Original Article

Phagocytosis plays a dual role in activating dendritic cells; digestive production of active Toll-like receptor ligands and cooperation with Toll-like receptor signaling

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ABSTRACT: Phagocytosis is an initial step in innate immunity, which is also stimulated by signals *via* Toll-like receptors (TLRs); however, the cooperation of phagocytosis with signals through TLRs to establish acquired immunity is unknown. We found that phagocytosis is an essential process to induce an immune reaction against an insoluble TLR ligand. Cell-wall skeleton prepared from *Mycobacterium bovis* BCG (BCG-CWS), an insoluble TLR2 ligand, activated and matured murine splenic dendritic cell (DC) line BC-1 as well as a soluble TLR2 ligand, Pam3CSK4. Surprisingly, BC-1 maturation with BCG-CWS was completely suppressed by inhibiting phagocytosis, while that with Pam3CSK4 was not affected. Moreover, BCG-CWS induced intense delayed-type hypersensitivity (DTH) reactions against mitomycin C-inactivated Lewis lung carcinoma cells but Pam3CSK4 did not. These results suggested that the phagocytosis process enables the insoluble TLR2 ligand to activate DCs *via* TLR2 comparable to a soluble TLR2 ligand *in vitro*, and stimulating TLR2 alone is not sufficient to establish T cell-mediated immunity *in vivo*. It is therefore conceivable that the process of phagocytosis induces additional effects on TLR2-stimulated DCs to activate cell-mediated immunity *in vivo*.

Keywords: Adjuvant, phagocytosis, TLR2, BCG-CWS, SMP-105

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1. Introduction

Phagocytosis is the process of endocytosis involving the vesicular internalization of solid particles, such as bacteria, and is therefore an essential step for eliciting effective innate immunity in mammals. Macrophages and DCs make use of a variety of surface receptors to internalize microbes, including direct pattern-recognition receptors and receptors for opsonins (*1*). TLRs are pathogen-associated molecular pattern recognition receptors (PRRs), and assume an important role in induction of the immune response *via* activation of innate immunity (*2*,*3*). Recent studies have demonstrated that signaling by TLRs can modulate phagocytosis (*4*) and moreover a complex interaction between phagocytosis and TLR signaling. Phagocytosis comprises the processes of recognition of particles by phagocytic receptors, phagosome formation, phagosome maturation and transcriptional responses in phagocytosis. Although many reports suggest TLRs do not act directly as phagocytic receptors (*5-8*), some reports insist that TLRs regulate phagocytosis (*1*,*9-14*).

BCG-CWS, cell-wall skeleton prepared from *Mycobacterium bovis* BCG as an insoluble fraction, is known to be an activator of innate immunity (*15*) and has been examined in several clinical studies (*16-19*). We originally prepared CWS from *M. bovis* BCG Tokyo 172 strain with purity of more than 97% (SMP-105) and investigated. SMP-105 is an insoluble TLR2 ligand and elicited immune reactions such as inducing interferon-γ-producing cells and cytotoxic T lymphocytes (CTL) and prevented the growth of tumors through TLR2 (*20*). SMP-105 was observed at the draining lymph nodes phagocytosed by macrophages and DCs (*21*). We therefore hypothesize that SMP-105 requires phagocytosis by macrophages or DCs for immune activation and it shows different effects from Pam3CSK4, a soluble TLR2 ligand, *in vivo*.

In this report, we investigated the maturation and activation of BC-1, a mouse immature DC line, by analyzing surface antigens and measuring cytokines *in vitro*. We found that cytocharasin B, an inhibitor of phagocytosis, suppressed BC-1 maturation by BCG-CWS, but a soluble TLR2 ligand, Pam3CSK4, did not. Acquired immunity established by BCG-CWS or Pam3CSK4 was also addressed as DTH reactions and is discussed in the context of the contribution of phagocytosis to immune reactions in this paper.

2. Materials and Methods

2.1. *Preparation of SMP-105*

SMP-105 was prepared as described previously (*17*,*22*). Contaminated endotoxin was less than 0.005 endotoxin units/mg. For *in vitro* experiments, SMP-105 was suspended in saline containing 0.01% polysorbate 80. SMP-105 labeled with fluorescein 5-isothiocyanate (FITC; Sigma) was prepared by incubating the mixture of FITC (0.5 mM) and SMP-105 suspension (2 mg/mL). For *in vivo* experiments, an oil-in-water emulsion of SMP-105 was prepared with the following formulation: 0.6 mg/mL SMP-105, 1.6% squalane, 1.0% polysorbate 80, and 5.0% mannitol (*21*,*23*). Vehicle preparation used the same formulation, except for SMP-105.

2.2. *Reagent*

Pam3CSK4 was purchased from Calbiochem (Merck, Tokyo, Japan). *Escherichia coli* J5 lipopolysaccharide (LPS) was purchased from LIST Biological Laboratories (Campbell, CA, USA). LPS was further purified using phenol extraction methods (*24*-*26*). Cytochalasin B and cytochalasin E were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Muramyl dipeptide (MDP-Lys (L18)) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). CpG1826 (*27*) was synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan).

2.3. *Cells*

BC-1, a mouse immature DC line (*28*,*29*), was kindly provided by Drs. Onoe and Yanagawa from the Institute for Genetic Medicine, Hokkaido University, Japan. The BC-1 cell line was maintained as described previously (*28*,*29*). In brief, BC-1 cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal calf serum (FCS), 30% NIH/3T3 supernatant, mouse recombinant GM-CSF, 4 mM L-glutamine, 50 μg/mL streptomycin, 50 U/mL penicillin, and 50 μM 2-mercaptoethanol. Lewis lung carcinoma 3LL was obtained from the Cancer Institute for the Japanese Foundation for Cancer Research (Tokyo, Japan). 3LL cells were maintained in RPMI-1640 medium supplemented with 10% FCS, 50 μg/mL streptomycin, and 50 U/mL penicillin. To prepare inactivated 3LL, cells were incubated for 20 min at 37°C in culture medium containing 200 μg/mL mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan), followed by repeated washing with sufficient culture medium.

2.4. *Animals*

C57BL/6J female mice were purchased from Charles River Japan (Kanagawa, Japan). BALB/c-[Tg]DO11.10-[KO]Rag2 mice (*30*) were obtained from Taconic (NY, USA). Mice were maintained under specific pathogen-free conditions. All animal experiments were conducted according to the guidelines of the Animal Care and Use Committee at Dainippon Sumitomo Pharma.

2.5. *IL-12 p40 induction assay*

BC-1 cells were cultured with SMP-105, Pam3CSK4 or MDP-Lys (L18) overnight. The concentrations of IL-12 p40 in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (BIOSource). In order to examine the effect of phagocytosis inhibitor, BC-1 cells were pretreated for 1 h in the presence of cytochalasin B (1, 10 μg/mL) or cytochalasin E (0.1, 1 μg/mL).

2.6. *Flow cytometry*

BC-1 cells were cultured with SMP-105 (10 μg/mL), CpG1826 (10 μg/mL), LPS (1 μg/mL), or Pam3CSK4 (1 μg/mL) overnight. Cells were collected and stained with FITC-conjugated anti-mouse CD11c, CD40, CD80, CD86, I-A^d , H-2K^d supplied by BD PharMingen (San Diego, CA) for 30 min in the dark at 4°C. After washing with PBS, samples were analyzed on a Coulter Epics XL (Beckman Coulter, Tokyo, Japan). Data were analyzed and presented using FlowJo Ver. 8.8.4 (Tree Star, Inc., Ashland, Oregon, USA).

2.7. *DO11.10 T-cell activation by BC-1 cells*

BC-1 cells were treated with 100 nM ovalbumin peptide, OVA323-339 (ISQAVHAAHAEINEAGR; Bachem), along with SMP-105, Pam3CSK4 or CpG1826 overnight. DO11.10 T cells were enriched from spleen cell suspensions prepared from BALB/c-[Tg]DO11.10-[KO]Rag2 by AutoMACS (Miltenyi Biotech K.K., Tokyo, Japan) with CD90 (Thy1.2) Microbeads (Miltenyi Biotech K.K.). DO11.10 T cells were co-cultured with pre-treated BC-1 cells overnight. The concentrations of IL-2 and IL-12 p40 in the supernatants were determined by ELISA (BIOSource).

2.8. *Fluorescence microscope*

BC-1 cells were cultured with 20 μg/mL FITC-

labeled SMP-105 for 1 h and fixed with 4% (w/v) paraformaldehyde/PBS. Lysosomes were labeled with Lyso Tracker Red DND-99 (Molecular Probes, Leiden, The Netherlands) as recommended by the manufacturer. BC-1 cells were analyzed by confocal microscopy (Leica TCS NT, Leica).

2.9. *Delayed-type hypersensitivity (DTH) reaction*

A mixture of inactivated 3LL cells (3×10^4 cells) and vehicle, SMP-105 (12.5 μg) or Pam3CSK4 (12.5 μg) were administered into the left flank region of C57BL/6J mice twice intradermally with a 7-day interval. Seven days after the second administration, inactivated 3LL cells were inoculated at 10^5 cells in 50 μL HBSS into the left footpads. Just before and 24 h after inoculation, the thickness of the left footpad was measured by dial gauge (Mitsutoyo Co., Kanagawa, Japan). The percentage of swelling was calculated according to the following equation:

Footpad swelling (%)
= (thickness of post-injected footpad (mm) – thickness of pre-injected footpad (mm))/ thickness of pre-injected footpad (mm) × 100

3. Results

3.1. *SMP-105 induces BC-1 maturation*

BC-1 is a phenotypically and functionally immature myeloid DC line (*29*). Exposure of BC-1 cells to various soluble ligands to TLRs, such as FSL-1, Poly(I:C), LPS and CpG1826, up-regulates the expression of CD86, a co-stimulatory molecule, and induces IL-12 p40 production (*31*). SMP-105 stimulated TLR2 (*20*) and we investigated whether SMP-105 matures BC-1 cells and induces IL-12 p40 protein.

BC-1 cells were incubated with SMP-105 or other TLRs ligands (Pam3CSK4, LPS or CpG1826) and expression levels of co-stimulatory and MHC molecules were analyzed by flow cytometry (Figure 1). SMP-105 up-regulated the expressions of CD40, CD80, CD86, I-A^d and H-$2K^d$ on BC-1 to the same level as Pam3CSK4 and LPS. TNF-α induced IL-12 p40 production slightly in BC-1, as previously reported (Figure 2a) and SMP-105 induced a significant amount of IL-12 p40 dose-dependently (Figure 2b), as also observed by treatment with Pam3CSK4 and MDP-Lys (L18) (Figures 2c and 2d). It was shown that SMP-105 induces DC maturation and activation, such as TLRs ligands and NOD2 ligand.

3.2. *SMP-105 activates DCs as antigen-presenting cells*

We tested whether SMP-105 activates DCs as antigen-presenting cells. BC-1 cells were treated with SMP-105 or TLR ligands along with OVA peptide (OVA 323-339) before incubation with $CD4^+$ T cells from DO11.10 mice. These T cells express a transgenic T-cell receptor that recognizes the OVA peptide in the context of BALB/c MHC class II (I-A^d) (*32*). DO11.10 T cells did not produce IL-2 when co-cultured with BC-1 cells without the OVA peptide, or in the presence of SMP-105 alone (data not shown), but a small quantity was observed in the culture of T cells with OVA-peptide pulsed BC-1. When BC-1 cells were pre-treated with SMP-105, IL-2 production was increased similar to pre-treatment with soluble TLRs ligands (Figure 3a). Moreover, we confirmed BC-1 activation under this condition by measuring IL-12 p40 (Figure 3b). These data suggested that DCs activated by SMP-105 function as antigen-presenting cells.

3.3. *SMP-105 is localized in lysosomes after phagocytosis*

Previously, we observed SMP-105 engulfed in phagocytes in regional lymph nodes when SMP-105 was injected intradermally (*21*,*23*). SMP-105 might be phagocytosed by macrophages or DC to elicit tumor immunity in the regional lymph nodes. We therefore investigated the uptake of SMP-105 by DCs employing BC-1. To observe SMP-105 localization in cells, fluorescent imaging was performed using FITC-labeled SMP-105 by confocal microscope (Figure 4). SMP-105 was localized in the intracellular region, especially in lysosomes, which prompted us to investigate the effect of inhibiting phagocytosis. Pre-treatment with cytochalasin B completely blocked the capture of SMP-105 by BC-1 and there was no SMP-105 in the cytoplasm or lysosomes of BC-1.

3.4. *Activation of BC-1 by SMP105 requires phagocytosis*

We investigated whether SMP-105 needs to be phagocytosed to activate BC-1 cells by pre-treatment with cytochalasin B. Cytochalasin B completely inhibited the production of IL-12 p40 by SMP-105, while stimulation by Pam3CSK4 and MDP-Lys (L18) was not affected (Figure 5a). Moreover, cytochalasin E, which also inhibits phagocytosis, abolished the production of IL-12 p40 by SMP-105, but not by other reagents (Figure 5b). These data suggested that phagocytosis is an indispensable step for activating BC-1 by SMP-105, different from the soluble TLR2 ligand and NOD2 ligand.

3.5. *SMP-105, but not Pam3CSK4, significantly induces delayed-type hypersensitivity (DTH) reactions against 3LL*

To investigate the activation and maturation of DCs by

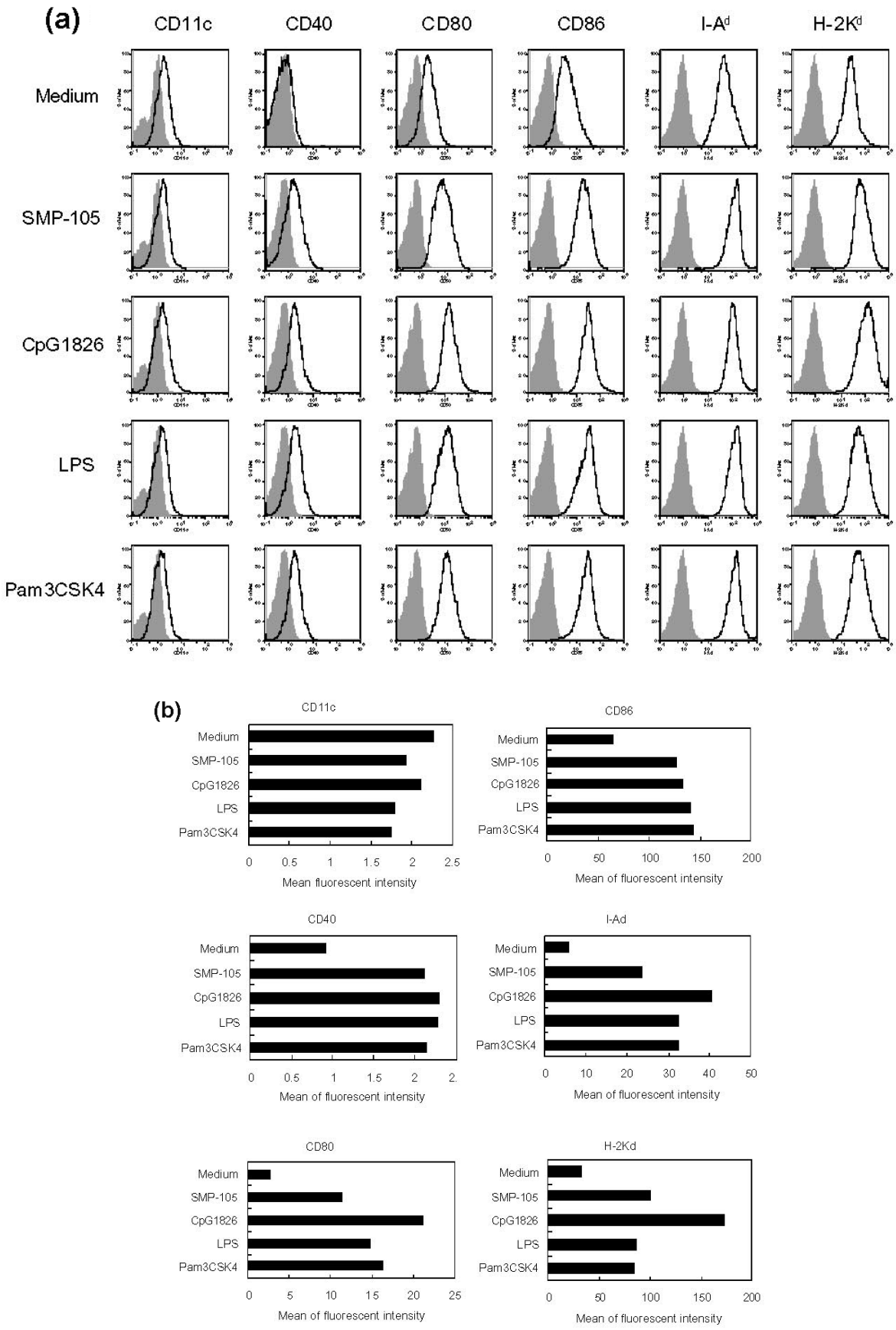

Figure 1. SMP-105 up-regulated CD40, CD80, CD86, I-A^d, and H-2K^d in BC-1. BC-1 cells were stimulated with medium, SMP-105 (10 μg/mL), CpG1826 (10 μg/mL), LPS (1 μg/mL), or Pam3CSK4 (1 μg/mL) overnight. (**a**) Histogram of expression intensities analyzed by flow cytometry are shown concerning CD11c, CD40, CD80, CD86, I-A^d, or H-2K^d (thick lines) or isotype control antibodies (shaded area). (**b**) Mean expression levels were demonstrated in graphs. The experiments were performed twice and the same results were obtained.

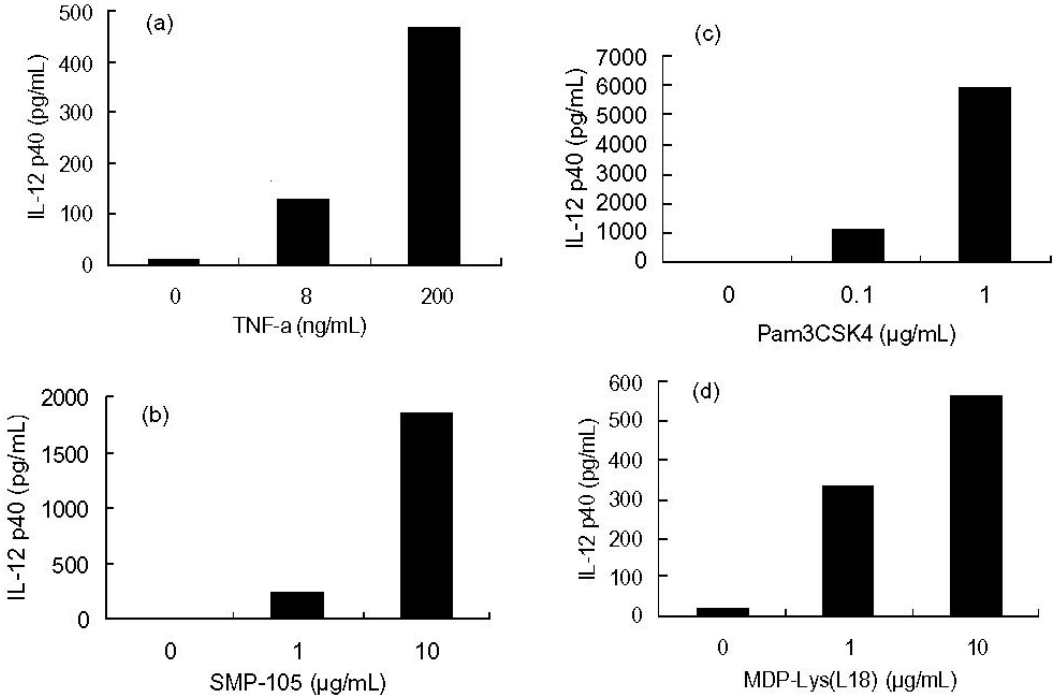

Figure 2. SMP-105 induced IL-12 p40 production by BC-1. BC-1 cells were stimulated with (**a**) TNF-α (8, 200 ng/mL), (**b**) SMP-105 (1, 10 μg/mL), (**c**) Pam3CSK4 (0.1, 1 μg/mL), (**d**) MDP-Lys (L18) (1, 10 μg/mL) or medium alone overnight. IL-12 p40 in the supernatants was measured by ELISA. Shown are representative data of more than four experiments.

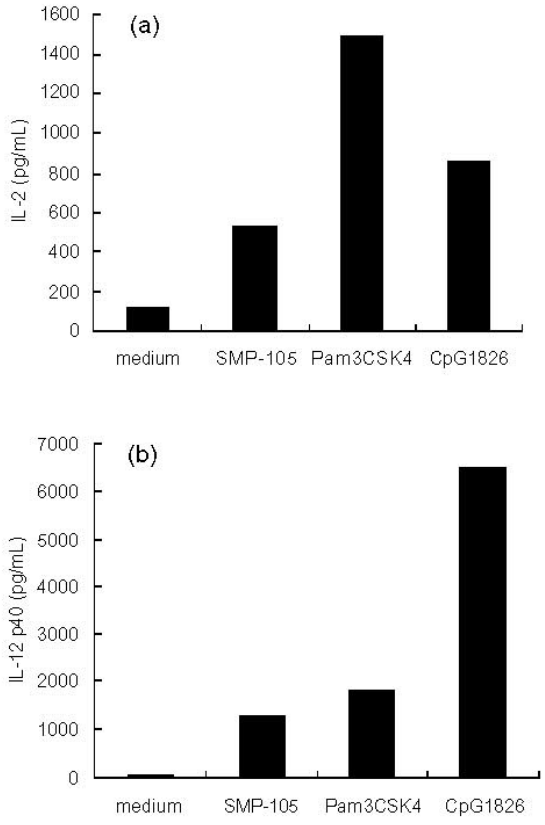

Figure 3. SMP-105 promoted (a) IL-2 and (b) IL-12 p40 secretion in DO11.10 T cells co-cultured with antigen-presenting BC-1 cells. BC-1 cells were pre-treated with SMP-105, Pam3CSK4 or CpG1826 at 10 μg/mL, 1 μg/mL, 1 μg/mL, respectively, and pulsed with 100 nM OVA peptide (OVA 323-339). The cells were co-incubated with T cells derived from DO11.10 mice overnight. IL-2 and IL-12 p40 in the supernatant were assessed by ELISA. The experiments were performed twice and the same results were obtained.

SMP-105 and Pam3CSK4 *in vivo*, we evaluated DTH reactions elicited when injected with an antigen at immunization. Mice were immunized with inactivated 3LL cell suspension admixed with each emulsified reagent, before injecting inactivated 3LL alone in the hind footpad, and edema was measured (Figure 6). SMP-105 evoked footpad swelling clearly when immunized with 3LL cells, whereas Pam3CSK4 exhibited no effect. The lack of an *in vivo* effect of Pam3CSK4, which is a potent TLR2 ligand that strongly activated BC-1 *in vitro*, suggested that activities other than TLR2 stimulation are required to establish strong acquired immunity to elicit DTH reactions.

4. Discussion

TLRs are important and major PRRs and recognize distinct microbial components and directly activate immune cells. Various PRR ligands, such as LPS, and peptidoglycan, stimulated immune systems (*2*,*3*). SMP-105 activates TLR2 and enhances immune responses, such as the number of interferon-γ-producing cells and CTL (*20*). We investigated the activation of DCs by SMP-105 employing BC-1, a mouse immature DC line and investigated the maturation and activation of BC-1 cells by measuring the expression intensity of co-stimulatory and MHC molecules and the production of IL-12 p40, respectively. As expected, SMP-105 up-regulated the expression of surface antigens (Figure 1) and induced IL-12 p40 production dose-dependently (Figure

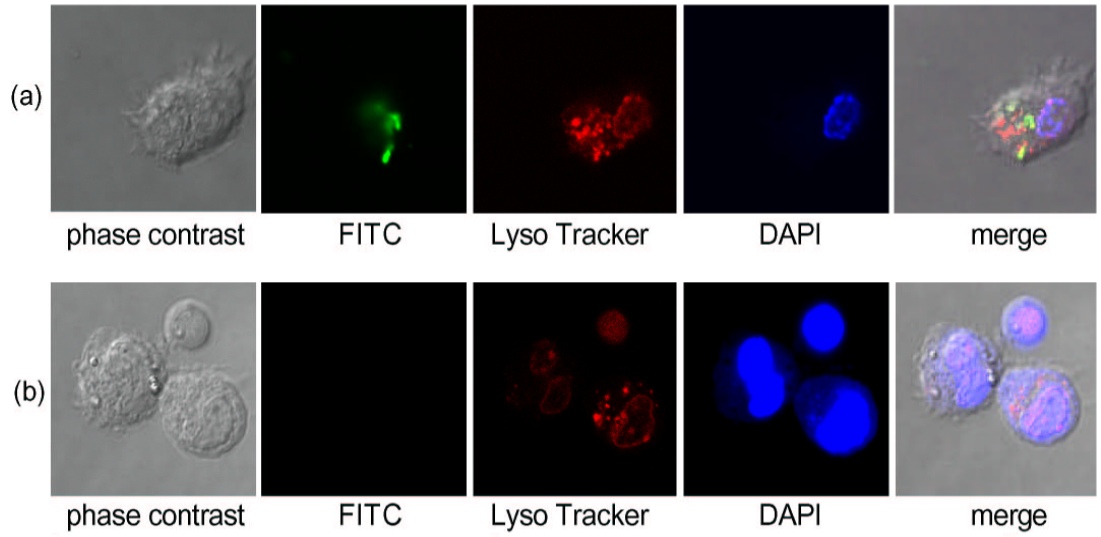

Figure 4. Cytochalasin B, an actin polymerization inhibitor, inhibited phagocytosis of SMP-105 by BC-1. BC-1 cells were pre-treated with (**a**) medium alone or (**b**) cytochalasin B for 1 h and then further incubated with FITC-labeled SMP-105 (10 μg/mL) overnight. BC-1 cells were examined with a fluorescence microscope (Leica TCS NT, Leica) for the distribution. Panels from left: phase contrast, SMP-105: FITC, lysosome: Lyso Tracker, DNA: DAPI, merge.

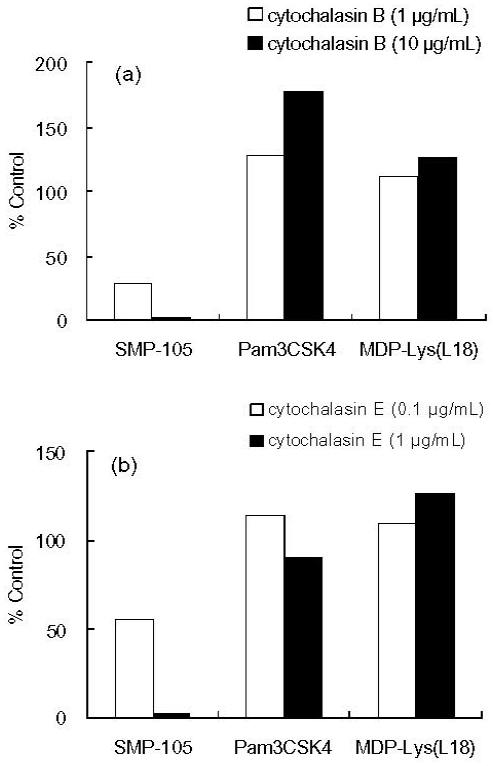

Figure 5. Cytochalasin B inhibits cytokine production of BC-1 by SMP-105, but not by Pam3CSK4 and MDP-Lys (L18). BC-1 cells were pre-treated with cytochalasin B (1, 10 μg/mL) (**a**) or cytochalasin E (0.1, 1 μg/mL) (**b**) for 1 h, and then stimulated with SMP-105 (10 μg/mL), Pam3CSK4 (0.1 μg/mL) or MDP-Lys (L18) (10 μg/mL) overnight. IL-12 p40 in the supernatants were measured by ELISA. Relative production is shown in the figure. Cytochalasin B or E treatment did not affect cell viability at the concentration used. Shown are representative data of five experiments.

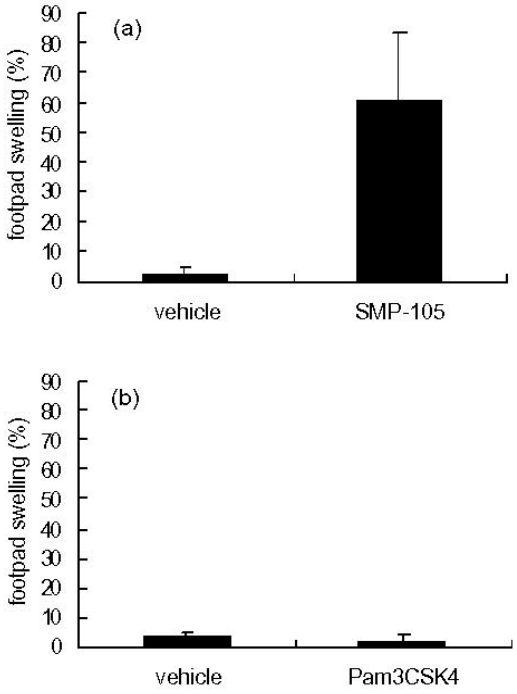

Figure 6. SMP-105, but not Pam3CSK4, induced strong delayed-type hypersensitivity reactions. A mixture of inactivated 3LL cells (3×10^4 cells) and SMP-105 (12.5 μg) (**a**) or Pam3CSK4 (12.5 μg) (**b**) was administered intradermally into the left flank region of C57BL/6J mice twice at a 7-day interval. Seven days after the second administration, inactivated 3LL cells were inoculated into the left footpads and swelling was monitored by measuring the thickness 24 h after inoculation, and the relative swelling was calculated. Means ± S.D. of six mice are shown.

2). Moreover, we confirmed the antigen-presenting function of BC-1 cells matured by SMP-105 by analyzing OVA-specific IL-2 production from T cells derived from DO11.10 mice (Figure 3).

The effects of SMP-105 on the maturation and activation of BC-1 cells were completely abolished by cytochalasin B, whereas those of Pam3CSK4 and MDP-Lys (L18) were not affected (Figure 5), indicating that cytochalasin B did not inhibit the TLR2/MyD88/NF-κB/IL-12 p40 pathway or the NOD2/NF-κB/IL-12 p40 pathway *per se*. SMP-105 might need to be phagocytosed before exhibiting its activity. Analysis with a fluorescence microscope demonstrated the localization of SMP-105 in lysosomes (Figure 4), which suggested that SMP-105 might be converted to active ligands by digestive enzymes in the acidic organella. SMP-105 has a gigantic molecular structure composed of a peptidoglycan linked to arabinogaractan and mycolic acids. Mycoloyl-arabinans, parts of SMP-105, showed potent TNF-α-inducing activities *in vitro* in almost the same order as CWS itself (*33*). Moreover, SMP-105 in physiological saline takes a double-folded sheet form, resulting in a mycolic acid layer on the inner side (*34*). It is conceivable that the digestion of SMP-105 following phagocytosis produces some immunoactive fragments. Okamoto reported that OK-432 activates TLR4 mediated by phagocytosis (*35*). The intact form of OK-432 is not able to stimulate TLR4, but after capture and digested by DCs or macrophages, it is converted to a soluble ligand for TLR4, OK-PSA, and shows antitumor effects accordingly (*35*,*36*). Our results concerning BCG-CWS additionally support that phagocytosis is an important step in activating the immune system *via* PRRs. Mycoloyl-arabinans are suggested as more direct ligand structures for TLR2, but further analysis is required.

It is well-known that preexisting immune T cells cause DTH reactions. We therefore investigated the establishment of antigen-specific T cell immunity *in vivo* by analyzing DTH reactions. Pam3CSK4 matured and activated BC-1 cells *in vitro* and induced the migration of a significant number of DCs and macrophages into a draining lymph node, which is dependent on TLR2 stimulation (*20*); however, although SMP-105 induced strong DTH reactions, surprisingly, Pam3CSK4 failed (Figure 6). These results prompted us to consider that it is not sufficient to stimulate TLR2 alone to establish T cell-mediated immunity *in vivo*. Comparing Pam3CSK4 with SMP-105 as to the effect of cytochalasin B to activate BC-1 cells, it is likely that the process of phagocytosis induces additional effects on TLR2-stimulated DCs to activate cell-mediated immunity *in vivo*. Actually, TLR2 and other signaling act synergistically (*37*,*38*). In addition, we reported in the previous paper that SMP-105 resided for a long time at the inoculation site and activated T cells in guinea pigs (*23*). As probable insoluble particles resistant to digestion, SMP-105 is retained in the skin and activates DCs for a long period, whereas Pam3CSK4 is soluble and probably easily excreted from the body, having only a transient effect on DCs. We think that the difference in retention at the inoculation site explains the strong activity of SMP-105 for the DTH reactions but not a lack of activity of Pam3CSK4, because rather a larger number of DCs were accumulated in the draining lymph node by Pam3CSK4 than SMP-105 (*20*).

We showed previously that SMP-105 induces an immune response against tumors dependent on TLR2/MyD88, but other mechanisms are not known. In this paper we newly showed that phagocytosis is an indispensable process for SMP-105 to activate TLR2. In addition, phagocytosis may also be important to mature and activate immature DCs together with TLR2 signaling to establish T cell immunity *in vivo*. The study of phagocytosis in the context of activating DCs will be necessary.

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