 complex, and that the expression of TLR4 and MD-2 may be a useful marker to discriminate between responders and nonresponders to OK-432-based immunotherapy. Furthermore, these findings strongly support our opinion that the molecule which makes the largest contribution to the OK-432-induced anti-cancer immunity, is OK-PSA.

Table 3. Requirement of TLR4 and MD-2 in OK-432-induced IFN-γ in sera from oral cancer patients

patient No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
TLR4	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
MD-2	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	-
Serum IFN-γ	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	-

Peripheral blood mononuclear cells were prepared from 28 patients with oral cancer. Total RNA was extracted from the cells, then expression of TLR4 and MD-2 mRNAs was examined by semi-quantitative RT-PCR analysis. Densitometric analysis for the RT-PCR band patterns was performed by using NIH Image 1.59 software. The relative density (RD) of each specific band was expressed as a ratio to the density of GAPDH used as an internal control, and RD<0.1 was defined as -, 0.1≦RD<0.5 as ±, and RD≧0.5 as +. Sera were collected from these patients at 5 h before and at 24 h after the peritumoral administration of OK-432, then IFN-γ was analyzed by ELISA. IFN-γ was not detected in the sera collected before OK-432 administration in any patients (data not shown). Therefore, the case in that IFN-γ protein was detected in the sera collected after OK-432 administration, was expressed +.

5) Isolation of a 55-kDa protein and its anti-tumor effect via TLR4 signaling

Aeginetia indica L. (AIL), a plant parasitic on roots of Japanese pampa grasses or sugar canes, has been used as a tonic and an anti-inflammatory herb agent in China and Japan. We previously reported that the butanol extract from seeds of AIL mediates potent anti-tumor immunity in tumor-bearing mice (106-108). We recently isolated a 55-kDa protein from the seed extract of the plant, and designated AILb-A. We reported that AILb-A was the protein with a molecular weight of 55-kDa not containing any carbohydrate determinants and markedly induced Th1-type cytokines and apoptosis-inducing factors such as TNF-α, TNF-β, Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL) and perforin on human PBMCs *in vitro* (108, 109), and that AILb-A induced Th1-dominant state and elicited marked anti-tumor effects in syngeneic Meth-A tumor-bearing BALB/c mice in which the Th2 response is genetically dominant. It is strongly suggested that AILb-A may be a useful immunotherapeutic agent for patients with malignancies (110).

We examined the role of TLR4-mediated signaling in AILb-A-induced anti-tumor immunity. In the luciferase assay using NF-κB-dependent reporter plasmid, AILb-A induced NF-κB activation in the cells transfected with the plasmid containing TLR4 gene in a dose-dependent manner. In the cells transfected both with TLR4 and with MD-2 genes, higher activity of NF-κB was observed by AILb-A stimulation than that in the cells expressing only TLR4. These cells transfected with TLR4 and/or MD-2 genes were provided by Drs. Miyake and Akashi, The Institute of Medical Science, The University of Tokyo. AILb-A did not induce cytokines (TNF-α and IL-12) in the peritoneal macrophages derived from TLR4-deficient mice (provided by Drs. Akira and Takeuchi, Research Institute for Microbial Diseases, Osaka University), while cytokines were markedly produced by AILb-A-stimulated macrophages from wild-type and TLR2-deficient mice (provided by Drs. Akira and Takeuchi). Further, in wild-type and TLR2-deficient mice bearing syngeneic LL/2 tumor, AILb-A treatment resulted in marked inhibition of tumor growth, but AILb-A was not effective in LL/2-bearing TLR4-deficient mice (Okamoto and Sato, manuscript in preparation). These findings suggest that AILb-A induces anti-cancer immunity via TLR4 signaling.

DISCUSSION AND FUTURE PERSPECTIVES

Although the major therapies for malignancies are surgical resection, chemotherapy and radiotherapy, the accumulated evidence demonstrates that the host immune response is essential for eliminating cancer cells completely. Augmentation of anti-cancer immunity in patients is significant to cure the diseases in addition to increase the quality of life of patients with cancer. Recent studies strongly suggest that TLR ligands are useful applications for immunotherapy for cancer patients. The schema of TLR ligand-induced anti-cancer immunity is shown in Fig. 3. In tumor tissues, bacterial components such as BCG-CWS, CpG-DNA and OK-PSA, in addition to a plant-derived protein, AILb-A, activate APCs which have captured tumor antigens, via TLRs. TLR-mediated signaling stimulates the maturation of DCs. The matured DCs in which expression of MHC and costimulatory

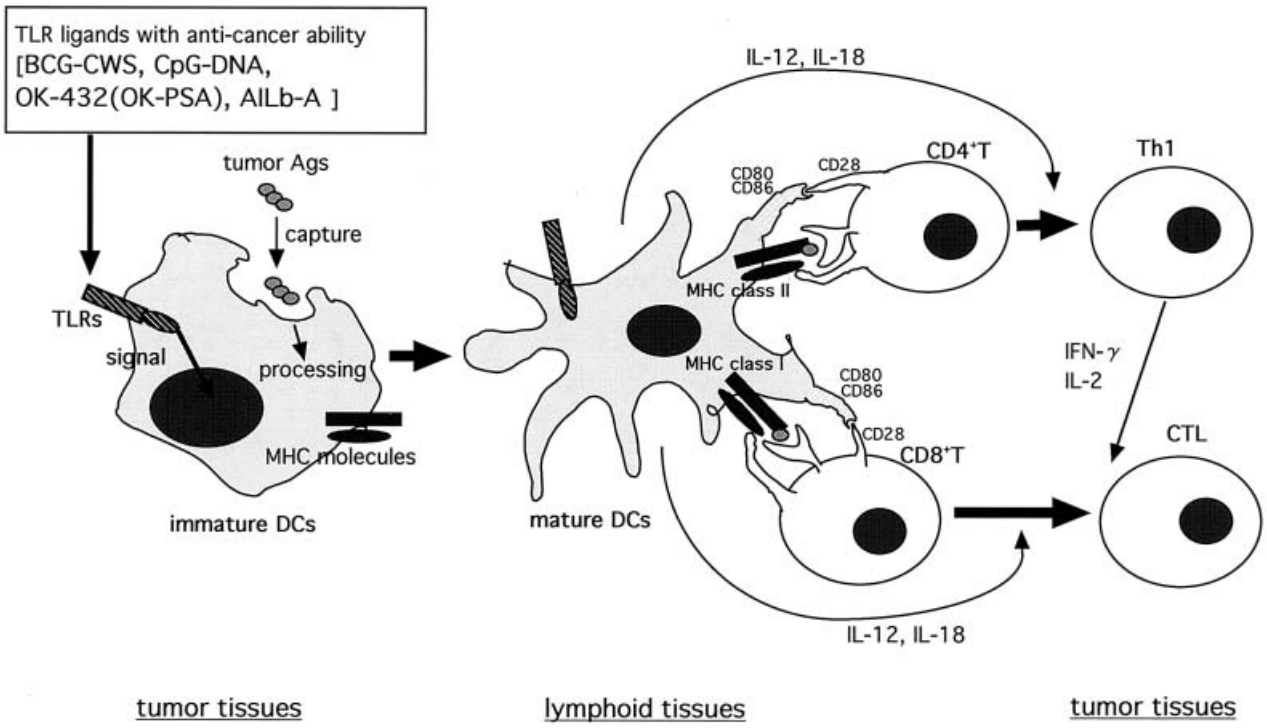


Fig. 3. TLR-mediated anti-cancer host response.

molecules is increased by TLR stimulation, migrate to the regional lymph nodes, then present antigen(s) to T cells. TLR-stimulated DCs also enhanced the producing ability of cytokines such as IL-12 and IL-18, potential Th1-inducing cytokines. Therefore, TLR-stimulated DCs may effectively induce tumor-antigen specific Th1 and CTL by presenting antigens to $CD4^+$ and $CD8^+$T cells while promoting a Th1-leading situation. It is possible that some ligands of TLRs are able to be effective immunotherapeutic agents for patients with cancer. Based on the results of our and other studies with regard to the role of TLR ligands on DC function as described above, TLR ligands may be useful applications as adjuvants in DC-based cancer immunotherapy. Clinical trials are expected.

As described by many investigators and oncologists, the therapy to increase the host response in cancer patients should be effective, while it is a critical problem for clinical use of the immunoadjuvants that the molecular mechanism by which the immunotherapeuitic agents activate immune cells, has remained uncertain. Discovery of TLRs as immunoadjuvant receptors is a great progress to use the immunoadjuvants to treat human cancer. Furthermore, when TLR ligands are used in therapeutic applications, the expression of TLRs in the patients may be a useful marker to discriminate between responders and nonresponders to the therapy using the agents. For example, if a patient does not express TLR4/MD-2, CpG-DNA but not OK-PSA should be selected as a therapeutic application. CpG-DNA should not work in patients in whom TLR9 is not expressed. In addition, stimulation of both TLR4 and TLR9 by the combination therapy using OK-PSA and CpG-DNA may be more effective to cure cancers in patients expressing both TLR4/MD-2 and TLR9.

The residual problem is that all TLR ligands and all types of signaling mediated by TLRs do not induce the Th1-type T-cell response. The next objective of these studies is to clarify the Th1-inducing mechanism(s) via TLRs, in addition to find Th1-inducing ligand(s) of TLRs. Establishment of the methodology to specifically induce the Th 1 response by utilizing TLR signaling is expected for future immunotherapy against cancers, allergic diseases in addition to infectious diseases. Further, if TLR ligand(s) that can selectively induce the Th2-type T-cell response will be found, it may be effective for the treatment of Th1-associated diseases such as autoimmune diseases.

With regard to our work, both the OK-432-derived component OK-PSA and plant-derived 55-kDa protein AILb-A augment anti-cancer immune response by acting as potent Th1 inducers mediated by TLR4/MD-2 signaling. In an attempt to apply these agents for the treatment of human cancers, the determination of the chemical structure of OK-PSA

and the isolation of the gene encoding AILb-A should be completed. It is currently under investigation in our laboratories. We believe that OK-PSA as well as AILb-A will be useful immunotherapeutic agents for patients with malignant diseases.

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