Original Article

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Optimization of cell-wall skeleton derived from *Mycobacterium bovis* BCG Tokyo 172 (SMP-105) emulsion in delayed-type hypersensitivity and antitumor models

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ABSTRACT: Cell-wall skeleton prepared from *Mycobacterium bovis* BCG (BCG-CWS) is known as a potent adjuvant and has been shown to possess antitumor activity in many non-clinical and clinical studies. As there are no approved BCG-CWS formulations for cancer therapy, we investigated the potential for cancer immunotherapy of SMP-105, our originally produced BCG-CWS. For optimizing SMP-105 emulsion, we compared the effects of drakeol- and squalane-based SMP-105 emulsions on IFN-γ production in rats and evaluated their ability to induce skin reaction in guinea pigs. Both emulsions had the same activity in both experiments. We selected squalane as base material and produced two types of squalane-based formulations (vialed emulsion and pumped emulsion) that can easily be prepared as oil-in-water emulsions. Although the vialed emulsion showed the same pattern of distribution as a usual homogenized emulsion, the pumped emulsion showed more uniform distribution than the other two emulsions. Whereas both emulsions enhanced strong delayed type hypersensitivity (DTH) reaction in a mouse model, the pumped emulsion induced slightly smaller edema. Data on oil droplet size distribution suggest that few micrometer oil droplet size might be appropriate for oil-in-water microemulsion of SMP-105. The antitumor potency of SMP-105 emulsion was stronger than that of some of the launched toll-like receptor (TLR) agonists (Aldara cream, Picibanil, and Immunobladder). Aldara and Picibanil showed limited antitumor effectiveness, while Immunobladder had almost the same effect as SMP-105 at the highest dose, but needed about 10 times the amount of SMP-105. These findings first indicate that SMP-105 has great potential in cancer immunotherapy.

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1. Introduction

With the recent approval of sipuleucel-T (Provenge®) and ipilimumab (Yervoy®) by the FDA (*1*), cancer immunotherapy seems to be well underway, and is increasingly attracting a great deal of attention. Cell-wall skeleton prepared from *Mycobacterium bovis* BCG (BCG-CWS) is known as an activator of innate immunity (*2*) and has been studied in many clinical studies (*3-6*). We have previously reported that SMP-105, a highly pure cell-wall skeleton prepared from *Mycobacterium bovis* BCG Tokyo 172 strain, exhibits potent immunostimulatory activity and strong antitumor effect in animal models (*7,8*). SMP-105 is an insoluble toll-like receptor 2 (TLR2) ligand that elicits immune reactions, including induction of interferon-γ (IFN-γ) producing cells and cytotoxic T lymphocytes (CTL), and prevents tumor growth through TLR2 (*9*). SMP-105 requires phagocytosis by macrophages or dendritic cells (DCs) for immune activation and shows different *in vitro* and *in vivo* effects from those of Pam3CSK4, a soluble TLR2 ligand (*10*). Intradermal injection of an oil droplet of emulsified SMP-105 shows that this antigen is readily engulfed by phagocytes at the draining lymph nodes (*11*).

Freund's Complete Adjuvant (FCA), an immuno-potentiator composed of inactivated and dried mycobacteria in mineral oil, is known to stimulate cell-mediated immunity, but may cause severe side effects, which effectively excludes it from clinical use. BCG-CWS on the other hand is used as oil-in-water emulsion, because the immunostimulatory effect of this adjuvant depends on its emulsion form (*12*). Antitumor activity of a drakeol-base oil-in-water emulsion in clinical research has already been reported (*13*). On the other hand, there are reports showing that BCG-CWS emulsified with squalane induces safer antitumor immunity (*14,15*). Squalane-based emulsions

and their hydrogenated derivatives have been shown as potent and safe vaccine adjuvant in preclinical and clinical studies (*16-19*). In fact, MF59^{TM}, an adjuvant emulsion based on squalane, has already been approved for human use (*20*).

Studies that investigated BCG-CWS adjuvant particle size and their phagocytosis have indicated that particles with a size ranging from 0.5-2 μm are readily engulfed by macrophages (*21,22*). Labeled squalane oil-based formulations have been shown to be taken up by macrophages and DCs at the site of injection (*23*). Ideal particles size has also been investigated for drug delivery systems and as a contributing factor to generation of immune response (*24-26*). Particles made from poly lactic-co-glycolic acid (PLGA) can be used as a delivery system that provides adjuvant activity (*27,28*). These polymeric particulate delivery systems are able to present antigens and activate both humoral and cellular responses (*29,30*). A 5 μm PLGA particle containing hepatitis B virus surface antigen elicited higher immune response than a larger particle of about 12 μm (*31*). Although we have previously shown that SMP-105 requires phagocytosis by immune cells for enhancing delayed type hypersensitivity (DTH) reaction *in vivo* (*10*), there are no studies on the correlation between oil droplet size of BCG-CWS emulsion and immune activation.

In this report, we prepared several SMP-105 emulsions and compared their DTH reaction and effects in a lymph node metastasis model. We also characterized oil droplet size distribution in each emulsion. Finally, we investigated antitumor effect of originally optimized SMP-105 emulsion and compared it to that of launched TLR agonists in clinical use.

2. Materials and Methods

2.1. *Preparation of SMP-105*

SMP-105 was prepared as previously described (*4,32*). Contamination with endotoxin was less than 0.005 endotoxin units/mg. The oil-in-water emulsion of SMP-105 (homogenized emulsion) with squalane or drakeol was prepared by homogenization with a Potter-type homogenizer as previously described (*8*). The first original SMP-105 formulation, an oil-in-water emulsion of SMP-105 (vialed emulsion), was prepared and lyophilized on the thousand-vial scale (*7,11*). The vialed emulsion can be prepared by adding only water and vortexing for several seconds. Vehicle preparation used the same formulation as the vialed emulsion, except for SMP-105. The second original SMP-105 formulation, a pumped emulsified oil-in-water emulsion of SMP-105 (pumped emulsion), was prepared by pumping with an SPG pump connector (SPG techno, Miyazaki, Japan). Oil droplet size of the uniformly-sized emulsion was modulated by changing the frequency of pumping (once to 10 times). The pumped emulsion and vialed emulsion had identical content.

2.2. *Materials*

Aldara cream (5% imiquimod) was purchased from 3M Pharmaceuticals (3M Pharmaceuticals, Minnesota, USA), and Immunobladder was purchased from Alfresa Corporation (Japan BCG Laboratory, Tokyo, Japan). Picibanil (OK-432) 5KE was purchased from KSK Co., Ltd. (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan).

2.3. *Oil droplet distribution*

Oil droplet distribution was analyzed by laser diffraction using a SALD-3000J (Shimadzu Corporation, Kyoto, Japan). Oil droplet size is reported as mean diameter.

2.4. *Cells*

Lewis lung carcinoma 3LL cell line was obtained from the Cancer Institute of the Japanese Foundation for Cancer Research (Tokyo, Japan). The 3LL tumor 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, the 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. Line 10 hepatocellular carcinoma cells were implanted intraperitoneally, and a range of cell stocks were prepared and stored in liquid nitrogen. In each experiment, the cells were freshly thawed before intradermal inoculation.

2.5. *Animals*

LEW/Crj male rats and C57BL/6J female mice were purchased from Charles River Japan (Kanagawa, Japan). Strain 2 male guinea pigs were obtained from Japan SLC Inc. (Shizuoka, Japan) and used at 6 weeks of age. All animals were maintained under specific pathogen free conditions, and all animal experiments were conducted according to the guidelines of the Animal Care and Use Committee of Dainippon Sumitomo Pharma.

2.6. *Rat IFN-γ production*

The oil-in-water emulsion of SMP-105 (60 μg/0.1 mL) with squalane or drakeol was administrated into the back paws of LEW/Crj rats 3 times daily. Blood was withdrawn 6 h after the third administration, and the concentration of rat IFN-γ in the serum was determined by enzyme-linked immunosorbent assay (genzyme TECHNE, Invitrogen Japan, Tokyo, Japan).

2.7. *Skin reaction*

The oil-in-water emulsion of SMP-105 (30 μg/0.1 mL) with squalane or drakeol was intradermally administrated once into the backs of Strain 2 guinea pigs. The size of skin reactions was measured at day 7, 13, 21, 27, 34, and 39 after administration.

2.8. *DTH reaction*

DTH reaction was evaluated as previously described (*8,10*). In brief, a mixture of inactivated 3LL cells (3×10^4 cells) and the vehicle; SMP-105 (12.5 μg), was intradermally administered into the left flank region of C57BL/6J mice twice 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 of the mice. Just before, and 24 h after inoculation, the thickness of the left footpad was measured using a dial gauge. Percentage footpad 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.

2.9. *Antitumor effect in guinea pigs*

SMP-105 antitumor effect was evaluated as described previously (*7,11*). In brief, line 10 hepatoma cells were inoculated intradermally at 1×10^6 cells in 0.1 mL into the right thoracic flank region of guinea pigs. SMP-105 and Immunobladder or vehicle was injected intradermally into sites distal to the site of tumor inoculation on days 0, 7, and 14. Picivanil was injected intradermally into sites distal to the site of tumor inoculation on days 0, 3, 7, 10, and 14. As in clinical use, Aldara cream was applied onto the inoculation sites. Animals were sacrificed by anesthesia with a high concentration of carbon dioxide and the axillary lymph nodes were collected and weighed. For pathological study, metastasis was scored from 0 to 4 based on the area occupied by the tumor cells. Metastasis rate (%) was calculated according to the following equation: Metastasis rate (%) = the number of animals with score 1 to 4 /the total number of animals in the group × 100. Metastasis Score: 0, no tumor cells observed; 1, tumor cells found as small clusters; 2, clusters composed of a significant number of tumor cells; 3, clusters composed of a large number of tumor cells, some of which are undergoing mitosis; 4, tumor clusters occupy more than half the area of the lymph node.

2.10. *Statistical analysis*

Results from all experiments are expressed as mean ± standard deviation (SD). Significant differences in skin reaction, DTH reaction, and antitumor effects were assessed using Dunnett's multiple comparison. Only one data of DTH (Figure 3A) was assessed using T-test. Statistical analysis was performed using the SAS system for Windows (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. *Immunostimulatory effect of drakeol-based and squalane-based SMP-105 emulsions*

Drakeol and squalane are commonly used to emulsify BCG-CWS for clinical and research use. To determine the immunostimulatory effect of drakeol-based and squalane-based SMP-105 emulsions, we measured IFN-γ concentration in serum from rats administered each of the SMP-105 emulsions. As expected, vehicle and SMP-105 dispersed in saline had no effect on IFN-γ production. On the other hand, both SMP-105 emulsions induced equally potent IFN-γ production (Figure 1A).

Because BCG-CWS has the ability to trigger skin reaction, we measured the size of skin reactions (ulcer, edema, and induration) formed after administration of each SMP-105 emulsion. Both SMP-105 emulsions dose-dependently and significantly induced skin reactions (Figure 1B). The size of the skin reactions was about the same for both emulsions. These results suggest that drakeol and squalane have similar properties as base material for SMP-105 emulsion.

3.2. *Oil droplet size distribution of oil-in-water SMP-105 emulsions*

To characterize the prepared SMP-105 emulsions, we

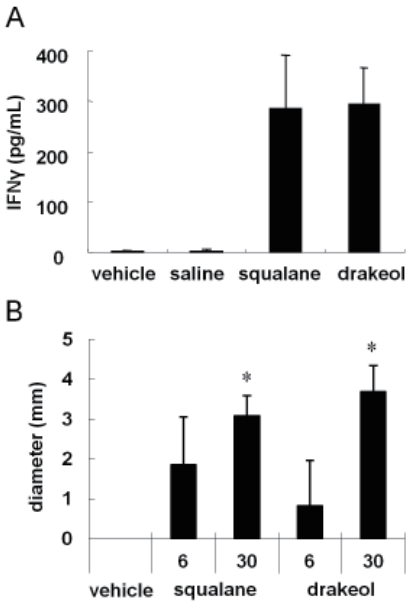

Figure 1. Immunostimulatory effect of drakeol-based and squalane-based SMP-105 emulsions. (A) IFN-γ concentration in serum from rat intradermally administrated each oil based emulsion dispersed in saline. Results are given as means ± SD of 4-5 mice. **(B)** Size of skin reactions induced by intradermal administration of each emulsion to guinea pigs. Results are given as means ± SD of maximum skin reaction size in 5 guinea pigs. * $p < 0.05$ when compared to vehicle.

measured oil droplet size distribution in each emulsion (Figure 2). Oil droplet size of SMP-105 homogenized oil-in-water emulsion with squalane and drakeol was distributed from sub-micrometer to several tens micrometers with the median about 2-3 μm (Figure 2A). Both SMP-105 emulsions (squalane-based and drakeol-based) showed the same oil droplet size distribution. The vialed emulsion had an oil droplet size close to that of the homogenized emulsion that was distributed from sub-micrometer to several tens micrometers with the median about 2-3 μm. On the other hands, the pumped emulsion showed uniform oil droplet size as compared to the homogenized and vialed emulsions (Figure 2B). Oil droplet size in the pumped emulsion differed depending on the pumping frequency (once to 10 times).

3.3. *SMP-105 emulsions enhancement of DTH in mouse model*

To investigate the immunostimulatory effect of each SMP-105 emulsion, we evaluated DTH reaction with each emulsion injection elicited by 3LL cell in mice. Mice were immunized with inactivated 3LL cell suspension admixed with each emulsion, before being injected with inactivated 3LL alone in the hind footpad, and edema was measured. As expected, the homogenized emulsion strongly enhanced swelling at the high dose (12.5 μg) of SMP-105 (Figure 3A). Although both original emulsions evoked strong footpad swelling around the high dose (12.5 μg), the edema produced by the 10 times pumped emulsion tended to be slightly weaker than that evoked by the vialed emulsion (Figures 3B and 3C). Because the pumped emulsion was composed of approximately the same material as the vialed emulsion, adequate oil droplet size seems to be required for acquired immunity against DTH reactions.

To further investigate oil droplet size, we prepared a vialed emulsion with pumping ten times that showed similar oil droplet size distribution to pumped emulsion and evaluated its induction of DTH. The pumped vialed emulsion enhanced footpad swelling induction but the swelling at highest dose was slightly weaker than that enhanced by the vialed emulsion (Figure 3D). These findings suggest that SMP-105 emulsion oil droplet size affects its immunostimulatory effect.

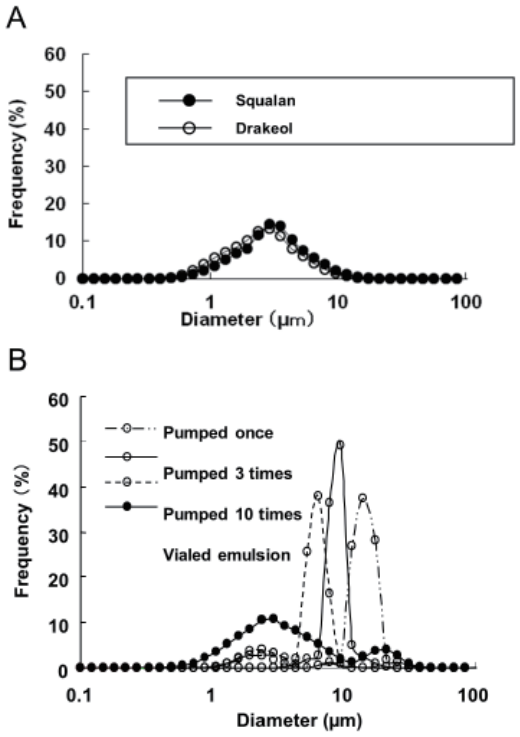

Figure 2. Analysis of oil droplet size distribution in SMP-105 emulsion. Oil droplet size distribution in SMP-105 emulsion was analyzed by laser diffraction particle size analyzer. **(A)** Squalane-based emulsion (black circle) and drakeol-based emulsion (white circle). **(B)** Vialed emulsion (black circle) and pumped emulsion (white circle) in diameter by laser diffraction particle size analyzer.

3.4. *Antitumor effect of SMP-105 emulsions in guinea pig metastasis model*

Guinea pigs were inoculated with line 10 hepatoma cells and each SMP-105 emulsion: homogenized emulsion, vialed emulsion, (10 times) pumped emulsion or vehicle. Inoculations were carried out at sites different from tumor inoculation site in order to avoid damage to tumor cells by local inflammation.

All emulsions (homogenized, vialed, and pumped) demonstrated prominent antitumor activity at 60 μg as indicated by in lymph node metastasis rate (Figures 4A and 4C) and metastasis score (Figures 4B and 4D). Growth of primary implanted tumor decreased in some animals about 2 weeks after the first dose of SMP-105.

3.5. *Antitumor effect of several TLR agonists in guinea pig model*

SMP-105 emulsions enhanced potent DTH reaction and showed antitumor activity. To confirm the antitumor activity of SMP-105, we evaluated the adequacy of the prepared SMP-105 vialed emulsion by comparing its antitumor effect on guinea pig lymph node metastasis to that of marketed TLR agonists (Aldara; TLR7 agonist, OK-432; TLR4 agonist and Immunobladder; live BCG). SMP-105 showed potent antitumor effect with a rate of lymph node metastasis about 20% (Figures 5A and 5B). Aldara inhibited lymph node metastasis by only 14.3%, but reduced the score significantly. Picivanil showed no antitumor effect on the rate of lymph node metastasis (Figures 5C and 5D). On the other hand, Immunobladder showed a dose-dependent potent antitumor effect (Figures 5E and 5F). The maximum effect of Immunobladder was almost equal to that of SMP-105, but required ten times the amount.

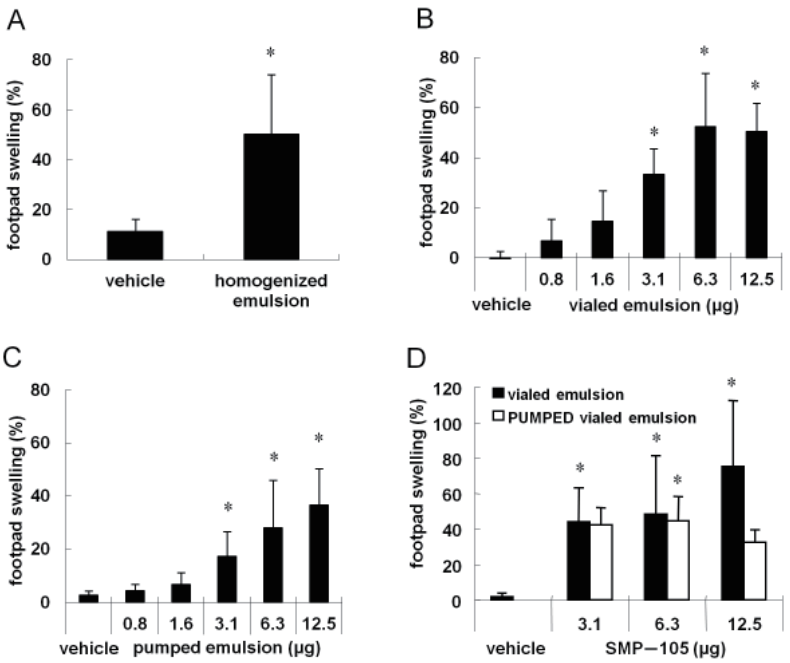

Figure 3. DTH reaction experiment with SMP-105 emulsions. A mixture of inactivated 3LL cells (3×10^4 cells) and each SMP-105 emulsion (0.8-12.5 μg) was intradermally administered into the left flank region of C57BL/6J mice twice at a 7-day interval. (**A**) Homogenized emulsion of 12.5 μg, **(B)** vialed emulsion, **(C)** pumped emulsion at each dose, **(D)** vialed emulsion and pumped vialed emulsion. Seven days after the second administration, inactivated 3LL cells were inoculated into the left footpads and swelling was monitored by measuring footpad thickness 24 h after inoculation. Relative swelling is calculated as means ± SD of 6 mice. * $p < 0.05$ when compared to vehicle.

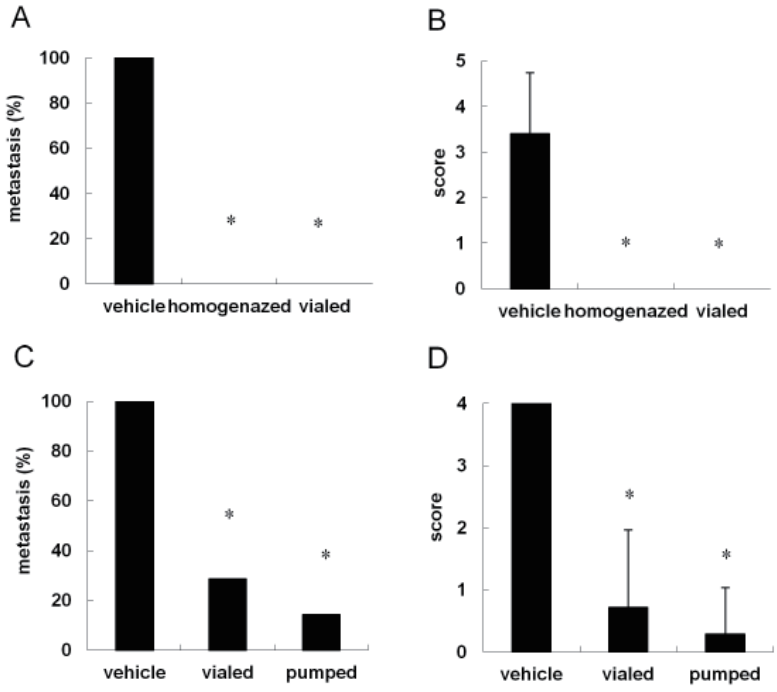

Figure 4. Antitumor effect of SMP-105 emulsions. After inoculation with line 10 hepatoma cells, SMP-105 emulsion or vehicle was injected into the same side as the tumor on days 0, 7, and 14. **(A)** Rate (%) of lymph node metastasis in the case of homogenized emulsion (60 μg) or vialed emulsion (60 μg). **(B)** Score (mean ± SD) of lymph node metastasis in the case of homogenized emulsion (60 μg) or vialed emulsion (60 μg). **(C)** Rate (%) of lymph node metastasis in the case of vialed emulsion (60 μg) or pumped emulsion (60 μg). **(D)** Score (mean ± SD) of lymph node metastasis in the case of vialed emulsion (60 μg) or pumped emulsion (60 μg). Lymph node metastasis rates are given as mean of 4-8 guinea pigs. * $p < 0.05$ when compared to vehicle.

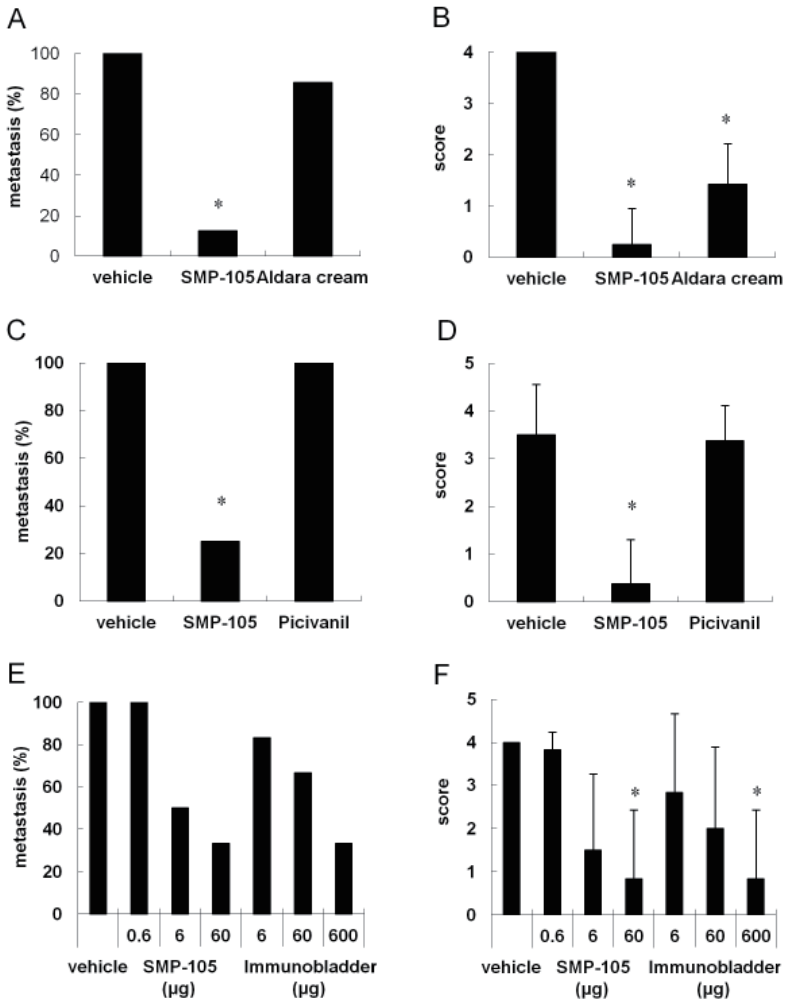

Figure 5. Antitumor effect of SMP-105 emulsion and TLR agonists on line 10 hepatoma in guinea pigs. After inoculation of line 10 hepatoma cells, SMP-105 emulsion, TLR agonist, or vehicle was injected into the same side as the tumor on days 0, 7, and 14. **(A)** Rate (%) of lymph node metastasis in the case of vialed emulsion (60 μg) or Aldara cream (100 μg). **(B)** Score (mean ± SD) of lymph node metastasis in the case of vialed emulsion (60 μg) or Aldara cream (100 μg). **(C)** Rate (%) of lymph node metastasis in the case of vialed emulsion (6 μg) or Picibanil (2KE). **(D)** Score (mean ± SD) of lymph node metastasis in the case of vialed emulsion (6 μg) or Picibanil (2KE). **(E)** Rate (%) of lymph node metastasis in the case of vialed emulsion (0.6, 6, 60 μg) or Immunobladder (6, 60, 600 μg). **(F)** Score (mean ± SD) of lymph node metastasis in the case of vialed emulsion (0.6, 6, 60 μg) or Immunobladder (6, 60, 600 μg). Lymph node metastasis rates are given as mean of 6-8 guinea pigs. * $p < 0.05$ when compared to vehicle.

4. Discussion

In this study, we prepared original BCS-CWS (SMP-105) emulsions with drakeol and squalane and showed their immunostimulatory effect and antitumor activity in animal models. We successfully generated two types of original SMP-105 formulations that can easily be made into oil-in-water emulsions. With these results, we adopted squalane as base material for SMP-105 emulsion, because of its immunostimulatory activity and its suitable properties for clinical use (Figure 1). Our original squalane-based emulsions, can easily be prepared at use, and successfully enhanced both potent DTH reaction in mouse model and antitumor activity with guinea pig lymph node metastasis model.

In this study, SMP-105 pumped emulsion induced potent footpad swelling but tended to be weaker than the vialed emulsion in enhancing DTH reaction. Interestingly, when the vialed emulsion was pumped with a connector, edema decreased compared to that observed with the vialed emulsion. We also confirmed roughly emulsified SMP-105 by voltex that showed about the same oil droplet size to once pumped emulsion enhanced DTH reaction with comparable swelling to 10 times pumped emulsion (data not shown). All emulsions were composed of almost the same material, but had different oil droplet size. The fact that both emulsions enhanced DTH reaction indicates that oil droplet size affects immunostimulatory activity. In particular, oil droplet size of the vialed emulsion seems to be an advantage for easy uptake by phagocytes. Oil droplet size distribution in the vialed emulsion ranged from sub-micrometer to over ten micrometers in diameter (Figure 2) with a mean size (about 2-3 μm) close to

the adequate size for easy uptake by macrophages (*21,22*). As previously indicated, SMP-105 requires phagocytosis by macrophages or DCs for immune activation both *in vitro* and *in vivo* (*10*). This indicates that both emulsions prepared in this study, although having different oil droplet size, were engulfed by phagocytes, suggesting that small micro droplet (about 2-3 μm) is easily devoured by phagocytes compared to large micro droplet (10 μm or larger). This consideration is consistent with the findings of other studies that show that several micrometer particles can be taken by phagocytes, but induce significant immune response (*21,22,31*). Because it is difficult to evaluate SMP-105 emulsion *in vitro*, we could not show in this study oil droplet uptake quantitatively and directly. Further studies are required to elucidate the mechanism of oil droplet uptake.

Our experiment on DTH reaction showed slight difference in immunostimulatory effect between the prepared emulsions. On the other hand, evaluation of the antitumor effect of both emulsion showed similar effect (Figure 4). Because the difference in footpad swelling between the two emulsions was slight, we could not detect difference in the antitumor effect on lymph node metastasis in guinea pig model. These results indicate that DTH experiment is sensitive enough to evaluate BCG-CWS formulations immunostimulatory effect *in vivo*.

Our group previously reported that SMP-105 is a TLR2 ligand that prevents tumors growth through TLR2 (*9*). Accordingly, we compare in this study the antitumor effect of SMP-105 emulsion to that of three launched TLR agonists (Aldara, Picibanil, and Immunobladder) (Figure 5). Although Aldara and Picibanil were administrated in sufficient amount and adequate route as indicated in drug package insert, they showed limited antitumor effect (Figure 5). SMP-105 emulsion showed potent antitumor effect equivalent to that of Immunobladder at low doses. This finding indicates that SMP-105 has promising clinical antitumor use.

Overall, we showed that squalane can be appropriate base material for SMP-105 emulsion and produced two types of squalane-based formulations that can be easily made into oil-in-water emulsions at the time of administration. The results of the prepared emulsions oil droplet distribution and DTH experiment suggest that small micrometer droplet (about 2-3 μm) can induce more potent immune reaction than large micrometer droplet (10 μm or larger). It is believed that small micrometer droplet is easily engulfed by phagocytes. This study is first to show that oil droplet size of few micrometer is optimal for SMP-105 microemulsion with squalane. We also showed for the first time that SMP-105 has more potent antitumor effect than launched TLR agonists in guinea pig metastasis model. These findings indicate that SMP-105 is a promising candidate for clinical investigation.

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