



# Enhancement of IFN- $\gamma$ production for Th1-cell therapy using negatively charged liposomes containing phosphatidylserine

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## Abstract

We have developed an efficient method of interferon- $\gamma$  (IFN- $\gamma$ ) induction for Th1-cell therapy. OVA (ovalbumin)-specific Th1 clone 42-6A cells cocultured with antigen presenting cells (APCs) from spleen resulted in high levels of OVA-specific IFN- $\gamma$  production by the treatment of phosphatidylserine (PS), but not phosphatidic acid (PA), liposomes-encapsulated OVA (OVA-liposomes). The IFN- $\gamma$  production was increased in a manner dependent on the PS content of the liposomes and inhibited by the addition of annexin V, a PS binding protein. Furthermore, coadministration of Th1 cells plus OVA-liposomes in mice strikingly enhanced IFN- $\gamma$  levels in serum and in spleen cells compared with that of Th1 cells plus OVA. In addition, serum levels of IL-12 p70 increased and ongoing OVA-specific IgE immune response was dramatically attenuated. These results first suggest that antigen delivery using negatively charged liposomes containing PS is very useful for the enhancement of IFN- $\gamma$  production in Th1-cell therapy.

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**Keywords:** Negatively charged liposome; Phosphatidylserine; Antigens; IFN- $\gamma$ ; Th1-cell therapy

## 1. Introduction

Liposomes are potentially useful as drug carriers to deliver pharmacologically active agents into cells [1]. Many investigators have used liposomes as an immunoadjuvant and targeting agents for delivery of antigens to macrophages and dendritic cells (M $\phi$ /DCs), which are antigen-presenting cells (APCs). However, the immunoadjuvant activity of liposomes against tumor, infection and allergy is not sufficient and need to be improved.

CD4<sup>+</sup> Th cells have been subdivided into at least two subsets on the basis of their patterns of cytokine secretion. Th1 cells produce Interleukin (IL)-2 and IFN- $\gamma$ , whereas, Th2 cells produce IL-4, IL-5 and IL-13 [2]. Production of IL-2 and IFN- $\gamma$  is associated with resistance to intracellular pathogens and tumor rejection. In contrast, the production of IL-4, IL-

5 and IL-13 is associated with humoral immunity and the development of allergic diseases [3–7].

Adoptive immunotherapy using antigen-specific T cells (T-cell therapy) has been expected as a potential strategy for immunotherapy against tumor and allergy and T cells facilitate the induction of the immune responses against tumor and allergy through upregulation of IFN- $\gamma$ -mediated signaling. [8–10]. However, its application to immunotherapy has been hampered by difficulties in activating antigen-specific T cells in vitro and in vivo. We recently reported that liposomes encapsulating antigens results in high levels of IFN- $\gamma$  production in the coculture of antigen-specific Th1 clone cells with APCs, such as M $\phi$ /DCs in vitro [11].

In this study, we determined whether negatively charged liposomes encapsulating antigens effectively induce IFN- $\gamma$  production in Th1 cells in vitro and in vivo. Our results suggest that antigen delivery using negatively charged liposomes containing PS is useful for the enhancement of IFN- $\gamma$  induction in Th1-cell therapy. We point out the importance of efficient antigen delivery system for T-cell therapy.

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## 2. Materials and methods

### 2.1. Mice

Balb/c mice were purchased from Japan SLC Co. Ltd. (Shizuoka, Japan). All mice were between 6 and 10 weeks of age. Animal use and relevant experimental procedures were approved by the Tokyo University of Pharmacy and Life Science Committee on the care and use of laboratory animals.

### 2.2. Monoclonal antibody (mAb) and reagents

Murine recombinant IL-2 was obtained from Genzyme (Cambridge, MA). Egg phosphatidylcholine (PC) was obtained from Nippon Oil and Fat Co. Ltd. (Tokyo, Japan). Phosphatidylserine (PS) from bovine brain and phosphatidic acid (PA) from egg yolk, ovalbumin (OVA), conalbumin (COA), were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO). Cholesterol (Chol) was purchased from Wako Pure Chemicals (Osaka, Japan). Bovine serum albumin (BSA) was purchased from Boehringer Mannheim (GmbH, Germany).

### 2.3. Preparation of liposomes

Liposomes were prepared using a dehydration–rehydration method. Briefly, 5  $\mu$ mol of total lipids (PC:PS:Chol at 1:1:2 or PC:PA:Chol at 1:1:2 mol ratios) were dried in a test tube under N<sub>2</sub> gas and desiccated under vacuum for at least 2 h. The dry lipid films were hydrated by the addition of 1 ml of water containing 300  $\mu$ g OVA, COA or BSA. The mixture was vortexed at room temperature for 5 min and sonicated to form small vesicles. The small vesicles were then frozen and lyophilized overnight, then rehydrated by the addition of 0.05 ml water. The mixture was vortexed at room temperature for 5 min and then 0.95 ml of 0.9% NaCl solution was added. Non-encapsulating OVA, COA or BSA was removed by centrifugation (15,000 rpm, 10 min, three times), and the liposomes encapsulating OVA, COA or BSA were suspended in saline. Liposomes were passed through a membrane filter (0.45  $\mu$ m; Corning Glassworks, Corning, NY). The amount of encapsulated OVA, COA or BSA in liposomes was determined by a Micro BCA assay kit (Pierce Co., Ltd, Rockford, IL) following the solubilization of liposomes with 0.1% SDS. The encapsulation efficiency is  $79 \pm 2.8\%$ .

### 2.4. Preparation of splenic adherent cells (SAC)

Spleen cells from Balb/c mice were incubated in 100 mm-plastic dishes (Iwaki glass, Chiba, Japan) for 90 min at 37 °C in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 0.28% NaHCO<sub>3</sub>, 10 mM HEPES, 50  $\mu$ M 2-mercaptoethanol, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (complete medium). Non-adherent cells were removed by washing the

dishes five times with ice-cold PBS. The SAC were recovered with 0.5% lidocaine-PBS (Astrazeneca Co., Ltd, Osaka, Japan). SAC were washed twice and resuspended in complete medium. In this study, SAC were used as APCs for direct antigen presentation to Th1 cells in vitro.

### 2.5. Th1 clone cells

OVA-specific Th1 clone 42-6A cells were established from Balb/c mice immunized with OVA, as described previously [12]. 42-6A cells were maintained in complete medium supplemented with 10 U/ml murine recombinant IL-2.

### 2.6. Liposome treatment of cells

42-6A cells ( $4 \times 10^4$  cells/well in 96 well culture plate) were incubated with SAC ( $4 \times 10^4$  cells/well in 96 well culture plate) in the presence of liposomes encapsulating OVA (10  $\mu$ g/ml) in 0.2 ml of complete medium in humidified 5% CO<sub>2</sub> at 37 °C for 18 h. The cells and culture supernatants were harvested.

### 2.7. Determination of murine IFN- $\gamma$ , IL-4 and IL-12 p70

The levels of IFN- $\gamma$ , IL-4 and IL-12 p70 in the cell-free supernatants and serum were routinely assayed by sandwich ELISA using pairs of purified capture and biotinylated detection mAb recognizing murine IFN- $\gamma$ , IL-4 and IL-12 p70.

### 2.8. Fluorescence labeling of cells

42-6A cells were washed twice with serum-free medium before staining. The cells were then resuspended in a loading buffer (an aqueous, osmolarity-regulating solution containing no Ca<sup>2+</sup> or other physiological salts; Sigma, St. Louis, MO) and incubated for 5 min with freshly prepared 2  $\mu$ M PKH26 (Sigma) at room temperature. The staining reaction was stopped by the addition of FCS. After centrifugation, the cell was transferred to a fresh 15 ml tube and washed twice with complete medium and twice with ice-cold PBS. The cells were then resuspended in ice-cold PBS.

### 2.9. OVA-specific IgE Ab levels

Blood samples from mice were collected 4 weeks after OVA-alum immunization. A doubling dilution of mouse serum were poured into an OVA-coated ELISA plate, and left overnight to confirm that the immune reaction took place. After four washes with PBS containing 0.05% Tween-20 (PBS-T), biotin-conjugated anti-mouse IgE monoclonal antibody (PharMingen), the plate was left to stand at 4 °C overnight. After further washing with PBS-T, avidin-conjugated peroxidase was added and the absorbance at 450 nm was determined.

### 3. Results

#### 3.1. Negatively charged liposomes containing PS, but not PA, effectively induced antigen-specific IFN- $\gamma$ production

PS and phosphatidic acid (PA) are major negatively charge phospholipids in the plasma membrane and negative charged liposomes containing PS or PA (PS- or PA-liposomes) are preferentially taken up by M $\phi$ . Thus, we first investigated the effect of PS- or PA-liposomes on the production of IFN- $\gamma$ . OVA-specific Th1 clone 42-6A cells were incubated with SAC in the presence of PS- or PA-liposomes encapsulating OVA (10  $\mu$ g/ml). Supernatants were collected after 18 h and assayed for IFN- $\gamma$  production by ELISA. As shown in Fig. 1A, high levels of IFN- $\gamma$  production were observed in the treatment of the cells with PS-liposomes, but not with PA-liposomes. Next, to investigate whether IFN-

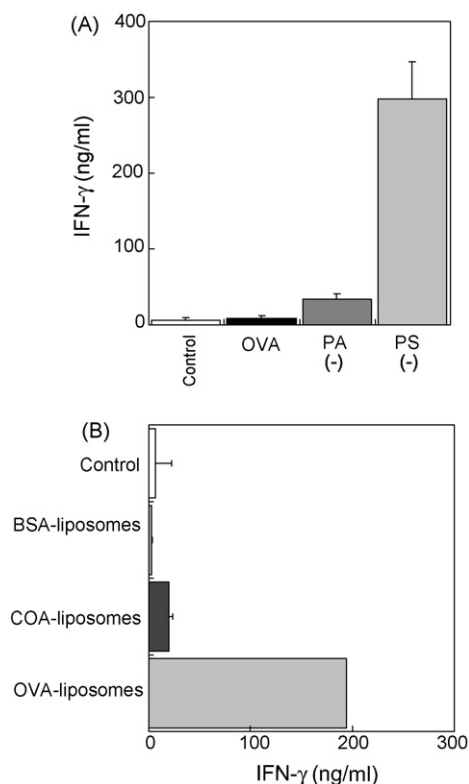


Fig. 1. (A) Effect of lipid composition of liposomes on IFN- $\gamma$  production. Lipid compositions of liposomes were PS:PC:Chol = 1:1:2 (PS-liposomes, negatively charged liposomes) and PA:PC:Chol = 1:1:2 (PA-liposomes, negatively charged liposomes) (mol ratio), respectively. OVA-specific Th1 clone 42-6A cells ( $4 \times 10^4$ /well) were incubated with SAC ( $4 \times 10^4$ /well) in the presence of each liposome encapsulating OVA (10  $\mu$ g/ml) or OVA alone (10  $\mu$ g/ml) for 18 h. (B) Antigen specificity of IFN- $\gamma$  production. 42-6A cells ( $4 \times 10^4$ /well) were incubated with SAC ( $4 \times 10^4$ /well) in the presence of PS-liposomes encapsulating OVA, BSA or COA (10  $\mu$ g/ml) (OVA-liposomes, BSA-liposomes or COA-liposomes) for 18 h. IFN- $\gamma$  productions in the supernatants were evaluated by ELISA, as described in Section 2. The values are the means  $\pm$  S.D. of triplicate cultures from three independent experiments.

$\gamma$  production by PS-liposomes shows antigen specificity, Th1 cells were incubated with SAC in the presence of PS-liposomes encapsulating OVA, bovine serum albumin (BSA) or conalbumin (COA) (OVA-liposomes, BSA-liposomes or COA-liposomes). OVA-liposomes effectively induced IFN- $\gamma$  production, but not BSA- or COA-liposome (Fig. 1B). These findings suggest that negatively charged liposomes containing PS effectively induce antigen-specific IFN- $\gamma$  production in vitro.

#### 3.2. PS recognition

PS-liposomes are preferentially taken up by M $\phi$ /DCs through receptors such as class A and B scavenger receptors [13,14], CD68 [15], vitronectin receptor [16], and PS-specific receptor [17]. To clarify whether PS recognition by APCs contributes to IFN- $\gamma$  production induced by PS-liposome-encapsulated antigens, we investigated the effect of the PS content in OVA-liposomes. As shown in Fig. 2A, the IFN- $\gamma$  production increased in a PS content dependent manner. PS appears on the surface of apoptotic cells and contributes to their phagocytosis by M $\phi$ /DCs [17–19]. Annexin V binds with high affinity to PS on the surface of apoptotic cells, and inhibits apoptotic cell uptake by M $\phi$  through interference with the availability of PS for recognition [20–23]. We thus investigated the effect of annexin V on IFN- $\gamma$  production. Annexin V inhibited IFN- $\gamma$  production induced by OVA-liposomes in a dose dependent manner (Fig. 2B). These results show that PS recognition on the surface of PS-liposomes by APCs play a critical role for the activation of Th1 cells leading to IFN- $\gamma$  production.

#### 3.3. The effect of the coadministration of OVA-specific Th1 cells plus OVA-liposomes in vivo

We investigated the effect of the coadministration of antigen-specific Th1 cells with PS-liposomes encapsulating antigens in vivo. Th1 cells were i.v. transferred and OVA-liposomes or OVA alone were i.p. injected. After 24 h, a marked elevation in serum IFN- $\gamma$  was observed in mice immunized with Th1 cells plus OVA-liposomes but not Th1 cells plus OVA or OVA-liposomes alone (Fig. 3). Furthermore, 5 days after injection, spleen cells obtained from mice were restimulated with OVA (50  $\mu$ g/ml) for 48 h and the production of IFN- $\gamma$  in the culture supernatant was determined by ELISA. As shown in Fig. 4A, spleen cells from mice immunized with Th1 cells plus OVA-liposomes produced higher levels of IFN- $\gamma$  compared with that of Th1 cells plus OVA. In order to demonstrate the distribution of Th1 cells into the spleen, we have used the tracking dye PKH26. In contrast to IFN- $\gamma$  production, the number of PKH26 positive cells in spleen cell from mice administered PKH26-labeled Th1 cells plus OVA-liposomes is lower than that of PKH26-labeled Th1 cells plus OVA (Fig. 4B). These results suggest that IFN- $\gamma$  production in antigen-specific Th1

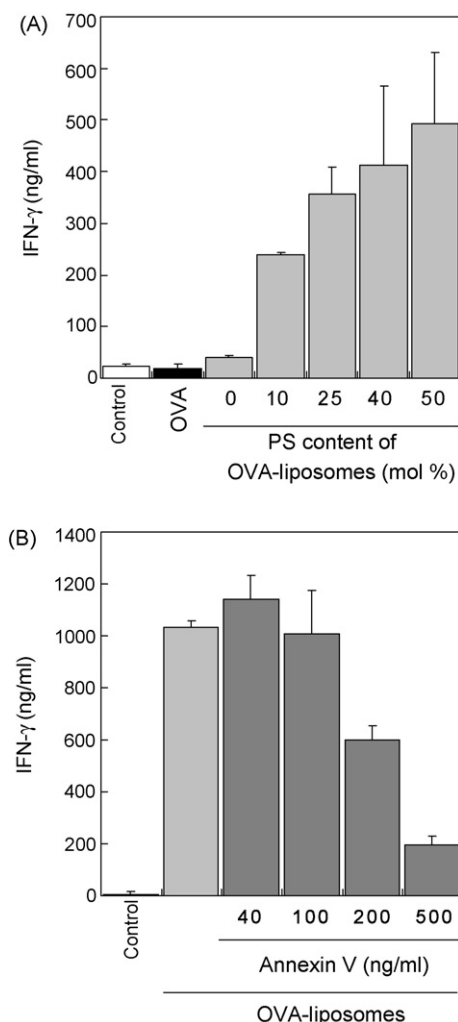


Fig. 2. (A) Effect of PS content of PS-liposomes on IFN- $\gamma$  production. 42-6A cells ( $4 \times 10^4$ /well) were incubated with SAC ( $4 \times 10^4$ /well) in the presence of OVA alone ( $10 \mu\text{g/ml}$ ) or PS-liposomes encapsulating OVA (OVA-liposomes,  $10 \mu\text{g/ml}$ ) for 18 h. IFN- $\gamma$  productions in the supernatants were evaluated by ELISA, as described in Section 2. The composition of OVA-liposomes were PS:PC:Chol = 0:1:1 (PS content 0%), PS:PC:Chol = 1:4:5 (PS content 10%), PS:PC:Chol = 1:1:2 (PS content 25%), PS:PC:Chol = 4:1:5 (PS content 40%), PS:PC:Chol = 1:0:1 (PS content 50%). (B) Effect of annexin V on IFN- $\gamma$  production. The coculture of 42-6A cells ( $4 \times 10^4$ /well) with SAC ( $4 \times 10^4$ /well) were incubated with PS-liposomes (PS content 25%) encapsulating OVA (OVA-liposomes,  $10 \mu\text{g/ml}$ ) in the presence of various concentration of annexin V for 18 h. IFN- $\gamma$  productions in the supernatants were evaluated by ELISA, as described in Section 2. The values are the means  $\pm$  S.D. of triplicate cultures from three independent experiments.

cells are effectively induced by the coadministration of PS-liposome-encapsulated antigens in vivo.

### 3.4. Inhibition of antigen-specific IgE production by Th1-cell therapy

IgE play an important role in the pathogenesis of allergic disease, in which there is a polarization of T-lymphocyte responses toward Th2 cells that produces cytokines such as

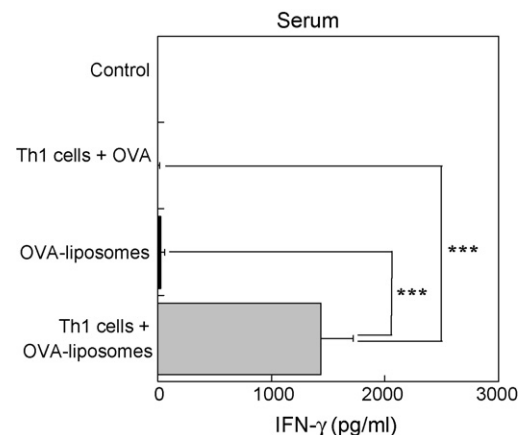


Fig. 3. Effect of PS-liposomes encapsulating antigens on serum IFN- $\gamma$  levels. Balb/c mice were intravenously immunized with 42-6A cells ( $5 \times 10^6$ /mouse, i.v.) plus OVA ( $50 \mu\text{g}$ /mouse, i.p.), PS-liposomes encapsulating OVA (OVA-liposomes,  $50 \mu\text{g}$ /mouse, i.p.) alone or 42-6A cells plus OVA-liposomes. Twenty-four hours later, serum IFN- $\gamma$  were evaluated by ELISA, as described in Section 2. Each value represents the mean  $\pm$  S.D. for three mice. \*\*\* $P < 0.005$ .

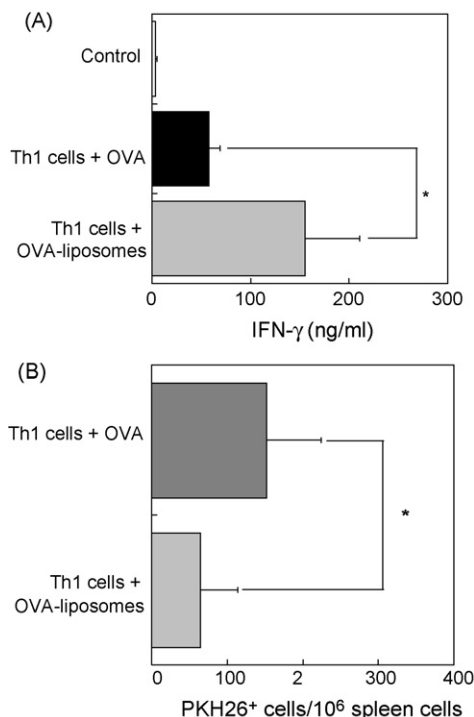


Fig. 4. Effect of PS-liposomes on the production of IFN- $\gamma$  by spleen cells. (A) Balb/c mice were immunized with 42-6A cells ( $5 \times 10^6$ /mouse, i.v.) plus OVA ( $50 \mu\text{g}$ /mouse, i.p.), 42-6A cells ( $5 \times 10^6$ /mouse, i.v.) plus OVA-liposomes ( $50 \mu\text{g}$ /mouse, i.p.). On day 5, spleen cells ( $5 \times 10^5$ /well) were incubated with OVA ( $50 \mu\text{g/ml}$ ) for 48 h. IFN- $\gamma$  productions in the supernatants were evaluated by ELISA, as described in the Section 2. \* $P < 0.05$ . (B) Balb/c mice were immunized with PKH26-labeled 42-6A cells ( $5 \times 10^6$ /mouse, i.v.) plus OVA ( $50 \mu\text{g}$ /mouse, i.p.) or PKH26-labeled 42-6A cells ( $5 \times 10^6$ /mouse, i.v.) plus OVA-liposomes ( $50 \mu\text{g}$ /mouse, i.p.). Twenty-four hours after, spleen cells were prepared and analyzed by flow cytometry. Each value represents the mean  $\pm$  S.D. for three mice. \* $P < 0.05$ .

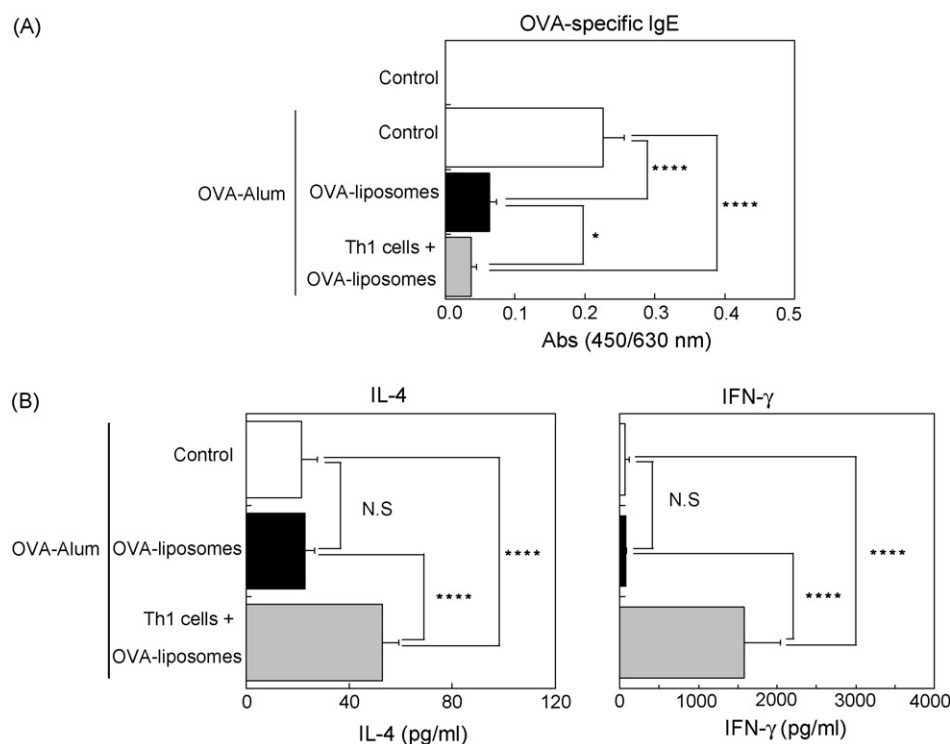


Fig. 5. In vivo inhibition of antigen-specific IgE production by Th1-cell therapy using PS-liposomes. Balb/c mice were intraperitoneally injected with PBS or OVA (50  $\mu$ g/mouse)-Alum. After 2 weeks, the mice were administered with 42-6A cells ( $5 \times 10^6$ /mouse, i.v.) plus OVA (50  $\mu$ g/mouse, i.p.), OVA-liposomes (50  $\mu$ g/mouse, i.p.) alone or 42-6A cells ( $5 \times 10^6$ /mouse, i.v.) plus OVA-liposomes (50  $\mu$ g/mouse, i.p.). For another 2 weeks, (A) the changes in IgE production were evaluated by ELISA. (B) Spleen cells ( $5 \times 10^5$ /well) were prepared and incubated with OVA (50  $\mu$ g/ml) for 48 h. IFN- $\gamma$  and IL-4 productions in the supernatants were evaluated by ELISA. Each value represents the mean  $\pm$  S.D. for four mice. \* $P < 0.05$ ; \*\*\*\* $P < 0.001$ .

IL-4 and IL-5 [24–26]. Th1-type cytokine IFN- $\gamma$  acts as a counterpart of Th2-type cytokine IL-4 and suppress the production of IgE [27,28]. We next examined the effect of Th1-cell therapy on the production of antigen-specific IgE in mice which were already immunized with OVA-Alum. Th1 cells and/or OVA-liposomes were administered 2 weeks after the injection of OVA-Alum. For another 2 weeks, the changes in IgE levels in serum were evaluated by ELISA. As shown in Fig. 5A, OVA-specific IgE levels increased in the mice immunized with OVA-Alum and decreased following the administration of OVA-liposomes or Th1 cells plus OVA-liposomes. IgE levels significantly decreased following the administration of Th1 cells plus OVA-liposomes compared with that of OVA-liposomes alone. We also determined the production of IFN- $\gamma$  and IL-4 in spleen cells obtained from immunized mice. As shown in Fig. 5B, unexpectedly, the enhancement of both IFN- $\gamma$  and IL-4 productions was observed in spleen cells from mice immunized with Th1 cells plus OVA-liposomes. The administration of OVA-liposomes alone did not affect. These results demonstrated that the coadministration of antigen-specific Th1 cell plus PS-liposomes encapsulated antigens attenuates drastically the production of IgE in mice undergoing an IgE immune response.

### 3.5. The coadministration of OVA-specific Th1 cells plus OVA-liposomes increased serum IL-12 p70

IL-12 p70 is a heterodimeric cytokine that is produced primarily by APCs such as M $\phi$ /DCs, and plays an important role in the regulation of both innate and adaptive immune responses [29]. Previously studies have shown that treatment with IL-12 inhibits IgE production in vivo [30,31]. Thus, the amount of serum IL-12 p70 in vivo was investigated. A marked elevation in serum IL-12 p70 was observed in mice immunized with Th1 cells plus OVA-liposomes but not Th1 cells plus OVA or OVA-liposomes alone (Fig. 6).

## 4. Discussion

The functional responses of T cells are initiated by the recognition of antigen peptide-major histocompatibility complex (MHC) complexes on the surfaces of APCs. There are two main types of MHC gene products, called MHC-classI molecules and MHC-classII molecules. CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) and CD4<sup>+</sup> Th cells recognize peptides bound to MHC-classI molecules and MHC-classII molecules, respectively [32]. Nakanishi et al. reported that positively

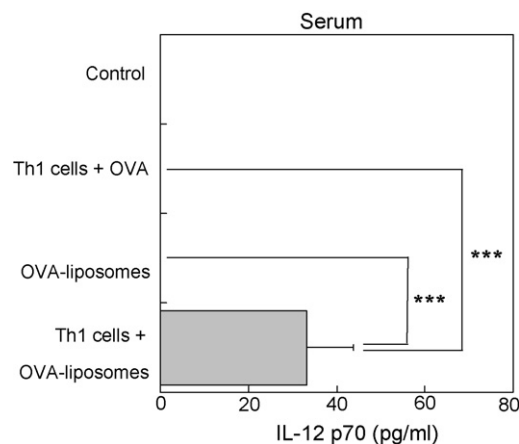


Fig. 6. Effect of PS-liposomes encapsulating antigens on serum IL-12 p70 levels. Balb/c mice were immunized with 42-6A cells ( $5 \times 10^6$ /mouse, i.v.) plus OVA ( $50 \mu\text{g}$ /mouse, i.p.), OVA-liposomes ( $50 \mu\text{g}$ /mouse, i.p.) alone or 42-6A cells ( $5 \times 10^6$ /mouse, i.v.) plus OVA-liposomes ( $50 \mu\text{g}$ /mouse, i.p.). Twenty-four hour later, serum IL-12 p70 were evaluated by ELISA, as described in Section 2. Each value represents the mean  $\pm$  S.D. for four mice. \*\*\* $P < 0.005$ .

charged liposome-encapsulated antigens effectively induced antigen-specific CTL responses, but did not negatively charged and neutral liposomes [33], suggesting that positively charged liposome-encapsulated antigens are presented to T cell receptors (TCR) on CD8<sup>+</sup>T cells via MHC-classI on APCs. On the other hand, in present study, we show that negatively charged liposome-encapsulated OVA resulted in high levels of OVA-specific IFN- $\gamma$  production in Th1 cells. It is likely that negatively charged liposome-encapsulated antigens are effectively presented to TCR on Th1 cells via MHC-classII on APCs, and the charge of liposomal surface results in the difference of the immunoresponse.

PS and PA are major negatively charge phospholipids in the plasma membrane. We previously reported that negatively charged liposomes containing PS or PA (PS- or PA-liposomes) are preferentially taken up by M $\phi$  [34]. PS- and PA-liposome-encapsulated antigens induced antigen-specific IFN- $\gamma$  production in vitro. However, induction effect of IFN- $\gamma$  by PS-liposomes was higher than PA-liposomes, suggesting that not only negatively charge, but also lipid head group (serine moiety of PS) play an important role in the recognition of liposomes by APCs. However, the molecular mechanism of PS-recognition by APCs is not fully understood. Recent reports suggest that the interaction of the PS recognition receptors on M $\phi$ /DCs and PS on apoptotic cells plays a major role in the recognition and uptake of apoptotic cells by M $\phi$ /DCs [17–19]. In addition, the presentation by M $\phi$ /DCs of antigens derived from apoptotic cells is known to potently induce humoral and cellular immunity [35–37]. Our results suggest that PS-liposomes encapsulating antigens partly mimic the activity of apoptotic cells to activate immune cells.

In the present study, PS-liposome-encapsulated OVA was more effective than OVA alone on inducing the production

of IFN- $\gamma$ . However, the uptake of PS-liposome-encapsulated OVA via PS-recognition receptor on SAC was slightly lower than that of OVA alone (data not shown). These results indicate that the activation of T cells by APCs is correlated with not the quantity but the quality of antigen uptake. Consistent with our observation, Copland et al. reported that the uptake of negatively charged liposome-encapsulated OVA by DCs is lower than that of OVA alone [38]. Previously, T cell activation has been reported to be dependent on the expression of MHC-peptide complexes of sufficient density on APC [39]. These results raises the possibility that the expression of MHC-class II-OVA peptide complexes, but not MHC-classII alone, on APC induced by OVA-liposomes is higher than that of OVA alone.

It is well known that IgE synthesis is highly dependent on the balance in the production of IFN- $\gamma$  and IL-4 [27,28]. The administration of OVA-specific Th1 cells plus OVA-liposomes and OVA-liposomes alone significantly attenuated the production of IgE in mice ongoing IgE immune responses and enhanced IFN- $\gamma$  and IL-4 production. Although IFN- $\gamma$  can play a critical role for inhibiting ongoing IgE immune response, the change in the balance in IFN- $\gamma$  and IL-4 may not exclusively associate with the change in ongoing IgE immune response. Previous reports show that IL-12 p70 inhibits IL-4-induced IgE production from B cells in vivo and transiently elicits intense serum IFN- $\gamma$  in vivo [30,31]. In our study, the administration of OVA-specific Th1 cells plus OVA-liposomes inhibited on-going IgE response and enhanced the amount of serum IL-12 p70 and IFN- $\gamma$  compared with that of OVA-liposomes, suggesting that IL-12 p70 play an important role of IgE inhibition by Th1-cell therapy using PS-liposomes.

Nishimura et al. recently reported that transfer of OVA-specific Th1 cells into mice bearing MHC class II<sup>+</sup> OVA-expressing A20 tumor cells causes complete tumor rejection [40]. However, Th1-cell therapy alone was not effective against MHC class II<sup>+</sup> OVA-expressing E.G7 tumor cells. Interestingly, treatment of mice bearing established E.G7 tumors by i.v. transfer of a large number of Th1 cells combined with intratumoral high dose injection of the model tumor antigen OVA induced complete tumor rejection [41]. In this way, antigen immunization protocol has a great influence on the induction of antitumor activity of Th1-cell therapy. In this study, the coadministration of OVA-specific Th1 cells plus OVA-liposomes resulted in high levels of IFN- $\gamma$  and IL-12 p70 production, indicating that this coadministration may be a novel therapeutic method for Th1-cell therapy against tumor. Studies are also underway to clarify whether the coadministration of OVA-specific Th1 cells and PS-liposome-encapsulating OVA induces cellular immunity against E.G7 tumor.

In conclusion, PS-liposomes encapsulating antigens result in high levels of IFN- $\gamma$  production in Th1 cells in vitro and in vivo. We therefore first suggest that negatively charged liposomes containing PS are an efficient antigen delivery system for Th1-cell therapy.

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