Original Article

Immune response against cell-wall skeleton of *Mycobacterium bovis* BCG at the inoculation site and peripheral lymphoid organs

Yasuo Kashiwazaki*, Masashi Murata, Tetsuya Fujii, Masae Nakagawa, Akihisa Fukushima, Nobuyoshi Chiba, Ichiro Azuma, Takashi Yamaoka**

Pharmacology Research Laboratories, Drug Research Division, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan.

ABSTRACT: We reported in the previous paper that highly purified cell-wall skeleton of *M. bovis* BCG (SMP-105) eliminated lymph node metastases and primary implanted tumor, presumably by generating tumor immunity, employing guinea pigs. In this paper, we investigated the immune reactions to elucidate the mechanisms of antitumor activity. Twenty-four hours after intradermal injection, inflammatory cells were seen migrating to the inoculation site. Massive infiltrations of lymphocytes were observed on day 7, when a large amount of SMP-105 was still observed in the dermis. Several chemokines attracting neutrophils and monocytes, detected by TaqMan RT-PCR, were induced rapidly and declined 72 h post-injection, but most increased again on day 7, consistent with the pathological findings of lymphocyte infiltration. Activation of lymph node cells was investigated using mice. Upon stimulation by SMP-105 *in vitro*, the draining lymph node cells collected from mice treated with SMP-105 produced interferon-γ (IFN-γ), whereas, lymph node cells did not release IFN-γ when prepared from mice treated with OK-432. This evidence prompted us to assume that SMP-105 functioned as T cell antigens. Intracellular cytokine analysis demonstrated that IFN-γ was mainly attributable to $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells. In conclusion, oil-in-water emulsion of SMP-105 resided for a long time at the inoculation site and activated T cells, probably recognizing SMP-105 itself. The strong tumor eliminating activity of SMP-105 may be explained by the boost of generating tumor immunity *via* positive feed-back from T cells reacting to it, and $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells may distinguish SMP-105 from other synthetic adjuvants.

Keywords: Adjuvant, Cell-wall skeleton of BCG, Immunotherapy, $CD4^-CD8^-\alpha\beta T$ cells

1. Introduction

In the 1980s, bacteria and polysaccharides were intensively studied for cancer immunotherapy and some *e.g.* BCG (*1-4*), OK-432 (*5*), PSK (*6*) were approved as medicines. Recently, agonists of toll-like receptors (TLRs), such as imiquimod and CpG oligodeoxynucleotide (CpG ODN), have been under investigation for cancer treatments (*7-9*).

Progress of the science of innate immunity prompted us to reevaluate microbial fractions first examined about 30 years ago for cancer therapy, and we investigated the activity of mono-therapy with highly purified cell-wall skeleton of *M. bovis* BCG (SMP-105), employing strain 2 guinea pigs bearing line 10 hepatocarcinoma, as reported in the preceding paper.

In this report we analyzed immune reactions to elucidate the mechanisms of the strong antitumor activity of SMP-105. First, reactions elicited at the inoculation site are very informative for understanding the potential of agents. The inoculation site was therefore pathologically investigated and the induction of cytokines and chemokines was analyzed by TaqMan-PCR using strain 2 guinea pigs. Next, we inquired into peripheral lymphoid organs, which are specialized to trap antigens and enable the initiation of adaptive immune responses. We observed phagocytes, presumably macrophages or dendritic cells in the draining lymph node with engulfed SMP-105 in the preceding paper. In this report, activation of lymphocytes was investigated using mice by analyzing cytokine production after inoculation of SMP-105 with or without re-stimulation by SMP-105 *in vitro*.

**Correspondence to*: Dr. Yasuo Kashiwazaki, Pharmacovigilance Department, Dainippon Sumitomo Pharma Co., Ltd. 6-8, Doshomachi 2-Chome, Chuo-ku, Osaka 541-0045, Japan;
e-mail: yasuo-kashiwazaki@ds-pharma.co.jp
***Present address*: Hokkaido Pharmaceutical University School of Pharmacy, Otaru, Hokkaido 047-0264, Japan.

Two important clinical events reported by Hayashi *et al*. (*10,11*), *i.e*. delayed-type inflammation at the inoculation site of cell-wall skeleton of BCG (BCG-CWS) and transient IFN-γ in blood, will be discussed based on our findings.

2. Materials and Methods

2.1. *Animals*

Male strain 2 guinea pigs, 5 weeks of age, were obtained from Japan SLC, Inc., Shizuoka and used when they were 6 weeks old. Female C57BL/6N mice at 6 or 7 weeks of age were obtained from Japan SLC, Inc., or Charles River Laboratories, Inc. Yokohama, Japan. Animals were maintained under specific pathogen-free conditions. Their maintenance and experiments were conducted with the approval of the DSP Animal Care and Use Committee.

2.2. *Preparation of SMP-105*

SMP-105 is a product of Dainippon Sumitomo Pharma Co., Ltd. and chemical analysis data were reported by Uenishi *et al*. (*12*). Briefly, SMP-105 contained less than 3% (w/w) of sugars and amino acids assumed not to constitute CWS. Both DNA and trehalose dimycolate were removed to less than 0.05% (w/w) and lipopolysaccharide was about 0.0015 EU/mg by gel-clot technique. An oil-in-water emulsion of SMP-105 was prepared and lyophilized on the thousand-vial scale. Each vial contained 1.2 mg of SMP-105, 32 mg of squalane, 20 mg of polysorbate 80 and 100 mg of mannitol. Vehicle preparation used the same formulation except for SMP-105. SMP-105 in emulsified form was used for inoculation, and the suspended form in saline was prepared for *in vitro* use.

2.3. *Pathological investigation*

SMP-105 was inoculated intradermally into the flank region of strain 2 guinea pigs at 60 μg (n = 3). Animals were sacrificed with a high concentration of carbon dioxide at various time points over 7 days, and skin, including the center of the inoculation site was sampled and fixed with 10% of formaldehyde solution. The skin was cut through the inoculation site into two equal pieces and analyzed immunohistochemically using rabbit anti-*M. bovis* BCG antibody (DAKO Japan Co. Ltd., Kyoto). The reaction of anti-BCG antibody to SMP-105 was investigated by binding assay (data not shown).

2.4. *Analysis of induction of chemokines and cytokines at inoculation site by Taq-Man real-time RT-PCR*

SMP-105 was inoculated intradermally into the flank region of strain 2 guinea pigs at 60 μg (n = 5), and the skin at the inoculation site was excised in a circle of ca.1 cm diameter and collected into liquid nitrogen over 7 days. Total RNA was extracted and purified using TRIZOL (GIBCO) and Rneasy Mini kit (Qiagen), respectively, according to each manufacturer's instructions. The quality of extracted RNA was checked by agarose-gel electrophoresis. Complementary DNA was synthesized using TaqMan Reverse Transcription Reagent (Applied Biosystems,). Amplification was performed using an ABI PRISM 7700 Sequence Detection System (PerkinElmer, Applied Biosystems) using the 5'-nuclease method (TaqMan) with a three-step PCR protocol (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min). All primers and probes were designed with the aid of Applied Biosystems Japan, Co., Ltd. (Tokyo, Japan) from cDNA sequences in NCI data base. TaqMan probes were labeled with FAM as reporter dye and MGB as minor groove binder, except for the β-actin probe, which was labeled with VIC as reporter dye. The sequences of all primers and probes used in this study are shown in Table 1.

Relative quantitation of mRNA levels was performed using the standard curve method. The standard curves were created using five serial dilutions (1:2, $1:2^2$, $1:2^3$, $1:2^4$, and $1:2^5$) of cDNA from the skin of guinea pigs inoculated with either SMP-105 or vehicle. The samples were run in duplicate with primers and probes against β-actin and the target mRNA in the same well. Samples without reverse transcriptase treatment were also run to confirm no contamination of genomic DNA. The relative amount of mRNA in each sample was calculated as the ratio between the target mRNA and the corresponding endogenous control β-actin, and induction kinetic curves were drawn as logarithms of the ratio plus 1 against sampling times.

2.5. *Response in weight of draining lymph node and spleen*

SMP-105 was injected intradermally into the flank region of strain 2 guinea pigs at 60 μg and animals were sacrificed with a high concentration of carbon dioxide on day 14. Axillary lymph node and spleen were sampled and weighed. In experiments using mice, SMP-105 was injected into the footpad of C57BL/6N mice at 30 μg. Popliteal lymph nodes and spleen were sampled over 4 weeks.

2.6. *Cytokine production of draining lymph node cells by re-stimulation of SMP-105 in vitro*

SMP-105 or OK-432 (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) was injected intradermally into both sides of C57BL/6N mice (n = 3). Ten days later, axillary and inguinal lymph nodes were collected and pooled, and single cell suspensions were prepared. Lymph node cells from mice treated with SMP-105 were stimulated

Table 1. Sequences of probes and primers

MCP-1	
MGB probe	5'- CCCAGCAGAAACAG -3'
forward primer	5'- ATTGCCAAACTGGACCAGAGA -3'
reverse primer	5'- TTGAAGTTTGAGGTGCAGTTGAG -3'
MCP-3	
MGB probe	5'- CAGTCCTTCTGTGCCTAC -3'
forward primer	5'-CCTTCCACCATGCAAGTCATT -3'
reverse primer	5'-GCTGAAGTTGGCTGCTGTGA -3'
GM-CSF	
MGB probe	5'-CCCTGTCACCCAGTCC -3'
forward primer	5'-GCACTGTGGTTTGCAGCATCT -3'
reverse primer	5'- GTGGCATCCACGTGTTTCC -3'
IL-1β	
MGB probe	5'- CAATGGAGACAATCTGA -3'
forward primer	5'-CCATCACGGAACTGCACACT -3'
reverse primer	5'- CTCCTTGTACGAAGCTCATGGA -3'
IL-8	
MGB probe	5'-TGACAATCGACAGCTCT -3'
forward primer	5'- GACCACGTTGTGCCAACTCA -3'
reverse primer	5'-CACGTCCTGCACCCACTTCT -3'
VEGF	
MGB probe	5'- TCGGAGAGATGAGTTTC -3'
forward primer	5'- TGCAGATCATGCGGATCAA -3'
reverse primer	5'- TTGGTCGCATTCACATTTGC -3'
eNOS	
MGB probe	5'- CGCCCATGACTTTG -3'
forward primer	5'- GCGGCTGCATGACATTGA -3'
reverse primer	5'- GATGGTCGAGTTGGGAGCAT -3'
GRO	
MGB probe	5'- TCCCTTGGACATTTT-3'
forward primer	5'- ACCTTCATGGTATGCAGTCAACA-3'
reverse primer	5'- CAGTTATGGCTAAACAAGGCATTG-3'
RANTES	
MGB probe	5'- CCCACTGCTTAGCAAT -3'
forward primer	5'- TCTAGGTTCCCAGGCCTCTCA -3'
reverse primer	5'- TGCCTTGAAAGATGTGCTGACT -3'
TNF-α	
MGB probe	5'- TCACACTCAGATCAGC -3'
forward primer	5'- CAGCGGGAAGAGCAGTTCTC -3'
reverse primer	5'- GCCACCGGCTTGTCATTAT -3'
IL-12 p40	
MGB probe	5'- CCTGCAACACTGCTG -3'
forward primer	5'- CCTGGAGAGACGGTGGTTCTT -3'
reverse primer	5'- GAGGTCCATGTGATGCCATCT -3'
β-actin	
MGB probe	5'- TGCGTGACATCAAGGA -3'
forward primer	5'- GACGGAGCGTGGCTACAGTT -3'
reverse primer	5'- GCCATCTCCTGCTCGAAGTC -3'

with SMP-105 for 48 h and IL-2, IL-4, IL-10, IL-12 and IFN-γ in culture supernatants were determined using ELISA kits (Endogen Inc., MA) according to the manufacturer's instructions. Lymph node cells from mice treated with OK-432 were stimulated for 48 h with SMP-105 at 1 μg/mL or OK-432 at 0.01 KE/mL and IFN-γ was determined.

2.7. *Analysis of IFN-γ-producing cells*

C57BL/6N mice were treated with SMP-105 at 60 μg/side on days 0, 3 and 6, and axillary and inguinal lymph nodes were collected and pooled on day 10. Single cell suspensions were prepared and incubated in a 24-well plate at 10^7 cells/well for 20 h with or without SMP-105 at 10 μg/mL. Cell-surface antigens and intracellular IFN-γ were stained using BD Cytofix/Cytoperm™ plus the Fixation/Permeabilization kit (BD Biosciences, New Jersey) according to the manufacturer's protocol. Briefly, for the final 5 h, GolgiStop™ (protein transport inhibitor containing monensin) was added to the medium. The cells were then harvested and Fc receptors were blocked with anti-FcγII/III receptor antibody (clone 2.4G 2), followed by staining of FITC-labeled monoclonal antibodies for CD3ε (clone 145-2C11), CD4 (clone RAM4-5), CD8a (clone 53-6.7), CD8b.2 (clone 53-5.8), NK1.1 (clone PK136), TCRβ (clone H57-597), γδTCR (clone GL3), or isotype control antibodies in separate tubes. After a thorough washing, cells were fixed and permeabilized, stained with PE-labeled anti-IFN-γ antibody (clone XMG1.2), washed and analyzed by FACScan. The monoclonal antibodies described above were all purchased from BD Biosciences.

2.8. *Detection of IFN-γ in blood and analysis of producing peripheral lymphoid organs*

SMP-105 was injected into both fore-footpads at 30 μg per pad on days 0, 3 and 6. After the final injection, blood was collected by heart puncture under ether anesthesia at various time points over 24 h. The brachial and axillary lymph nodes and spleen were collected and lymph nodes from each animal were pooled. Cell suspension was prepared and incubated for 48 h with 10 U/mL of interleukin 2 (IL-2) (Genzyme Corporation) without re-stimulation with SMP-105. IFN-γ in the serum and culture supernatant was determined using ELISA kits (Endogen Inc., MA) according to the manufacturer's instructions.

2.9. *Statistical analysis*

Statistical analysis was performed using the SAS system for Windows (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. *Pathological investigation of inoculation site*

Tumor elimination, presumably by generating tumor immunity using SMP-105, was observed in strain 2 guinea pigs bearing syngeneic line 10 hepatocarcinoma. We first pathologically investigated the skin at the

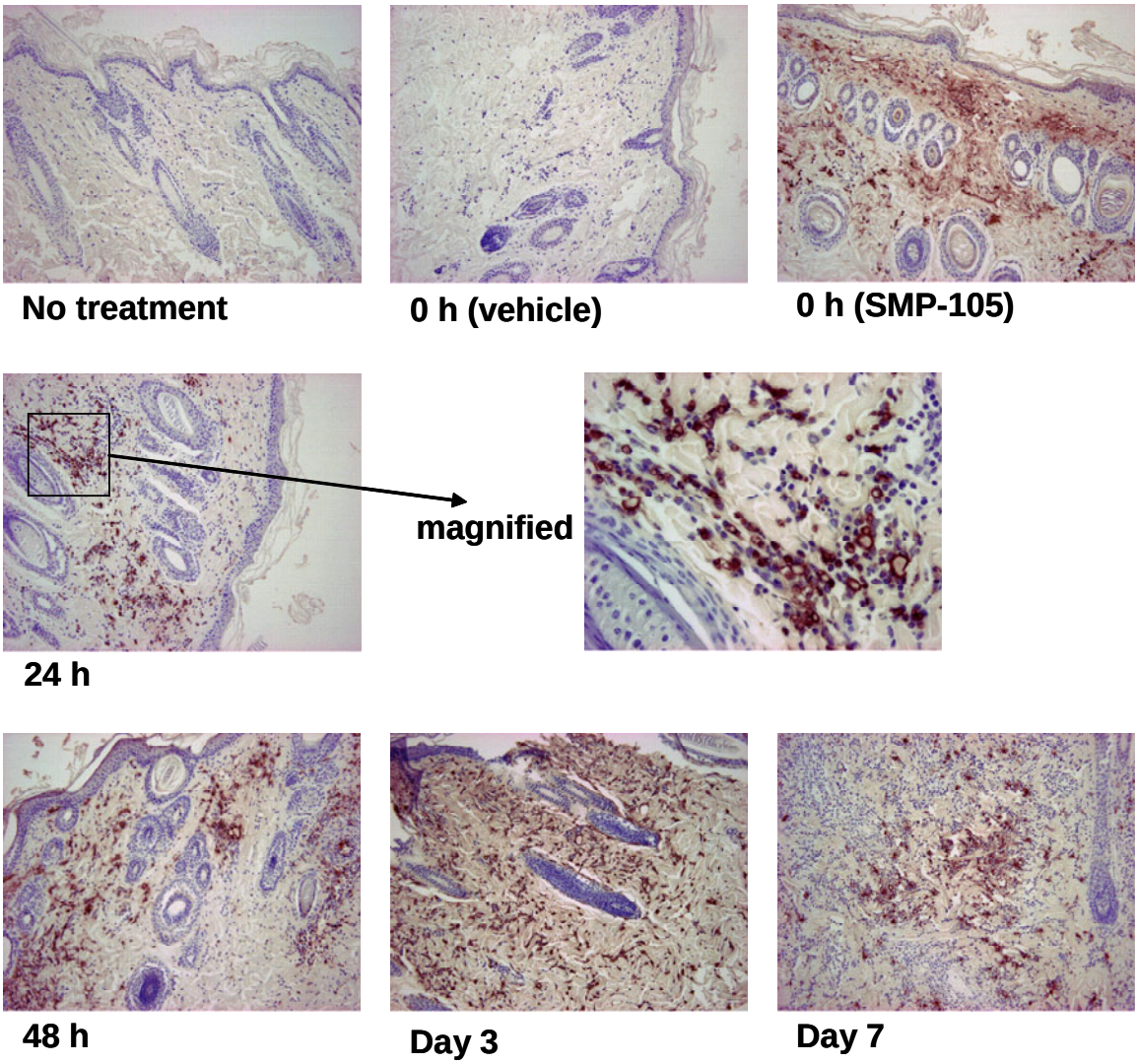

Figure 1. Distribution of SMP-105 at inoculation site (guinea pig). Paraffin-fixed skin tissues from guinea pigs injected with SMP-105 were stained with anti-BCG polyclonal antibody ($n = 3$). Representative sections are shown.

inoculation site using strain 2 guinea pigs. SMP-105 was injected intradermally into the flank region and the skin was sampled over 7 days. Twenty-four hours after injection, inflammatory cells were seen migrating to the inoculation site and engulfing SMP-105 (Figure 1). A large number of positive signals from SMP-105 were still observed on day 7 by immunohistochemical analysis and massive infiltration of lymphocytes was observed into areas where SMP-105 was distributed (Figure 1).

3.2. *Induction of cytokines and chemokines at inoculation site*

Gene expression of the skin at the inoculation site was investigated by the TaqMan quantitative RT-PCR approach. Tumor necrosis factor-α (TNF-α), IL-1β, growth-related oncogene (GRO), monocyte chemotactic protein 1 (MCP-1) and IL-8 were induced rapidly, and peaked 8 h after inoculation. Regulated on activation normal T cells expressed and secreted (RANTES), which is reported to be up-regulated by TNF-α and IL-1β (*13*), showed a somewhat delayed peak at 24 h (Figure 2). After decreasing once, TNF-α, IL-1β, RANTES, MCP-1 and IL-8 increased again 7 days after injection (Figure 2), suggesting that the immune reactions entered a new phase. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was induced significantly only 7 days after injection (Figure 2). MCP-1, IL-8, GRO, RANTES and endothelial nitric oxide synthase (eNOS) were induced also by vehicle (Figure 2) and in the early phase, the mRNA level of MCP-1 and eNOS stimulated by SMP-105 was explicable by the vehicle, probably due to squalane contained in the vehicle. The induction of chemokines and cytokines by the vehicle may involve adjuvant activity of squalane (*14*). A clear increase or decrease of MCP-3, vascular endothelial growth factor (VEGF and IL-12 p40 was not observed (Figure 2).

3.2. *Marked increase in weight of the draining lymph node but no change in the spleen*

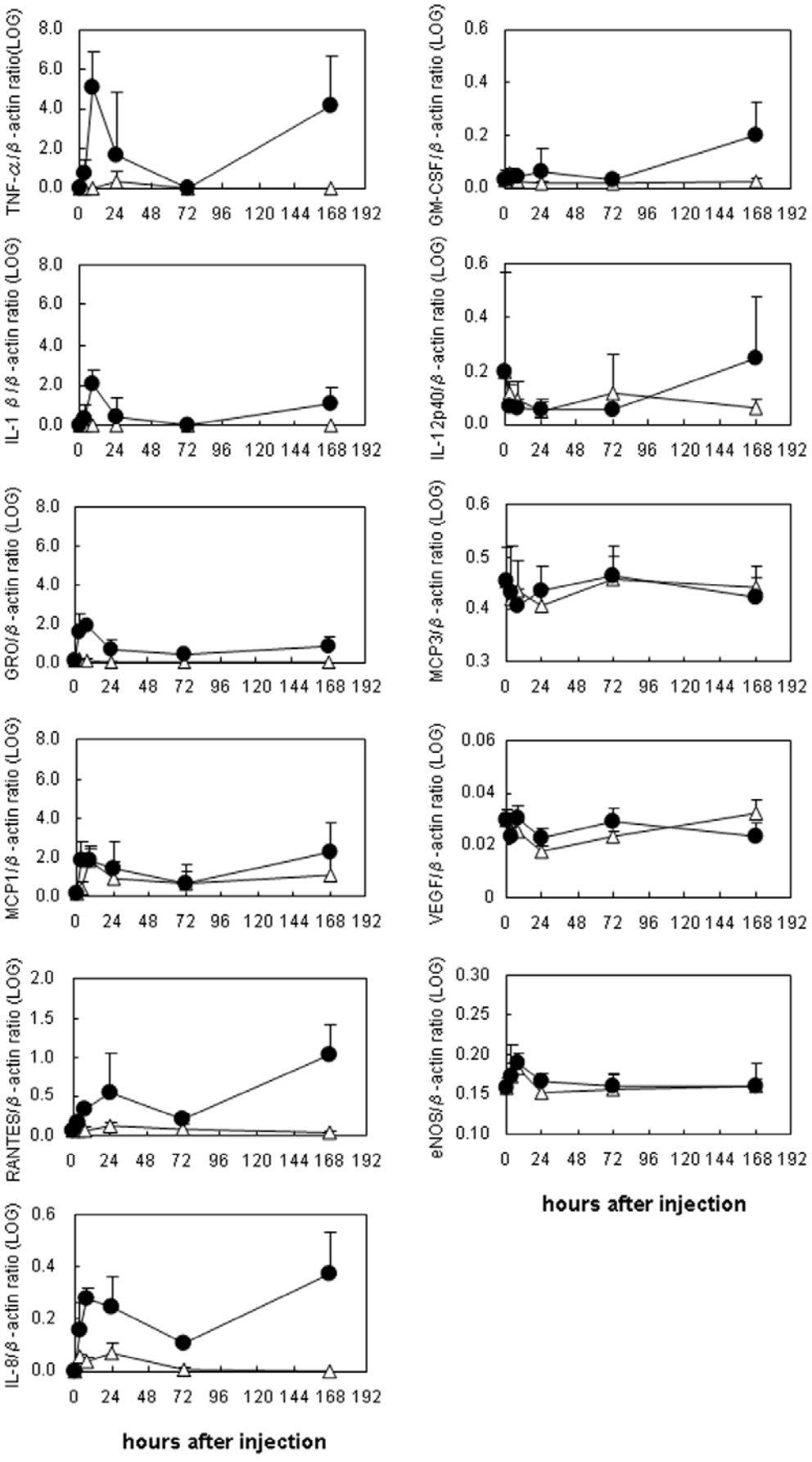

Figure 2. Induction of cytokines and chemokines at inoculation site (guinea pig). Induction of cytokines and chemokines indicated in each figure at the inoculation site was investigated by TaqMan quantitative RT-PCR approach. Average and SD (n = 5) are shown. Circle, SMP-105; triangle, vehicle.

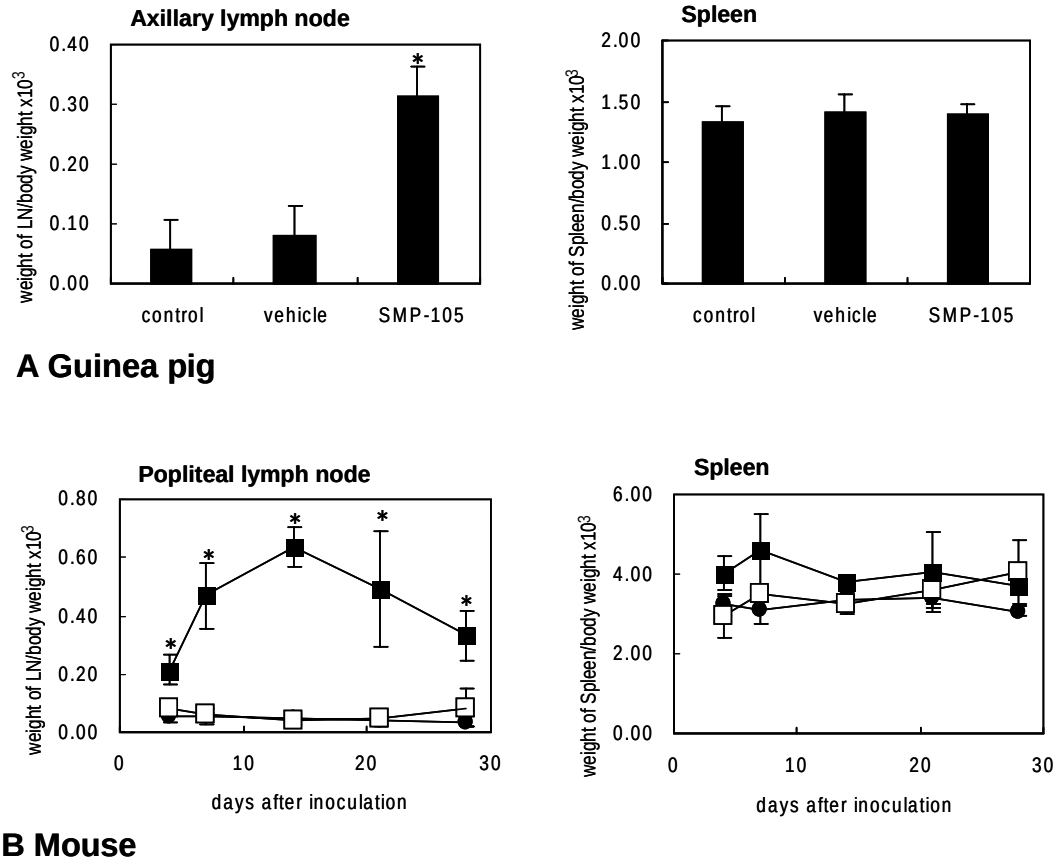

Figure 3. Increase in weight of the draining lymph node (guinea pig and mouse). A: Guinea pig. Wet weight of axillary lymph node and spleen 14 days after SMP-105 injection is demonstrated as the ratio to body weight ($n = 4$); B: Mouse. Wet weight of popliteal lymph node and spleen over 4 weeks is shown as the ratio to body weight ($n = 3$). Closed circle, control; open square, vehicle; closed square, SMP-105. Average and SD are indicated. *: $P < 0.05$ *T*-test (*vs* vehicle).

Next, peripheral lymphoid organs were analyzed. We noticed marked swelling of the draining lymph nodes and wet weight was therefore measured.

Two weeks after injection of SMP-105 into guinea pigs, the wet weight of the draining lymph nodes was remarkably increased, but there was no response in spleen weight (Figure 3A). In experiments using C57BL/6N mice, the same results were obtained, *i.e.* increase in weight of popliteal lymph node was observed for 4 weeks, peaked 2 weeks after inoculation, and there was no response in spleen weight (Figure 3B). Further analysis of lymph node activation was performed using mice, because various well-characterized antibodies were available.

3.3. *Production of IFN-γ, IL-2 and other cytokines in draining lymph node cells by re-stimulation of SMP-105 in vitro*

Activation of the draining lymph nodes by injecting SMP-105 was investigated by cytokine production upon re-stimulation with SMP-105 *in vitro*. The lymph node cells produced IFN-γ dependent on the pre-treatment doses (Figure 4A), demonstrating that SMP-105 injection induced the activation of draining lymph node cells. In addition to IFN-γ, IL-2 was also secreted (Figure 4B). Production of a marginal amount of IL-10 was observed but the induction of either IL-12 or IL-4 was not detected (Figure 4B).

The production of IL-2 indicated that T cells were differentiated in the lymph nodes. When lymph node cells from mice immunized with OK-432 were re-stimulated with OK-432 *in vitro*, a large amount of IFN-γ was released, but little was detected upon stimulation with SMP-105 (Figure 4C). When lymph node cells prepared from mice immunized with SMP-105 were re-stimulated with SMP-105 *in vitro*, an enormous amount of IFN-γ was produced (Figure 4C). This evidence suggested that some portion of IFN-γ is attributable to T cells activated by antigen presentation. This issue will be discussed later.

3.4. *Major populations of IFN-γ-producing cells*

Produced in abundance by re-stimulated lymph node cells, the major populations of IFN-γ-producing cells were investigated. Axillary and inguinal lymph node cells from C57BL/6N mice pre-treated with SMP-105 were re-stimulated *in vitro* with SMP-105, and IFN-γ in cytoplasm was analyzed by FACS using the cellular cytokine detection method. The major populations of IFN-γ-producing cells were $CD4^{-}CD8^{+}\alpha\beta$T cells and

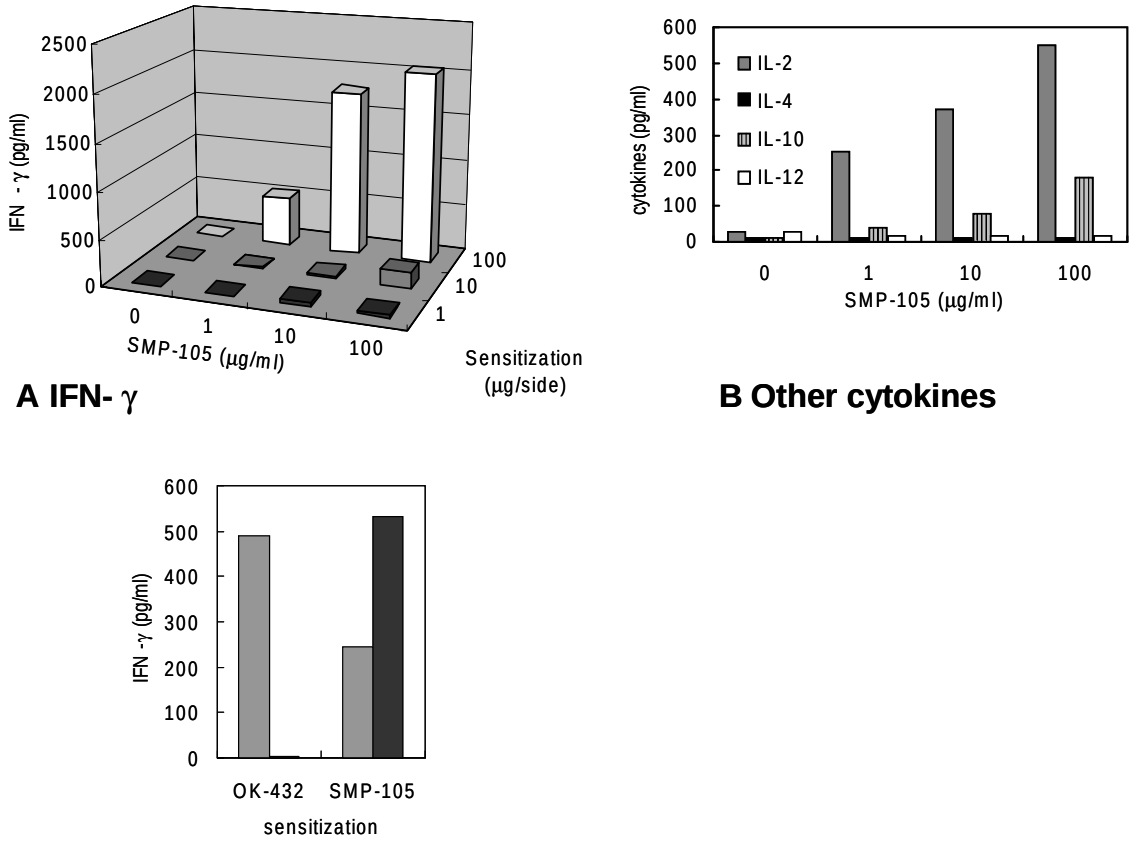

Figure 4. Production of cytokines from draining lymph node cells by re-stimulation of SMP-105 *in vitro* (mouse). Ten days after inoculation of SMP-105 at 1, 10 or 100 μg (1 μg; n = 5, 10, 100 μg; n = 3) or OK-432 at 0.1KE (n = 3), draining lymph nodes were sampled and pooled. Cell suspensions were prepared and stimulated by SMP-105 or OK-432 for 48 h (n = 3). IL-2, IL-4, IL-10, IL-12 and IFN-γ in the culture supernatants pooled from 3 wells were determined. A: Production of IFN-γ. Production of IFN-γ is demonstrated by 3-D figure. X-axis, concentration at re-stimulation *in vitro*; Y-axis, pre-treatment dose; Z-axis, IFN-γ secreted into medium. Shown are representative experiments of more than three; B: Production of other cytokines. Culture supernatant of lymph node cells prepared from mice treated with 100 μg of SMP-105 in the same experiments as described above was assayed for cytokines indicated in the figure; C: Antigen dependency of IFN-γ production. Cell suspensions were stimulated *in vitro* by 1 μg/mL of SMP-105 (black bar) or 0.01 KE/mL of OK-432 (gray bar) for 48 h (n = 3). IFN-γ in the culture supernatants pooled from 3 wells was determined.

$CD4^{-}CD8^{-}\alpha\beta$T cells, and few positive signals of IFN-γ were detected from $NK1.1^{+}$ or γδT cells (Figure 5).

3.5. *Transient IFN-γ detected in blood after repeated injection of SMP-105*

Hayashi *et al*. reported that IFN-γ was transiently detected in blood after the injection of BCG-CWS from cancer patients and that IFN-γ was an excellent prognostic marker (*10,11*). Detection of transient IFN-γ in blood was therefore attempted using mice and we succeeded by injecting SMP-105 into fore-footpads. IFN-γ was detected from 3 h, peaked at 6 h and then fell markedly 24 h after the final injection (Figure 6).

3.6. *Production of IFN-γ by draining lymph node cells but not the spleen upon incubation without SMP-105*

In order to investigate the origin of IFN-γ in blood, the draining lymph nodes and spleen were collected after repeated injection of SMP-105, incubated without re-stimulation by SMP-105 for 48 h, and the culture supernatant was assayed for IFN-γ by ELISA. IFN-γ was detected from the supernatant of the draining lymph node cells (Figure 7A), but not from that of spleen cells (Figure 7B), suggesting that IFN-γ in the blood was secreted mainly by draining lymph node cells.

4. Discussion

It is reported that BCG-CWS in the emulsified form induces delayed and long-lasting inflammation at the inoculation site (*10,11*). When SMP-105 was injected into the skin, slight redness developed, peaked from 24 to 48 h post-injection, and then disappeared by day 5. Erythema accompanied by edema then developed and lasted for more than a month (data not shown). Within 24 h, inflammatory cells migrated to the inoculation site, probably attracted by chemokines affecting neutrophils, *e.g.*, IL-8, GRO and macrophages, *e.g.*, MCP-1, RANTES and further activated by IL-1β

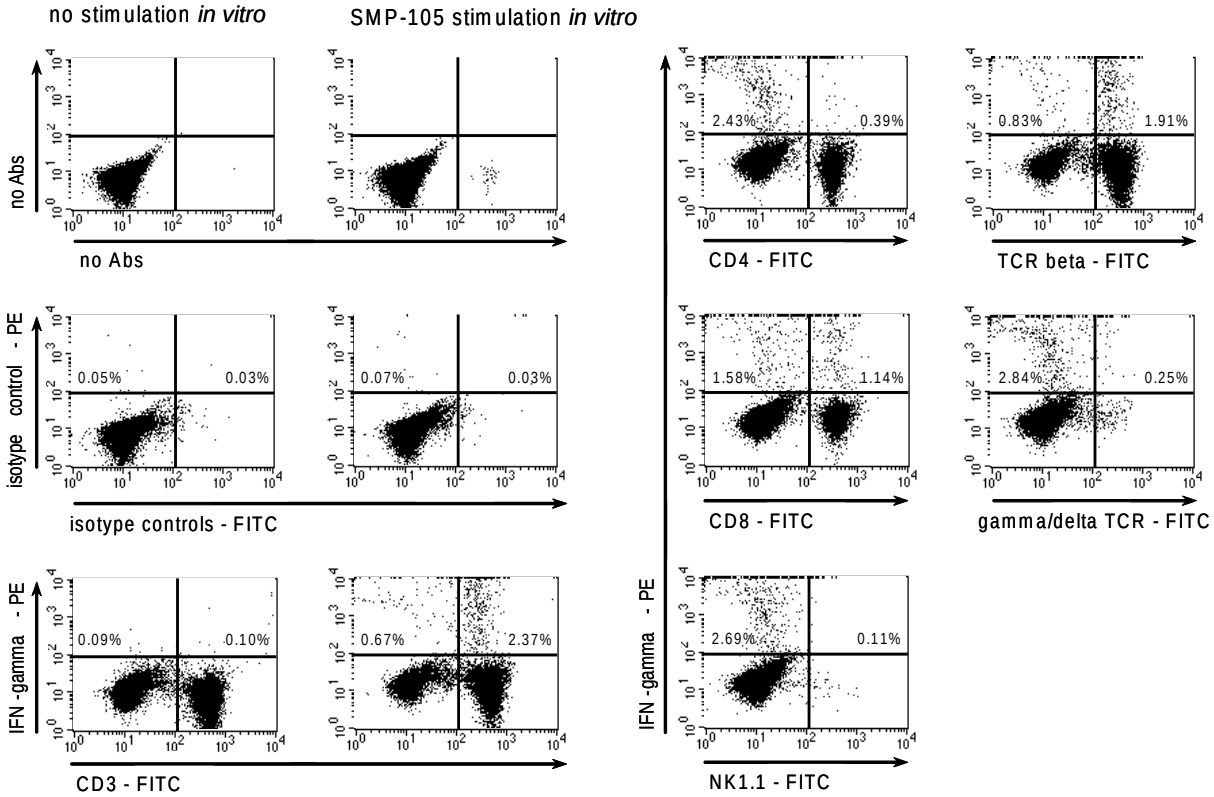

Figure 5. Major population of IFN-γ-producing cells (mouse). Draining lymph node cells from C57BL/6N mice pre-treated with SMP-105 were re-stimulated *in vitro* with SMP-105, and IFN-γ in cytoplasm was analyzed by FACS using the cellular cytokine detection method. Shown are representative experiments of more than five.

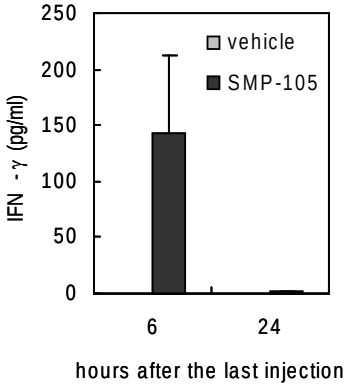

Figure 6. Transient IFN-γ detected in blood (mouse). Blood was collected from mice repeatedly treated with SMP-105 (n = 3), and serum was assayed for IFN-γ. Blood from vehicle-treated mice was collected 6 h after the final treatment. Average and SD are indicated.

and TNF-α (*15-17*) (Figure 2). On day 7, massive infiltration of lymphocytes was seen (Figure 1), probably associated with the secretion of RANTES (*13,15-17*), and RANTES is considered to involve T-cell-mediated chronic inflammation. In fact, at that time, a large amount of SMP-105 remained (Figure 1) and more than a month later, positive signals derived from SMP-105 were still observed (personal communication from Dr. Koji Hanai), consistent with skin reactions. Long-lasting stimulation of immune systems, from innate immunity to acquired immunity, could evoke tumor elimination activity, and

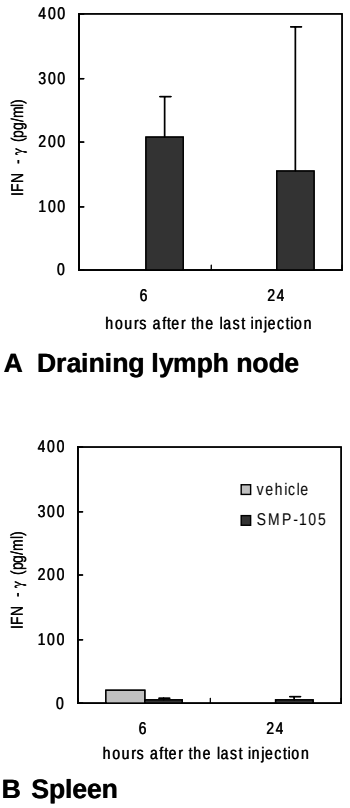

Figure 7. Secretion of IFN-γ by peripheral lymphoid organs (mouse). Brachial and axillary lymph node and spleen were collected from the same mice as described in Figure 6 after repeated inoculation of SMP-105 into fore-footpads (n = 3). Lymph node cells (A) and spleen cells (B) were incubated with 10 U/mL of IL-2 for 48 h and culture supernatant was assayed for IFN-γ. Average and SD are indicated.

the long residence of BCG-CWS emulsified with oil may explain the recurrence of inflammation at past inoculation sites upon fresh injection into the other arm (personal communication from Dr. Akira Hayashi).

The production of IL-2 by draining lymph node cells from mice pre-treated with SMP-105 indicated the differentiation of T cells (Figure 4B). Lymph node cells also produced a large amount of IFN-γ (Figure 4A). There are two pathways to activate T cells; direct activation *via* TCR ligation and indirect activation *via* macrophages stimulated by SMP-105. When lymph node cells from mice immunized with OK-432 were re-stimulated with OK-432 *in vitro*, a large amount of IFN-γ was released, whereas little was detected upon stimulation with SMP-105 (Figure 4C). If T cells from mice immunized with SMP-105 were only activated without antigen presentation, T cells from OK-432-immunized mice would also produce IFN-γ *via* macrophages stimulated with SMP-105. From these observations we consider that some portion of IFN-γ was attributable to T cells activated *via* TCR ligation.

Although proteinous antigens, except some living organisms (*18*), generally induce $CD4^+$ T cell activation, SMP-105 did not activate $CD4^+$ T cells (Figure 5). Furthermore, SMP-105 is a highly purified BCG-CWS containing only a small amount of amino acid residues not constituting peptidoglycan (1%, w/w) (*12*). There is a possibility that T cells releasing IFN-γ recognize non-proteinous antigens. In fact, there are papers reporting that $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells recognize mycobacterial lipid antigens (*18*).

This is one of the prominent features of SMP-105, when considering that there are no reports that synthetic TLR agonists, such as CpG ODN or imiquimod, function as antigens to induce IFN-γ or IL-2. T cells, even though not specific to tumors, will be able to boost the generation of tumor immunity through cell-to-cell interactions, *e.g.*, CD40L-CD40 and secreted cytokines, *e.g.*, IFN-γ (*19-21*). IL-2 activates other T cell populations, including precursor CTL to tumors. Multi-pathways for activating immune reactions may be necessary for patients suffering from malignant neoplasm in immuno-compromised conditions.

Hayashi *et al*. reported that transient IFN-γ in peripheral blood after inoculation of BCG-CWS was associated with survival (*10,11*). Our investigation demonstrated that IFN-γ was mainly produced by $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells in draining lymph nodes and suggested that transient IFN-γ in the blood was a marker indicating that lymph node functions to activate T cells were retained or retrieved. As for delayed-type skin inflammation, lymphocytes infiltrating tissues to which SMP-105 was distributed may include $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells. Delayed chronic inflammation therefore indicated immunophysiological states of the host, *e.g.*, draining lymph node retains or retrieves functions for differentiating T cells, and the immune system is not lost, at least at the site of chronic inflammation. In fact, skin inflammation was weak or not observed in guinea pigs, enabling the growth of implanted line 10 hepatoma. Analysis of the infiltrating lymphocytes is required.

In conclusion, oil-in-water emulsion of SMP-105 resided for a long time at the inoculation site and activated T cells, probably recognizing SMP-105 itself. The strong tumor-eliminating activity of SMP-105 may be explained by the boost of generating tumor immunity *via* positive feed-back from T cells reacting to it, in addition to direct activation of macrophages and dendritic cells. $CD4^-CD8^+\alpha\beta T$ and $CD4^-CD8^-\alpha\beta T$ cells may distinguish SMP-105 from other synthetic adjuvants. Further investigation is needed to clarify the mechanisms of T cell activation, including TCR-dependency, the antigen structures and presenting molecules, and then the contribution of T cells to the therapeutic effect.

Acknowledgement

We thank Ms. Erina Koga, Mr. Toshiyuki Sato and Ms. Yukari Ishitsubo for excellent technical assistance.

References

1. Heyn RM, Joo P, Karon M, Nesbit M, Shore N, Breslow N, Weiner J, Reed A, Hammond D. BCG in the treatment of acute lymphocytic leukemia. Blood 1975; 46:431-442.
2. Pines A. A 5-year controlled study of B.C.G. and radiotherapy inoperable lung cancer. Lancet 1976; 1:380-381.
3. Mavligit GM, Gutterman JU, Burgess MA, Khankhanian N, Seibert GB, Speer JF, Jubert AV, Martin RC, McBride CM, Copeland EM, Gehan EA, Hersh EM. Prolongation of postoperative disease-free interval and survival in human colorectal cancer by B.C.G. or B.C.G. plus 5-fluorouracil. Lancet 1976; 1:871-876.
4. Richman SP, Livingston RB, Gutterman JU, Suen JY, Hersh EM. Chemotherapy versus chemoimmunotherapy of head and neck cancer: report of a randomized study. Cancer Treat Rep 1976; 60:535-539.
5. Watanabe Y, Iwa T. Clinical value of immunotherapy with the streptococcal preparation OK-432 in non-small cell lung cancer. J Biol Response Mod 1987; 6:169-180.
6. Tsukagoshi S, Hashimoto Y, Fujii G, Kobayashi H, Nomoto K, Orita K. Krestin (PSK). Cancer Treat Rev 1984; 11:131-155.
7. Chen K, Huang J, Gong W, Iribarren P, Dunlop NM, Wang JM. Toll-like receptors in inflammation, infection and cancer. Int Immunopharmacol 2007; 7:1271-1285.
8. Paul S. Technology evaluation: CpG-7909, Coley. Curr Opin Mol Ther 2003; 5:553-559.
9. Garland SM. Imiquimod. Curr Opin Infect Dis 2003; 16:85-89.
10. Hayashi A. Interferon-γ as a marker for the effective cancer immunotherapy with BCG-cell wall skeleton. Proc Japan Acad 1994; 70 (Ser B):205-209.

11. Hayashi A, Doi O, Azuma I, Toyoshima K. Immuno-friendly use of BCG-cell wall skeleton remarkably improves the survival rate of various cancer patients. Proc Japan Acad 1998; 74 (Ser B):50-55.
12. Uenishi Y, Okada T, Okabe S, Sunagawa M. Study on the cell wall skeleton derived from Mycobacterium bovis BCG Tokyo 172 (SMP-105): establishment of preparation and analytical methods. Chem Pharm Bull (Tokyo) 2007; 55:843-852.
13. Rathanaswami P, Hachicha M, Sadick M, Schall TJ, McColl SR. Expression of the cytokine RANTES in human rheumatoid synovial fibroblasts. Differential regulation of RANTES and interleukin-8 genes by inflammatory cytokines. J Biol Chem 1993; 268:5834-5839.
14. Allison AC. Squalene and squalane emulsions as adjuvants. Methods 1999; 19:87-93.
15. Nelson PJ, Krensky AM. Chemokines, lymphocytes and viruses: what goes around, comes around. Curr Opin Immunol 1998; 10:265-270.
16. Ward SG, Bacon K, Westwick J. Chemokines and T lymphocytes: more than an attraction. Immunity 1998; 9:1-11.
17. Ward SG, Westwick J. Chemokines: understanding their role in T-lymphocyte biology. Biochem J 1998; 333 (Pt 3):457-470.
18. Kawashima T, Norose Y, Watanabe Y, Enomoto Y, Narazaki H, Watari E, Tanaka S, Takahashi H, Yano I, Brenner MB, Sugita M. Cutting edge: major CD8 T cell response to live bacillus Calmette-Guérin is mediated by CD1 molecules. J Immunol 2003; 170:5345-5348.
19. Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB. Recognition of a lipid antigen by CD1-restricted alpha beta^{+} T cells. Nature 1994; 372:691-694.
20. Yang Y, Wilson JM. CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. Science 1996; 273:1862-1864.
21. Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help *via* APC activation. J Exp Med 1996; 184:747-752.

(*Received March 9, 2008; Revised April 28, 2008; Accepted May 13, 2008*)