BRM cocktail treatment using PSK and OK432 significantly up-regulates the migration activity in human dendritic cells without losing effective CTL induction

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Abstract
One of the major factors for successful DCs immunotherapy is thought to be the maintenance of the migratory activity of matured DCs. We evaluated the effectiveness of PSK on OK432-activated DCs, in terms of maturation, migration and induction of CTLs. When DCs were treated with both OK432 and PSK, migration ability of the DCs were significantly high as compared to OK432 alone preserving the beneficial effect of OK432 treatment. BRM cocktail treatment with OK432 and PSK induced high level of migration activity in activated DCs, suggesting a potential protocol for more effective DCs immunotherapy for cancer.

Key Words: dendritic cell, OK432, PSK, migration activity, human

(Received December 5, 2010; Accepted December 13, 2010)

Introduction
Specific cancer immunotherapies using dendritic cells (DCs) for advanced human cancers are applied in many institutes1, 2), however patient outcomes are not yet acceptable3). In terms of the quality of the DC, it is important to obtain cells with good maturation, induction of cytotoxic T lymphocyte (CTL) and migration ability to take effective antitumor activity4-6).

OK432, known as one of the good manufacturing practice (GMP) grade biological response modifiers (BRMs), functions as an immune activator through the mechanism of multi-cytokine induction7-9) and maturation of DCs10-12). OK432 is widely applied for cancer treatment, mainly in Japan, and is also thought to have a negative effect on the migration ability in matured DCs13, 14).

PSK, protein-bound polysaccharide preparation, is also known as a GMP grade immune modulator. It is reported that PSK reduces or ameliorates the depression of immune-competence in cancer bearing patients15-18). PSK probably modifies DC activity, but its functions on DCs have not been clarified19-21).

In this report we investigated the mechanisms behind OK432 and PSK in immune modulation targeting to DC modifications. We revealed that OK432 and PSK had a crucial role on DCs, especially in a cocktail treatment of OK432 and PSK that significantly up-regulated the activity of migration of DCs without losing the effective tumor specific CTL response.

Materials and Methods

1. Reagents
OK432 was purchased from Chugai Pharmaceutical Co. (Tokyo, Japan), PSK from Sankyo (Tokyo, Japan), Ficoll-Conray from IBL (Fujioka, Gunma, Japan), Hanks balanced salt solution and phosphate buffered saline (PBS) from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan), and serum-free medium AIM-V from Invitrogen (Carlsbad, CA, USA). Cytokines that were used are as follows; recombinant human (rh) IL-4 and TNF-α (BD Biosciences, Bedford, MA, USA), rh GM-CSF (BD Pharmingen, San Diego, CA, USA) and rh IL-2 (Takeda chemical industries Ltd, Osaka, Japan).

2. Samples
The blood samples were obtained from 3 healthy volunteers (2 males, 1 female) and 4 patients with cancer (3: pancreatic carcinoma, 1: carcinoma of the bile duct).
3. Generation of DCs from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were separated from leukapheresis products (22) or peripheral blood by means of a standardized Ficoll-Conray density gradient centrifugation. After washing with Hanks balanced salt solution, PBMCs were plated onto 6-well culture plates (Greiner Bio-one, Frickenhausen, Germany) and cultured at 37°C in a humidified atmosphere in 5% CO2 for 2 hr. After floating cells were removed, adherent cells were harvested and plated onto 6-well culture plates at a concentration of 1 × 10^6 cells/well. Cells were then cultured for 6 days at 37°C in 5% CO2 in AIM-V supplemented with 50 ng/ml rh IL-4 and 50 ng/ml rh GM-CSF (23).

4. Activation of immature DCs

After cultivation, cells were harvested (immature DCs) and pulsed with peptides (MUC1 or CEA) for 12 hr. Thereafter, cells were washed with PBS and further cultured for 24 or 48 hr in AIM-V containing 50 ng/ml rh IL-4 and 50 ng/ml rh GM-CSF on 12-well culture plates (Becton Dickinson, Franklin Lakes, NJ, USA). To induce maturation, 0.1 KE/ml of OK432 alone (OK-DC), which was considered to be optimal from our previous study (24), 10-100 μg/ml of PSK alone (PSK-DC), 0.1 KE/ml of OK432 with 10-100 μg/ml of PSK (OK-PSK-DC), or 100 ng/ml of TNF-α (TNF-DC) was concomitantly added to the culture. 48 hours after stimulation, cells were harvested and analyzed.

5. Flow cytometric analysis

Phenotypic characterization of DCs were carried out by flow cytometry using FACS Caliber (BD Biosciences) and analysis was performed with CELL Quest ver. 3.1 (BD Biosciences). Cells (5 × 10^6) were incubated with specific antibodies or isotype-matched control mouse IgG in PBS containing 0.2% BSA for 30 min at 4°C. After being rinsed twice, cells were resuspended with 0.1% paraformaldehyde and examined by flow cytometry using FACS Caliber. The numbers of migrating DCs were calculated from the number of all migrating cells and the percentages of the cells positive for both CD11c and CD86.

6. In vitro chemotaxis assay

The in vitro chemotaxis assay was performed as described previously but with minor modifications (6). In brief, recombinant chemokine (rhMIP-3/β/CCL19), purchased from Genzyme/TECHNE (Minneapolis, MN), was diluted to appropriate concentrations with the serum-free assay medium to a final volume of 600 μl and applied to 24-well tissue culture plates (Corning Costar, Corning, NY, USA). Transwell culture inserts (Corning Costar, Corning, NY, USA) of 6.5 mm diameter with 5.0-μm pores were inserted into each well, and DCs (5.0 × 10^5 cells/well) were added to the top chamber in assay medium at a final volume of 100 μl. The plates were incubated for 4 hr and then the cells in the bottom chambers were recovered. Harvested cells were counted and an aliquot was stained with PE-conjugated anti-CD11c and FITC-conjugated anti-CD86 mAbs for analysis by FACS Caliber. The numbers of migrating cells were calculated from the number of all migrating cells and the percentages of the cells positive for both CD11c and CD86.

7. CTL induction

Mature DCs were pulsed with CEA or MUC1 peptide and were matured by 0.1 KE/ml of OK432 alone, 100 μg/ml of PSK alone, 0.1 KE/ml of OK432 and 100 μg/ml of PSK, or 100 ng/ml of TNF-α for 48 hr. Fresh PBMCs were obtained and resuspended in AIM-V at 5 × 10^5 cells/well on 24-well culture plates and were then co-cultured with 2 × 10^5 cells/well of OK-DC, PSK-DC, OK-PSK-DC or TNF-DC for once a week twice. The cultures were supplemented with 50 IU/ml rh IL-2 and further cultured. After cultivation, cells were collected as CTL (25).

8. CTL assay (LDH releasing assay)

Mature DCs and PBMCs were collected from a HLA A-24 healthy volunteer and DCs were pulsed with CEA peptide (HLA-A-24 restricted). The human gastric cancer cell line, MKN-45 (HLA-A-24, high level of CEA expressed, Riken Cell Bank), was used as target cells. Effector and target cells were plated onto v-bottomed 96-well microplates (Corning, Corning, NY, USA) at effector: target (E:T) ratios of 50, 25 or 12.5 : 1 and incubated for 4 hours at 37°C under 5% CO2. Cytotoxic activity was measured by release of LDH using CytoTox-OneTM (Promega, WI, USA) according to the manufacturer’s instructions. The percentage of specific lysis was determined according to the formula below:

\[
\frac{((\text{Experimental release}) - (\text{Spontaneous release}))}{((\text{Maximum release}) - (\text{Spontaneous release}))} \times 100 \% 
\]

(1) (Spontaneous release) was represented by measurements of the supernatant of target cells alone. Maximum release was measured after complete lysis with 9% Triton X.)

9. CD107a mobilization assay

As with the CTL detection assay, we examined cytotoxicity using the CD107a mobilization assay (26-28). When T-cells and NK-cells recognize target cells, the content of the lytic granules are secreted by degranulation. As a result of degradation, CD107 present on the cell surface leads to indirect evaluation of the secretion of the contents of lytic granules such as perforin, granzymes and granulysin, which enables rapid assess-
ment of cell-mediated cytotoxicity and detection of antigen specific cytotoxic T-cells.

In this method, the cells are cultured in the presence of CD107a monoclonal antibody after antigen stimulation, which enables sensitive detection of CD107a. CD107a mobilization assay using IMMUNOCYTO CD107a Detection Kit (MBL, Japan) was the method used for the detection of CD107a exposed on the cell surface after antigen stimulation.

The protocol outline is described in the product description. In brief, PBMCs were co-cultured with MUC1 (HLA-non-restricted peptide)-pulsed DCs. 3 × 10^5 cells/well were added to each well of a 96-well round microplate. MUC1 peptide 0.2 mg/ml (or medium) and FITC-labeled CD107a monoclonal antibody (or FITC-labeled mouse IgG1 isotype control) was added to each well and incubated for 4 hours in a 5% CO2 incubator at 37°C. After incubation, PE labeled CD8 was added and cells were analyzed by flow cytometry.

10. Quantification of cytokine production

Mature DCs were cultured on a 12-well microplate at a concentration of 2 × 10^5 cells/ml for 48 hr with 0.1 KE/ml of OK432 alone, 10-100 μg/ml of PSK alone, 0.1 KE/ml of OK432 and 10-100 μg/ml of PSK, or 100 ng/ml of TNF-α. After cultivation, the supernatants were collected and stored at −80°C until assayed. The concentration of IL-12 and IFN-γ were measured with corresponding human enzyme linked immunosorbent assay (ELISA) kits (BioSource Europe S.A., Nivelles, Belgium) according to the manufacturer’s instructions.

11. Statistical analysis

Statistical analysis was performed with SPSS v. 16.0 software (SPSS Inc.). To evaluate the correlations between two variables, linear regression analysis was performed. Statistical differences between two unpaired groups were evaluated by using the Mann-Whitney U-test. Statistical differences between two corresponding groups were evaluated by using Wilcoxon’s signed rank test. A p value of less than 0.05 was considered to be statistically significant.

Results

1. Expression of CCR7 is significantly increased in OK-PSK-DC.

The expression of cell surface markers was determined by flow cytometry. Immature DCs were stimulated by OK432, PSK, OK432 and PSK, or TNF-α for 48 hr, then cell surface expression was examined. The expression levels of surface markers were measured by mean fluorescence intensity (MFI) on PSK-DCs, OK-PSK-DCs, TNF-DCs or non-stimulated DCs. The expression of HLA-DR, CD83 and CD86 of OK-DCs were significantly higher than that of PSK-DCs, TNF or non-stimulated DCs and there was no significant difference between OK-DCs and OK-PSK-DCs (Fig. 1A, 1B).

Flow cytometric analysis of CCR7, which is one of the major indicators of migration ability, was also performed. The expression of CCR7 on PSK-DCs was gradually increased in a dose dependent manner with PSK. In the presence of OK432, potentiation of the expression of CCR7 in OK-PSK-DCs was less marked, but reached statistical significance at 100 μg/ml (Fig. 2).

2. OK-PSK-DCs showed high potency for MIP3β inducing migration.

To assess the migration ability of DCs treated by BRM and TNF-α, we performed a MIP3β inducing migration assay. The number of migrated cells were significantly increased in OK-PSK-DCs compared to non-treated DCs. The migration ability of OK-PSK-DCs tended to be high compared with OK- or TNF-DCs, although the difference did not reach statistical significance (Fig. 3).

3. OK-PSK-DC induced strong antigen specific CTL activity.

OK-DC-induced CTL showed strong cytotoxicity in the LDH cytotoxic assay targeting MKN45. OK-PSK-DC-induced CTL also showed comparable, although less marked, cytotoxicity to MKN45 (Fig. 4A). When CD107a mobilization of CD8+ cells was assessed using MUC-1 stimulation, OK-PSK-DCs showed potent activation of CTL compared with OK-DCs (Fig. 4B). These results indicated that additional PSK treatment of DCs together with OK-432 did not hamper generation of CTL which were augmented by OK-432 treatment.

4. OK-PSK-DCs produced high levels of Th1 cytokines.

We examined the production of Th1 type cytokines (IL-12 and IFN-γ) in DCs. DCs were induced by PBMCs obtained from healthy volunteers and patients with cancer and were pulsed with peptides then treated with OK432, PSK, OK-PSK or TNF-α. OK-DC produced significantly higher levels of IL-12 and IFN-γ compared with TNF-α or PSK. OK-PSK-DCs also produced high levels of IL-12 and IFN-γ (Fig. 5).

Discussion

Recent developments in molecular biology have enabled us to apply basic tumor immunology to a clinical setting, such as a cancer vaccine. One of the most promising approaches to cancer immunotherapy is the administration of antigen-presenting cells such as DCs loaded with tumor-associated antigens. DCs are one of the major antigen-presenting cells that regulate many steps of the immune system. Numerous kinds of DC immu-
Fig 1. Flow cytometric analysis of cell surface molecules on matured DC are shown.
A: The expression of HLA-DR, CD83 and CD86 in OK-DCs were significantly higher than that in PSK-DCs, TNF or non-stimulated DCs, and there was no significant difference between OK-DCs and OK-PSK- treated DCs. 
B: Average of the mean fluorescence intensity (MFI) values for each maturity marker are shown (Mean ± SD). Expression of the examined cell surface phenotypes was increased either on the surface of OK- or OK-PSK-treated DCs. No significant difference in the intensity of expression of cell surface phenotypes was noted between OK- and OK-PSK- treated DCs.
Fig 2. Flow cytometric analysis of CCR7 on matured DCs.
A: Expression level peaks of CCR7 on PSK-DCs and OK-PSK-DCs shifted to the right compared with those on TNF- and OK-DCs.
B: Average MFI values for CCR7 are shown. Expression of CCR7 was increased on the surface of PSK- and OK-PSK-DCs following a dose dependent exposure to PSK. Significant difference was seen between OK-DCs and PSK (100 μg/ml)-DCs, and between OK-DCs and OK-PSK (100 μg/ml)-DCs. *p = 0.027, **p = 0.034.

Fig 3. To assess the migration ability of DCs treated by BRM and TNF-α, we performed a MIP3β inducing migration assay. In vitro chemotaxis assay using MIP-3β, a CCR7 ligand, is shown. The number of migrated cells was significantly increased in OK-PSK-DCs as compared to non-treated DCs. The migration ability of OK-PSK-DCs tended to be high compared with OK- or TNF-DCs, although the difference did not reach statistical significance. *p = 0.034.

Fig 4. CTL induction ability of DCs was evaluated. Mature DCs were pulsed with CEA or MUC1 peptide and were matured by OK432 alone, 100 μg/ml of PSK alone, OK432 and 100 μg/ml of PSK, or TNF-α for 48 hr. Fresh PBMCs were obtained and were then co-cultured with OK-DCs, PSK-DCs, OK-PSK-DCs or TNF-DCs for once a week twice. After cultivation, cells were collected as CTL.
A: CTL cytotoxic activity against human gastric cancer cell line MKN-45 (HLA-A-24, high level of CEA expressed) using LDH releasing assay was examined. Mature DCs and PBMCs were collected from a HLA A-24 healthy volunteer, and DCs were pulsed with CEA peptide (HLA-A-24 restricted). OK-PSK-DC-induced CTL also showed comparable, although less marked cytotoxicity to MKN45.
B: As another CTL detection method, we examined using the CD107a mobilization assay. PBMCs which were co-cultured with MUC1 (HLA-non-restricted peptide)-pulsed DCs (using various stimulus), were exposed to MUC1 peptide and FITC labeled CD107a monoclonal antibody and incubated. After incubation PE labeled CD8 was added to the cells, that were then subsequently analysed using flow cytometry. OK-PSK-DCs showed potent activation of CTL as comparable with OK-DCs. These results indicated that additional PSK treatment of DC together with OK-432 did not hamper generation of CTL which was augmented by OK-432 treatment.
Annals of Cancer Research and Therapy Vol. 19 No. 1, 2011

immunotherapy are currently being used around the world for treatment of advanced solid tumors. However, a recent review of DC immunotherapy has shown disappointing data revealing unacceptably low response rates (e.g., 7.1%). To obtain an effective clinical response to DC immunotherapy, several reports have suggested that three major factors; DC maturation, DC migration and effective CTL induction, are very important. Two major phases occur in DCs due to a difference in maturation, namely the immature and mature phase. Basically, immature DCs have good migration ability to regional lymph nodes, whereas mature DC do not. On the contrary, matured DCs have high ability of effective CTL induction, whereas immature DCs do not. To date, there has been no effective protocol to maintain the migration ability of DCs with good maturation and capacity of CTL induction. In this report, we have clearly shown that highly matured DCs can be induced that maintain migratory ability and effective CTL induction by using a combination of clinical grade BRMs; OK432 and PSK.

OK432 is a biological preparation which is obtained from penicillin treated type A Streptococcus haemolyticus and is known as strong immuno-potentiating agent. OK432 has been marketed in Japan for more than 20 years and is applied for several types of cancer therapy, mainly in an adjuvant setting. OK432 is known as a biological response modifier via the induction of multiple cytokines, promoting the production of inflammatory cytokines and activating natural killer (NK) cells or macrophages. OK432 is able to induce the production of typical T-helper (Th) 1 type cytokines, such as IL-12, IFN-γ, and change the Th1 / Th2 balance in a state of Th1 dominance. It can mobilize the expression of many surface molecules present in DCs, for instance HLA class II, CD40, CD86, ICAM-1 and so on. OK432 also positively affects the proliferation of DCs and antiviral CTL responses in vitro. We and others also have reported that these effects of OK432 on DCs were superior to TNF-α and depend on the signal pathway linked to the Toll-like receptor (TLR). Thus, OK432 probably have a positive effect on the maturation of human DCs. However, several reports have mentioned the negative effect of OK432 on DC migration. Satoh et al. have reported that prostaglandin E2 (PGE2) may up-regulate the migration ability of DCs. Although they did not mention the CTL response in PGE2 treated DCs, encouraging data demonstrated that PGE2 may up-regulate the migration ability in mature DCs. Recently, a novel mechanism for migration was revealed. Migration of macrophages was up-regulated through the binding of β-Glucan to the DECTIN-1 receptor or through the TLR2 pathway. PSK, a clinical grade BRM, is also known as β-Glucan in oral medication. Therefore, to restore the ability of migration for functionally effective CTL inducible mature DCs, we focused on the activity of PSK.

PSK is extracted from the CM101 strain of the Kawaratake mushroom and underwent insurance adaptation as an anti-neoplastic medicine having non-specific immune activator activity in 1976. PSK was reported to demonstrate clinical benefits in the field of gastric cancer, colonic cancer and small cell lung cancer. Immunologically, PSK induces lymphocyte proliferation, NK activity, LAK activity and cytokine production. Evidence concerning the effect of PSK on DCs is also accumulating. It has been shown that PSK induces the maturation of DCs in both mice and humans. After considering these studies, we hypothesized that PSK could be a candidate for a possible modifier of DC migration through the β-Glucan - DECTIN-1 receptor or the TLR2 pathway. Although PSK alone failed to potentiate maturation, CTL induction and Th1 cytokine production in DCs, the expression of CCR7 was enhanced significantly as compared with OK432 treatment alone. Even in the presence of OK432, this beneficial effect was maintained. By contrast, the number of migrated cells was not increased by PSK alone. This may be explained.
by insufficient maturation of DCs with PSK alone. We have found that a combined treatment of OK432 with PSK (we named this a BRM cocktail) creates a highly matured DC phenotype with good migration ability. We have also shown that the "BRM cocktail" induces functional DCs with a high ability of CTL induction using relatively novel technique; a CD107 cytotoxicity assay for the detection of cytokotic CD8+ cells.

The dose of PSK is another important issue for use in a clinical setting. Applying the recommended dose of PSK for oral administration (3.0 g/day), the estimated concentration of PSK in human blood is 105 μg/ml\(^{59}\). Out in vitro study indicated that PSK could induce the migration at a dose of 10-100 μg/ml. In view of these findings, the effect of PSK on DCs found in this study may be useful for application to into clinical use. PSK has few toxic effects and is clinically tolerable\(^{60}\). Both OK432 and PSK, which we used in this study, are clinical grade BRMs and can be easily used for cancer therapy, at least in Japan. Our interesting results on DC maturation and migration with CTL induction need to be applied in clinical immunotherapy, not only in systemic but also in local DC therapy. Consideration needs to be given to clinical trials of immunotherapy using the "BRM cocktail".

Acknowledgements

We thank Mr. Hironori Igaragi and Ms. Yuko Sato (Department of Surgery I, Fukushima Medical University) for their skillful technical assistance.

References

1) Thurner, B., Haendle, I., Röder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von den Driesch, P., Bröcker, E.B., Steinman, R.M., Enk, A., Kämpgen, E., Schuler, G. (1999) Vaccination with mage-3A1 peptide-pulsed matura, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J Exp Med 190:1669-78.

2) Stift, A., Friedl, J., Dubsky, P., Bachleitner-Hofmann, T., Schueller, G., Zontisch, T., Benkoe, T., Radelbauer, K., Brostjan, C., Jakesz, R., Gnant, M. (2003) Dendritic cell-based vaccination in solid cancer. J Clin Oncol 21:135-42.

3) Rosenberg, S.A., Yang, J.C., Restifo, N.P. (2004) Cancer immunotherapy: moving beyond current vaccines. Nat Med 10:909-15.

4) Fong, L., Engleman, E.G. (2000) Dendritic cells in cancer immunotherapy. Annu Rev Immunol 18:245-73.

5) Ridge, J.P., Rosa, F.D., Matzinger, P. (1998) A conditioned den -

6) Nakazato, H., Koike, A., Saji, S., Ogawa, N., Sakamoto, J. (1994) Efficacy of immunomodulation as adjuvant treat-
men after curative resection of gastric cancer. Study Group of Immunomodulatorok with PSK for Gastric Cancer. The Lancet 343:1122-1126.

7) Ito, K., Nakazato, H., Koike, A., Takagi, H., Saji, S., Baba, S., Mai, M., Sakamoto, J., Ohashi, Y.; Study Group of Immunomodulatorok with PSK for Colon Cancer. (2004) Long-term effect of 5-flourouracil enhanced by intermittent administra-
tion of polysaccharide K after curative resection of colon cancer. A randomized controlled trial for 7-year follow-up. Int J Colorectal Dis 19:157-164.

8) Konno, K., Motomiya, M., Otsumi, K., Sato, M., Yamamoto, F., Tamiya, K., Hasuiki, T., Yokozawa, A., Ushiyama, T., Ogawa, N., Nakai, Y. (1998) Effects of Protein-Bound Polysaccharide Preparation (PSK) in Small Cell Carcinoma of the Lung. JLC 28:19-28.

9) Ogihara, T., Inuma, H., Okinaga, K. (2004) Usefulness of immu-
nomodulators for maturation of dendritic cells. Int J Oncol 25:453-9.

10) Okuzawa, M., Shinohara, H., Kobayashi, T., Iwamoto, M., Toyoda, M., Tanigawa, N. (2002) PSK, a protein-bound polysaccharide, overcomes defective maturation of dendritic cells exposed to tumor-derived factors in vitro. Int J Oncol 20:1189-95.

11) Kanazawa, M., Moroi, T., Yosihira, K., Iwadate, M., Suzuki, S., Endoh, Y., Okhi, S., Takita, K., Sekikawa, K., Takeshita, S. (2004) Effect of PSK on the maturation of dendritic cells derived from human peripheral blood monocytes. Immunol Lett 91:229-38.

12) Thurner, B., Röder, C., Dieckmann, D., Heuer, M., Kruse, M., Glaser, A., Keikavoussi, P., Kämpgen, E., Bender, A., Schuler, G. (1999) Generation of large numbers of fully mature and stable den-
dritic cells from leukapheresis products for clinical application. J Immunol Methods 223:1-15.

13) Romani, N., Gruner, S., Brang, D., Kämpgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P.O., Steinman, R.M., Schuler, G. (1994) Proliferating dendritic cell progenitors in human blood. J Exp Med 180:83-93.

14) Kanzaki, N., Terashima, M., Kashimura, S., Hoshino, M., Ohtani,
S., Matsuyama, S., Hoshino, Y., Kogure, M., Oshibe, I., Endo, H., Saito, T., Yaginuma, H., Gotoh, M., Ohno, H. (2005) Understanding the response of dendritic cells to activation by streptococcal preparation OK-432. Anticancer Res 25:4231-8.

25) Celis, E., Tsai, V., Crimi, C., DeMars, R., Wentworth, P.A., Chesnut, R.W., Grey, H.M., Sette, A., Serra, H.M. (1994) Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes. Proc Natl Acad Sci USA 91:2105-2109.

26) Mittendorf, E.A., Storrer, C.E., Shriver, C.D., Ponniah, S., Peoples, G.E. (2005) Evaluation of the CD107 cytotoxicity assay for the detection of cytolytic CD8+ cells recognizing HER2/neu vaccine peptides. Breast Cancer Research and Treatment 92:85-93.

27) Rubio, V., Stuge, T.B., Singh, N., Betts, M.R., Weber, J.S., Roederer, M., Lee, P.P. (2003) Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. Nat Med 9:1377-82.

28) Betts, M.R., Brenchley, J.M., Price, D.A., De Rosa, S.C., Douek, D.C., Roederer, M., Koup, R.A. (2003) Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods 281:65-78.

29) Steinman, R.M. (1991) The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 9:271-96.

30) Inaba, K., Steinman, R.M., Pack, M.W., Aya, H., Inaba, M., Sudo, T., Wolpe, S., Schuler, G. (1992) Identification of proliferating dendritic cell precursors in mouse blood. J Exp Med 175:1157-67.

31) Dieu, M.C., Vanbervliet, B., Vicari, A., Bridon, J.M., Oldham, E., Aït-Yahia, S., Brière, F., Zlotnik, A., Lebecque, S., Caux, C. (1998) Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in deferent anatomic site. J Exp Med 188:373-86.

32) Dhodapkar, M.V., Steinman, R.M., Krasovsky, J., Munz, C., Bhardwaj, N. (2001) Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med 193:233-8.

33) Okamoto, H., Shii, J., Mion, S., Koshimura, S., Shimizu, R. (1967) Studies on the anticancer and streptolysin S-forming abilities of hemolytic streptococci. Jpn J Microbiol 11:323-36.

34) Okamoto, M., Oshikawa, T., Ohe, G., Nishikawa, H., Furuichi, S., Tano, T., Mori, Y., Saito, M., Sato, M. (2001) Severe impairment of anti-cancer effect of lipoteichoic acid-related molecule isolated from a penicillin-killed Streptococcus pyogenes in toll-like receptor 4-deficient mice. Int Immunopharmacol 1:1789-1795.

35) Yadav, M., Schoeber, J.S. (2006) The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. Blood. 108:3168-75.

36) Tsukagoshi, S., Hashimoto, Y., Fujii, G., Kobayashi, H., Nomoto, K., Orita, K. (1984) Krestin (PSK). Cancer Treat Rev 11:131-55.

37) Kariya, Y., Okamoto, N., Fujimoto, T., Inoue, N., Kihara, T., Sugie, K., Yagita, M., Kanzaki, H., Mori, T., Uchida, A. (1991) Lysis of fresh human tumor cells by autologous peripheral blood lymphocytes and tumor-infiltrating lymphocytes activated by PSK. Jpn J Cancer Res 82:1044-1050.

38) Hirose, K., Zachariae, C.O., Oppenheim, J.J., Matsushima, K. (1990) Induction of gene expression and production of immunomodulating cytokines by PSK in human peripheral blood mononuclear cells. Lymphokine Res 9:475-483.

39) Freireich, E.J., Gehan, E.A., Rall, D.P., Schmidt, L.H., Skipper, H.E. (1966) Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. Cancer Chemother Rep 50:219-44.