

Analysis of transgene expression profile-dependent  
induction of transgene-specific immune response

**2014**

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## Preface

Gene therapy is expected to become a new treatment for refractory diseases such as cancers and virus infections [1-6]. The therapeutic effects of gene therapy depend on the expression profile and biodistribution of the transgene product and types of cells expressing transgene product [7-10]. Therefore, much effort has been made to develop methods that can regulate these factors. In previous studies performed in my laboratory, it was demonstrated that construction of plasmid vectors with reduced the number of CpG motifs and optimized promoter allows sustained transgene expression [11-13]. The transgene product is sometimes recognized by immune system, which causes immune response [14-16]. Induction of transgene-specific immune response could affect transgene expressing cells and cause adverse effects [17-18]. Induction of the immune response to transgene products or, in other words, the encoded therapeutic protein, is a serious concern in gene therapy [19-20].

It is generally known that induction of the immune response depends on several factors, including the antigenicity of the protein, types of transgene-expressing cells, transgene expression profile, and subcellular localization or secretion of the product [7, 9]. However, the exact nature of the relationship between the transgene expression profile and immune induction following gene transfer is unclear despite the fact that many efforts by our laboratory and others have been made to develop methods that regulates transgene expression profile [11-13].

In this study, I investigated the effects of transgene expression profile, type of transgene expressing cells and transgene expression level on the induction of transgene-specific immune response. In addition, I tried to identify the cells that work as antigen presenting cells (APCs) in the induction of transgene-specific immune response after gene delivery by the hydrodynamics-based procedure.

In Chapter I, the effect of transgene expression profile on the induction of immune response against the transgene product was examined. Firefly luciferase (fLuc) was selected as a model antigen and *Gaussia* luciferase (gLuc) was chosen as a reporter protein. Two types of fLuc-expressing plasmid DNA, a long-term expression plasmid pCpG-fLuc or a short-term expression plasmid pCMV-fLuc, were injected into mice by hydrodynamic injections along with a gLuc-expressing long-term plasmid (pROSA-gLuc) whose transgene expression was used to evaluate the transgene expression profile of fLuc-expressing plasmids in the liver affected by immune response because simultaneous injection of the fLuc- and gLuc-expressing plasmid DNAs resulted in the expression of fLuc and gLuc in the same cells. In addition, the effect of transgene expression in splenic cells and macrophages on the induction of immune response was evaluated by spleen removal and macrophage depletion.

In Chapter I, it was found that high and sustained transgene expression of fLuc induced the immune responses to the antigenic protein. However, it was not investigated whether total amount of transgene product or the amount of transgene product per cell is important for the induction of

transgene-specific immune response. In Chapter II, the relationship between the amount of transgene product in each cell and the induction of immune response against the transgene product was examined in more detail. *Cypridina* luciferase (cLuc), a secretory reporter protein that has antigenicity, was used as a model antigen. gLuc-expressing plasmid was delivered with cLuc-expressing plasmid as above by one high-dose hydrodynamic injection or by three low-dose injections. Then, I examined whether the total level of transgene expression or the level of transgene expression per cell is important for the induction of transgene-specific immune responses. Finally, I investigated whether hepatocytes work as APCs after hydrodynamic injection of plasmid vectors using ovalbumin (OVA) as a model antigen.

In this thesis, the results are presented in the following two chapters.

## Chapter I

# Elucidation of the effect of the duration of transgene expression on the induction of transgene-specific immune response

### I-1 Introduction

The therapeutic effects of gene therapy depend on the types of transgene expressing cells, as well as the expression profile and biodistribution of the transgene, and much effort has been made to develop methods that control these factors. In previous studies performed in my laboratory, it was reported that the plasmids with reduced numbers of CpG motifs or with optimized promoter and enhancer can be used for achieving sustained transgene expression [21]. It was also demonstrated that these novel plasmid vectors can be used to increase the therapeutic effects of interferon gene transfer against tumor metastasis, atopic dermatitis or hepatitis. A drawback of sustained transgene expression, however, is that it might increase the risk of induction of immune response to the transgene product. Immune response to the transgene product or, in other words, the encoded therapeutic protein, is a major concern in gene therapy because it would cause serious adverse effects and affect the transgene expression profile [22].

Induction of the immune response depends on several factors, including the antigenicity of the protein, the type of transgene-expressing cells, transgene expression profile, and the subcellular localization or secretion of the product. A previous study suggested that transgene expression in antigen-presenting cells (APCs) is a risk factor for eliciting immune response to the transgene [23]. On the other hand, studies investigating the relationship between the transgene expression profile and the immune response to the product are limited. Bates *et al.* reported that hydrodynamic injection of plasmid DNA expressing firefly luciferase (fLuc) driven by a cytomegalovirus (CMV) promoter, which resulted in a transient luciferase expression, hardly induced anti-fLuc antibody production, whereas administration of an fLuc expression vector driven by a ubiquitin promoter, which generated sustained transgene expression, induced anti-fLuc antibody production [24]. The same group also investigated whether incorporation of target sites for APC-specific microRNA (miRNA) in the plasmid and the use of cell type specific promoters would be effective approaches for reducing the risk of immune responses to the transgene product [25].

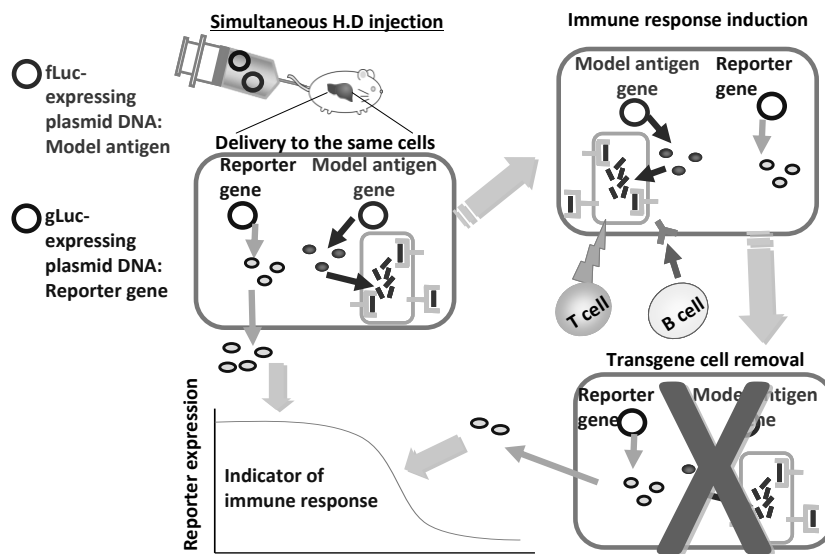
In general, the reduction in transgene expression could be accounted for by two different phenomena: the removal of transgene-expressing cells as a consequence of the immune response or a reduced efficiency of transgene expression by processes such as promoter inactivation. It is necessary to distinguish between these two phenomena to clearly understand the association between the transgene expression profile and the immune response. This issue, however, has not been fully addressed in previous



studies.

In the present study, I investigated the effect of the transgene expression profile on the induction of an immune response to the transgene product. Accordingly, different types of plasmid vectors were administered by hydrodynamic injections to obtain transient and sustained transgene expression profiles. fLuc was selected as a model antigen because (i) the amount of fLuc can be quantitatively determined using the luciferase assay and (ii) fLuc is immunogenic and elicits an immune response [26,27].

The immune response to fLuc-expressing cells was evaluated using *Gaussia* luciferase (gLuc) as a reporter, which was accomplished by co-administering a gLuc-expressing plasmid along with the fLuc-expressing vectors. This approach was employed for several reasons: (i) our preliminary study indicated that hydrodynamic administration of the gLuc-expressing vector, pROSA-gLuc, resulted in sustained, high gLuc expression for more than 1 year (Takahashi Y and Matsui Y, unpublished data); (ii) the distribution of two vectors co-administered by hydrodynamic injection almost completely overlapped [28]; (iii) the expression levels of gLuc can be quantitatively and reproducibly determined by measuring serum gLuc activity without sacrificing the mice; and (iv) the use of gLuc protein as a marker for transgene-expressing cells enables us to monitor the removal of these cells as a consequence of the immune response to fLuc because the reduction in serum gLuc activity is ascribable to the removal of the transgene-expressing cells and not to any phenomenon reducing the transgene expression levels in the cells. Plasmids that stably (pCpG-fLuc) or transiently (pCMV-fLuc) express fLuc were co-administered with the pROSA-gLuc vector to mice by hydrodynamic injections. The cytotoxic immune response to fLuc-expressing cells was continuously evaluated by measuring serum gLuc activity without sacrificing the mice. The schematic image of the experimental design is shown as Figure 1.



**Figure 1. Experimental image for the evaluation of immune response for transgene product.**

## I-2 Material and Methods

### Plasmid DNA

pCpG-mcs was purchased from Invivogene (San Diego, CA, USA). The fLuc-expressing plasmids, pCMV-fLuc and pCpG-fLuc, were constructed as described previously [21]. pROSA-gLuc and pROSA-fLuc, plasmid expressing gLuc and fLuc, respectively, were constructed using In-fusion Advantage polymerase chain reaction. pCMV-fLuc was amplified in the Escherichia coli strain DH5 $\alpha$ , whereas pCpG-mcs, pCpG-fLuc and pROSA-gLuc were amplified in the E. coli strain GT115. Plasmid DNA was purified using JETSTAR 2.0 Plasmid MAXI Plasmid Purification Kits (GENOMED GmbH, Löhne, Germany). Characteristics of the plasmid DNAs used are summarized in Table 1.

**Table 1. Properties of plasmid DNA used in Chapter I.**

Plasmid	Size (kbp)	Number of CpG *	Enhancer	Promoter	cDNA
pCMV-fLuc	7.1	846	hCMV	hCMV	Firefly luciferase
pCpG-mcs	3.0	0	hCMV	hEF1	None
pCpG-fLuc	4.7	194	hCMV	hEF1	Firefly luciferase
pROSA-gLuc	5.8	598	None	hROSA26	<i>Gaussia</i> luciferase
pROSA-fLuc	6.7	622	None	hROSA26	Firefly luciferase

\* The Numbers of CpG dinucleotides in plasmid DNA are indicated. CMV, human cytomegalovirus; EF1 human elongation factor 1.

### Mice and plasmid DNA administration

Four-week-old female ICR mice and 6-week-old female C57/BL6 mice, weighing approximately 20 g each, were purchased from Japan SLC (Shizuoka, Japan). C57/BL6 mice were used only in the experiment in which interferon (IFN)- $\gamma$  secretion from splenocytes was evaluated. All animal experiments were subject to deliberation and approval by the Ethics Committee for Animal Experiments at the

Graduate School of Pharmaceutical Sciences, Kyoto University. Plasmid DNA was administered to the mice using a hydrodynamics-based procedure, in which plasmid DNA dissolved in saline solution (whose volume equaled 8% of the total body weight) was injected into the tail vein of the mice within 5 s using a 26-gauge needle.

### **Cell culture**

Murine melanoma B16BL6 cells or B16BL6 cells stably expressing fLuc (B16BL6/fLuc) was cultured in Dulbecco's modified Eagle medium (DMEM; Nissui Co. Ltd., Tokyo, Japan) supplemented with 10 % heat-inactive fetal bovine serum (FBS). splenocytes was cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated FBS, 50  $\mu$ M 2-mercaptoethanol, 2 mM L-glutamine, gLucose, sodium pyruvate.

### **Luciferase Assay**

At indicated time points, blood was collected from the tail vein of mice. The blood samples were incubated at 4 °C for 2 h to allow clotting and then centrifuged at 8000  $\times$  g for 20 min to obtain serum samples. To measure luciferase activities in the liver, the liver was harvest and homogenized in 10 ml/g liver of lysis buffer (0.1 M Tris, 0.05 % TritonX-100, 2 mM EDTA, pH7.8), and the homogenates were centrifuged at 12000  $\times$  g for 10 min at 4 °C. Then, the supernatant was mixed with luciferase assay buffer (PicageneDual, Toyo Ink, Tokyo, Japan), and the chemiluminescence produced was measured in a luminometer (Lumat LB 9507; EG and G Berthold, Bad Wildbad, Germany).

### **Quantitation of antibody titers**

Serum samples were obtained as described above. The amount of fLuc-specific antibodies was measured by enzyme linked immunosorbent assay (ELISA) as described previously [29]. In brief, 96-well flat-bottom polystyrene plate was coated with 0.2 mg/ml of firefly luciferase (Promega, San Luis Obispo USA) by overnight incubation at 4 °C. The wells were blocked with 5% bovine serum albumin (BSA)-containing phosphate buffered saline with Tween-20 (0.5 % Tween-20 in phosphate buffer saline (PBS)) for 1 h at 37 °C. After washing, serially diluted serum samples were added to the wells. After 2 h incubation at 37 °C and subsequent washing, horse raddish peroxidase (HRP)-labeled rabbit anti-mouse IgG (1:2000 dilution; Zymed Lab, San Francisco, CA) was added to each well. After 1 h incubation at 37 °C and subsequent washing, freshly prepared o-phenylenediamine dihydrochloloride (Wako, Tokyo, Japan) solution containing H<sub>2</sub>O<sub>2</sub> was added to each well. After 10 min incubation at room temperature, 10 % H<sub>2</sub>SO<sub>4</sub> was added to each well to stop the reaction and measured absorbance at 490 nm.

### **IFN- $\gamma$ secretion from splenocytes**

C57BL6 mice received two injections of pROSA-gLuc with pCpG-mcs or pCpG-fLuc with a 3-week interval. One week after the second injection, splenocytes were isolated, purified and cultured in the presence of mitomycin C-treated murine melanoma B16BL6 cells or mitomycin C-treated B16BL6 cells stably expressing fLuc (B16BL6/fLuc) in 96-well culture plates for 2 days. The concentration of IFN- $\gamma$  in supernatant was determined by an ELISA (Ready-SET-Go! Mouse IFN- $\gamma$  ELISA; eBioscience, San Diego, CA, USA).

### **Measurement of serum alanine aminotransferase (ALT) activity**

At the indicated time points after plasmid DNA administration, serum was collected as described above. Serum ALT level was measured using a quantification kit (Transaminase CII test Wako; Wako Pure Chemical, Osaka, Japan).

### **Hematoxylin and eosin staining**

Mice were euthanized by cutting the vena cava, and the liver was gently infused with 2 ml of saline through the portal vein to remove the remaining blood. The liver was then fixed in 4 % paraformaldehyde in PBS, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE). The stained sections were examined using a microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan).

### **Histochemical analysis of the liver**

Mice were euthanized by cutting the vena cava, and the liver was gently infused with 2 ml of saline through the portal vein to remove the remaining blood. The liver was then embedded in Tissue-Tek OCT embedding compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen, and stored in 2-methyl butanol at -80 °C. Frozen liver sections (10  $\mu$ m thick) were made using a cryostat (Jung Frigocut 2800E; Leica Microsystems AG, Wetzlar, Germany) by the routine procedure. The sections were fixed with 4% paraformaldehyde in PBS.

To detect CD8<sup>+</sup> cells and CD4<sup>+</sup> cells in the liver, the fixed sections were blocked with 20% FBS in PBS for 1 h at 37 °C and incubated with biotinylated Abs specific to mouse CD8 (Acris antibody, Herford, Germany) or CD4 (ebioscience, San Diego, CA, USA) for 1 h at 37 °C. Samples were examined under a fluorescence microscope (Biozero BZ-8000).

### **Spleen removal and macrophage depletion**

For spleen removal, mice were anaesthetized and shaved. Then, a 2-cm incision was made in the skin at the left flank. The peritoneal membrane was opened, and the entire spleen was removed intact. The peritoneal membrane and the skin were closed separately with surgical silk-thread. This procedure ensures

that the spleen is removed in total and that no splenic fragments are left behind [30].

Macrophage depletion was performed by using clodronate-encapsulating liposome (clodronate liposome). Clodronate liposome was prepared by the method as described previously [31]. For macrophage depletion, mice received clodronate liposome administration 1 and 3 days before plasmid DNA administration, and then repeated every two days.

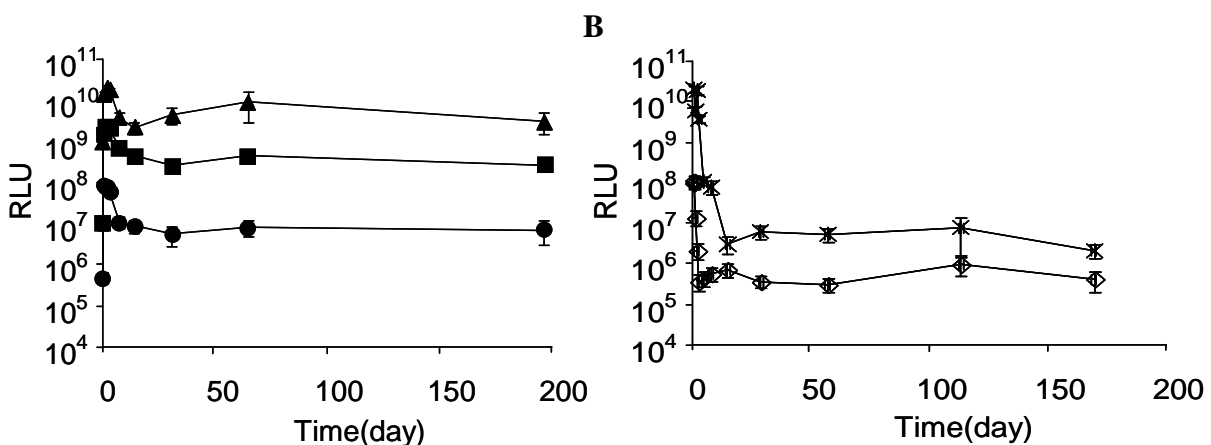
### Statistical analysis

Differences were statistically evaluated by Student's t-test. The level of statistical significance was set at  $P < 0.05$ .

## I-3 Results

### I-3-a Hydrodynamic injection of pROSA-gLuc resulted in stable gLuc activity in the serum

To evaluate the stability of gLuc expression after hydrodynamic injection of pROSA-gLuc, serum gLuc activity was measured over time after hydrodynamic administration of 0.1, 1 or 10  $\mu\text{g}$  of pROSA-gLuc, without sacrificing the mice. As shown in Figure 2A, stable gLuc activity was observed for more than 1 year, irrespective of the pROSA-gLuc dose administered. On the other hand, hydrodynamic injection of pCMV-gLuc (pCMV-based gLuc expressing plasmid) or pCpG-gLuc (pCpG-based gLuc expressing plasmid) also resulted in a long-term gLuc expression, although the time-dependent decline in gLuc activity was greater than that after pROSA-gLuc administration (Figure 2B).



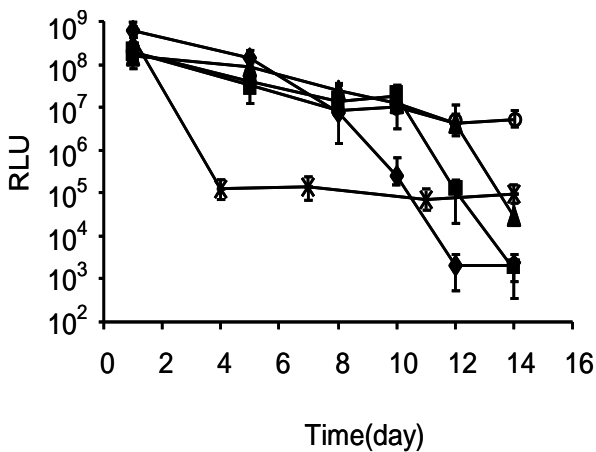
**Figure 2. gLuc activity in the serum after hydrodynamic delivery.**

(a) Time-course of gLuc activity in serum after hydrodynamic injection of 0.1 (circle), 1 (square) and 10  $\mu\text{g}$  (triangle) of pROSA-gLuc. (b) Time-course of gLuc activity in serum after hydrodynamic injection of

10 (asterisk) of pCpG-gLuc and 10  $\mu\text{g}$  (open diamond) of pCMV-gLuc. The results are expressed as the mean  $\pm$  SD of five mice.

### I-3-b Hydrodynamic injection of pCpG-fLuc resulted in the stable fLuc activity in the liver at early time points and reduction of fLuc activity at later time points

After the administration of fLuc-expressing plasmid DNAs that show stable and transient expression profile (pCpG-fLuc and pCMV-fLuc, respectively) to mice by hydrodynamic injection, fLuc activity in the liver was high at immediately after administration of pCMV-fLuc, and it started to decline within the first day after injection. After the initial decline, fLuc activity in the liver was constant after pCMV-fLuc administration (Figure 3). On the other hand, fLuc activity in the liver was constant for the first 1 week after the administration of pCpG-fLuc. Drastic decline in the fLuc activity was observed in the mice that received 30, 10 and 3  $\mu\text{g}$  of pCpG-fLuc at 10, 12, 14 days after the administration, respectively.

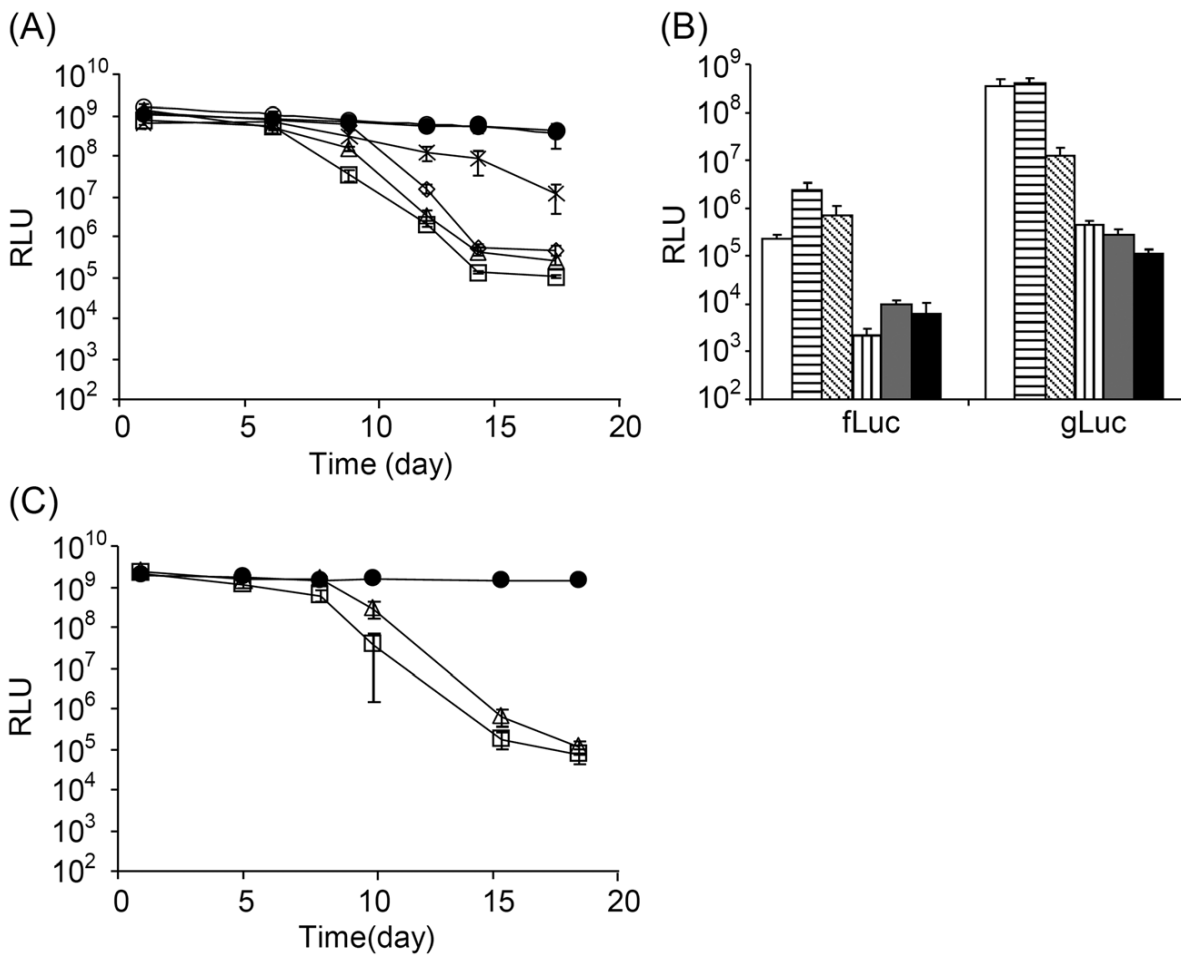


**Figure 3. Time-course of fLuc activity in the liver after hydrodynamic injection of fLuc-expression plasmids DNA at varying doses.** Mice received administration of 10  $\mu\text{g}$  of pCMV-fLuc (asterisk) or 1 (open circle), 3 (closed triangle), 10 (closed square) or 30  $\mu\text{g}$  (closed circle) of pCpG-fLuc. The results are expressed as the mean  $\pm$  SD of five mice. .

### I-3-c Co-administration of fLuc-expressing plasmid affected gLuc activity from pROSA-gLuc

pROSA-gLuc was co-administered with 10  $\mu\text{g}$  of pCMV-fLuc or 0.1, 0.3, 1, 3 or 10  $\mu\text{g}$  of pCpG-fLuc, and serum gLuc activity was measured as an indicator of the number of cells expressing both gLuc and fLuc (Figure 4A). The time course of serum gLuc activity after co-administration of pROSA-gLuc with 10  $\mu\text{g}$  of pCMV-fLuc or 0.1  $\mu\text{g}$  of pCpG-fLuc was similar to that obtained after administration of pROSA-gLuc alone. On the other hand, serum gLuc activity showed a sharp decline approximately 1 week after co-administration of pROSA-gLuc with 1, 3 or 10  $\mu\text{g}$  of pCpG-fLuc. This reduction in serum gLuc activity was initiated earlier as the dose of co-administered pCpG-fLuc was increased. A slight reduction in serum gLuc activity was also observed in mice that received pROSA-gLuc with 0.3  $\mu\text{g}$  of pCpG-fLuc. These results suggest that sustained fLuc expression from the co-administered pCpG-fLuc induces the reduction in serum gLuc activity. Sustained expression of fLuc, an antigenic

protein, is capable of inducing a fLuc-specific immune response; I therefore hypothesized that the number of fLuc-expressing cells (which also simultaneously express gLuc) is reduced because of the removal of the transgene-expressing cells by the fLuc-specific immune response, which in turn is induced by sustained fLuc expression. The activities of both the luciferases (gLuc and fLuc) in the liver were measured 17 days after co-administration (Figure 4B). fLuc activity in the liver was higher in mice that received a low dose (0.1 or 0.3  $\mu\text{g}$ ) of pCpG-fLuc or 10  $\mu\text{g}$  of pCMV-fLuc than in mice that received a higher dose (1, 3 or 10  $\mu\text{g}$ ) pCpG-fLuc, which suggests that the initial high and sustained expression obtained by a high dose pCpG-fLuc might induce a fLuc-specific immune response that removed the fLuc expressing cells. Similarly, gLuc activity in the liver of the mice that received pROSA-gLuc with 10  $\mu\text{g}$  of pCMV-fLuc or 0.1  $\mu\text{g}$  of pCpG-fLuc was much higher than the corresponding activity in mice that received higher doses of co-administered pCpG-fLuc, which strongly suggests the removal of transgene-expressing cells by fLuc-specific immune response. Next, pROSA-gLuc was coadministered with pROSA-fLuc. Serum gLuc activity showed a sharp decline approximately 10 days after the co-administration of pROSA-gLuc with pROSA-fLuc (Figure 4C).



**Figure 4. gLuc and fLuc activities in the serum and liver after co-administration of the respective**

### **plasmids by hydrodynamic injection.**

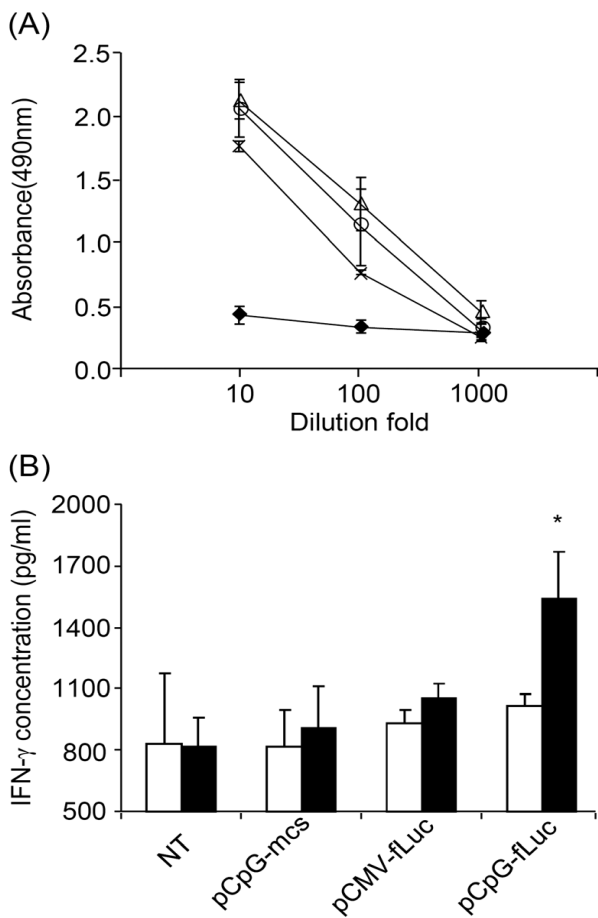
(A) Time course of serum gLuc activity after co-administration of 10 µg of pROSA-gLuc with 10 µg of pCMV-fLuc (closed circle) or 0.1 (open circle), 0.3 (asterisk), 1 (open diamond), 3 (open triangle) or 10 µg (open square) of pCpG-fLuc. (B) fLuc and gLuc activities in the liver 17 days after co-administration of 10 µg of pROSA-gLuc with 10 µg of pCMV-fLuc (open column) or 0.1 (horizontal lines), 0.3 (skewed lines), 1 (vertical lines), 3 (grey) or 10 µg (closed column) of pCpG-fLuc. (C) Time-course of gLuc activity in the serum after co-administration of 10 µg of pROSA-gLuc with 10 µg of pCpG-mcs (closed circle), pROSA-fLuc (open triangle) or pCpG-fLuc. The results are expressed as the mean ± SD of five mice

### **I-3-d fLuc-specific humoral and cellular immune response were induced in mice that received hydrodynamic injections of pCpG-fLuc**

As a next step, I evaluated the fLuc-specific immune response in mice that received hydrodynamic injections of the fLuc-expressing plasmid DNA by measuring fLuc-specific antibodies in the serum 14 days after pCpG-fLuc or pCMV-fLuc administration. As shown in Figure 5A, fLuc-specific antibodies were detected in the serum of mice that had been administered with 1, 3 or 10 µg of pCpG-fLuc. On the other hand, fLuc-specific antibodies were scarcely detected in the serum of mice receiving 10 µg of pCMV-fLuc.

To evaluate the cellular immune response specific for fLuc, I investigated IFN- $\gamma$  production in spleen cells of immunized mice in response to the stimulation with fLuc. Spleen cells from mice immunized with pCpG-fLuc generated a large amount of IFN- $\gamma$ . Splenocytes of mice receiving pCpG-fLuc produced significantly higher amounts of IFN- $\gamma$  in response to fLuc than the splenocytes of mice (Figure 5B).





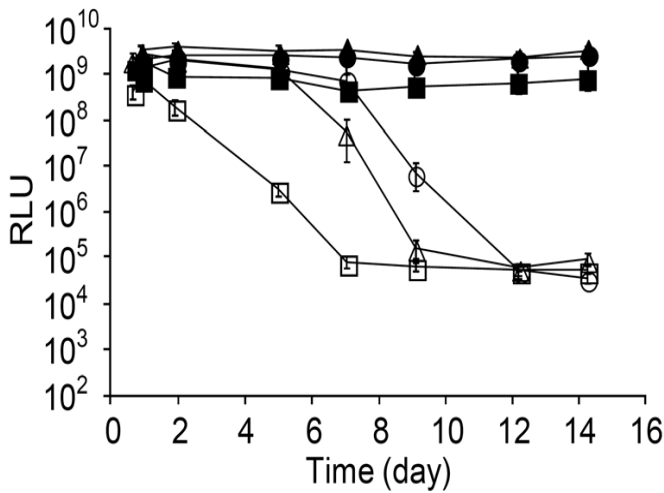
**Figure 5. Induction of humoral and cellular immune response specific for fLuc after hydrodynamic injection of fLuc-expression vector.**

(A) Mice received hydrodynamic injections of 10 μg of pCMV-fLuc (closed diamond), 1 (asterisk), 3 (open triangle) or 10 μg (open circle) of pCpG-fLuc. Fourteen days after plasmid DNA administration, serum samples from the mice were collected, and antibody titers were measured by ELISA. The results are expressed as the mean ± SD of five mice. (B) Splenocytes collected from untreated mice or mice receiving pDNA administration were cocultured with B16BL6 (open columns) or B16BL6/fLuc cells (closed columns) for 2 days. IFN-γ concentration in the culture medium was measured by ELISA. The results are expressed as the mean ± SD of five mice. \*p<0.05 compared to the B16BL6-stimulated group.

### **I-3-e Reduction in serum gLuc activity immediately after co-administration of pROSA-gLuc with pCpG-fLuc in mice pre-administered with pCpG-fLuc**

I hypothesized that the fLuc-specific immune response induced by sustained fLuc expression eliminated fLuc expressing cells, thereby also resulting in decreased gLuc activity. This hypothesis was tested by pre-administering fLuc-expressing plasmid DNA, and evaluated whether the fLuc-specific immune response thereby induced affects the profile of gLuc expression from pROSA-gLuc that was subsequently co-administered with fLuc-expressing plasmid DNA. Specifically, saline, pCpG-fLuc or pCMV-fLuc was pre-administered through hydrodynamic injections, followed by co-administration of pROSA-gLuc with pCpG-fLuc or pCMV-fLuc 14 days later. As shown in Figure 6, a reduction in the gLuc activity was observed as early as 2 days after pCpG-fLuc co-administration in mice preadministered with pCpG-fLuc. In addition, serum gLuc activity began to decrease 7 and 9 days after co-administration of pROSA-gLuc with pCpG-fLuc in mice pre-administered with pCMV-fLuc or saline, respectively. On the other hand, co-administration of pROSA-gLuc with pCMV-fLuc in mice pre-administered with

pCpG-fLuc scarcely altered serum gLuc activity.



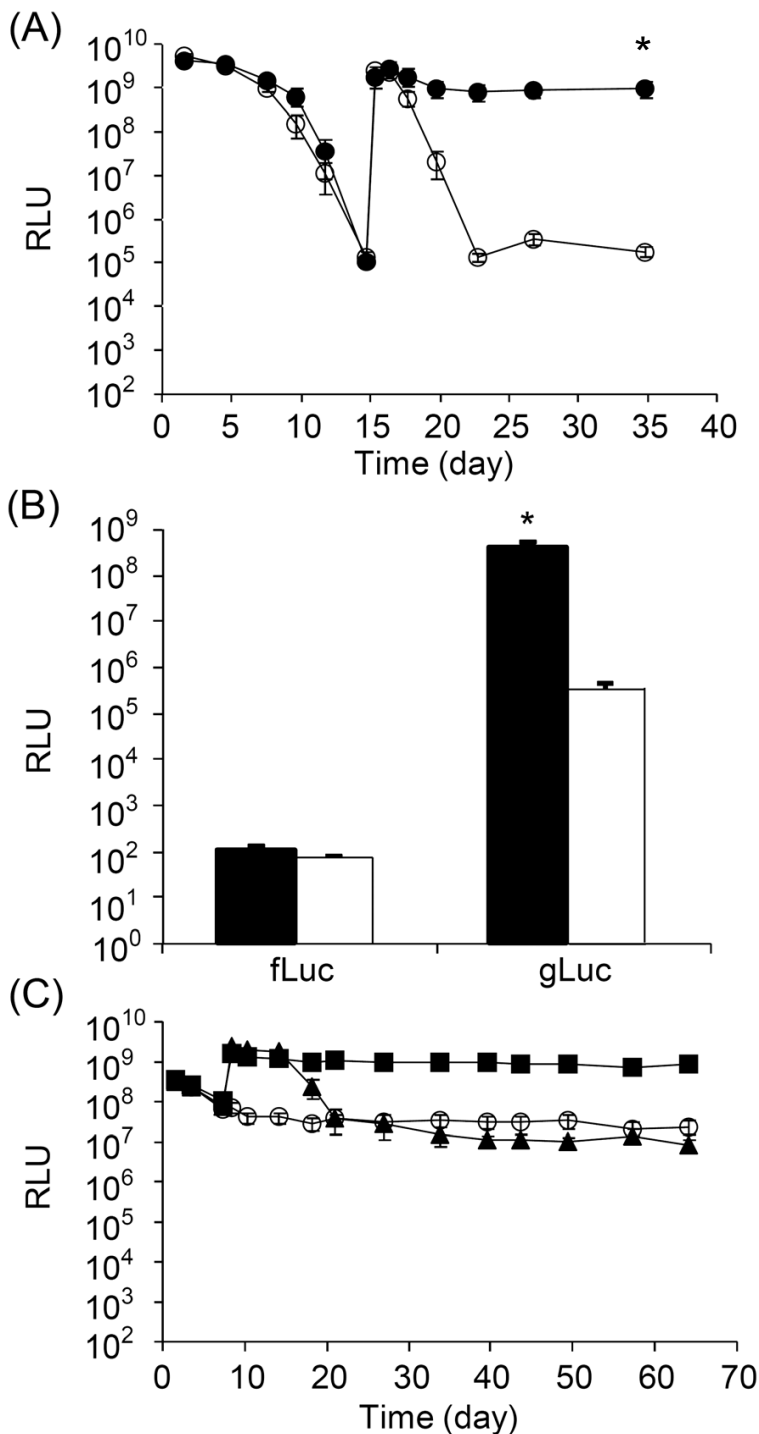
**Figure 6. Time course of serum gLuc activity after repeated hydrodynamic injections.**

The mice first received hydrodynamic injections of saline (circle), 10  $\mu$ g of pCMV-fLuc (triangle) or 10  $\mu$ g of pCpG-fLuc (square). Fourteen days after the first administration, the mice were co-administered 10  $\mu$ g of pROSA-gLuc with 10  $\mu$ g of pCMV-fLuc (closed symbols) or pCpG-fLuc (open symbols). The results are expressed as the mean  $\pm$  SD of five mice.

### I-3-f Serum gLuc activity decreased only upon simultaneous co-administration of pROSA-gLuc and pCpG-fLuc

Next, it was investigated whether co-expression of fLuc and gLuc in the same cell was required for the reduction in gLuc expression observed after co-administration of pROSA-gLuc with pCpG-fLuc. To avoid co-expression of fLuc and gLuc in the same cells, I sequentially injected the two plasmids instead of a simultaneous injection because it was shown that the former protocol resulted in the expression of the transgenes in different cells with little overlap [28]. Mice were first co-administered pROSA-gLuc with pCpG-fLuc. Fourteen days after the first injection, pROSA-gLuc and pCpG-fLuc were co-administered or sequentially administered; the latter was administered with a 12-h interval (Figure 7A). Serum gLuc activity decreased in mice that received co-administration and not sequential administration. On the other hand, fLuc activity in the liver 14 days after the second injection was comparable between these two groups (Figure 7B), which suggests that the fLuc-specific immune response was induced in both groups. I further investigated whether reduction in gLuc activity after co-administration of pROSA-gLuc with pCpG-fLuc accompanies the reduction in gLuc expression from separately administered pROSA-gLuc. The mice first received hydrodynamic administration of 1  $\mu$ g of pROSA-gLuc. One week after the first injection, the mice were administered saline (without any plasmid DNA) or coadministered 10  $\mu$ g of pROSA-gLuc with pCpG-mcs or pCpG-fLuc (Figure 7C). Co-administration of pCpG-mcs hardly affected the gLuc expression from pROSA-gLuc. Serum gLuc activity was enhanced by the second administration of pROSA-gLuc, which reflects the fact that the dose of pROSA-gLuc was 10-fold higher

in the second administration than in the first administration. In the mice that received a second injection of pROSA-gLuc with pCpG-fLuc, serum gLuc activity began to decrease 7 days after the second injection and reached the levels found in the control mice that received saline during the second administration. This result implies that gLuc-expressing cells generated by the first pROSA-gLuc administration were not affected by the immune response induced by the second injection of ROSA-gLuc with pCpG-fLuc.



**Figure 7. Time course of serum gLuc activity and activities of both gLuc and fLuc in the liver after simultaneous or sequential hydrodynamic delivery**

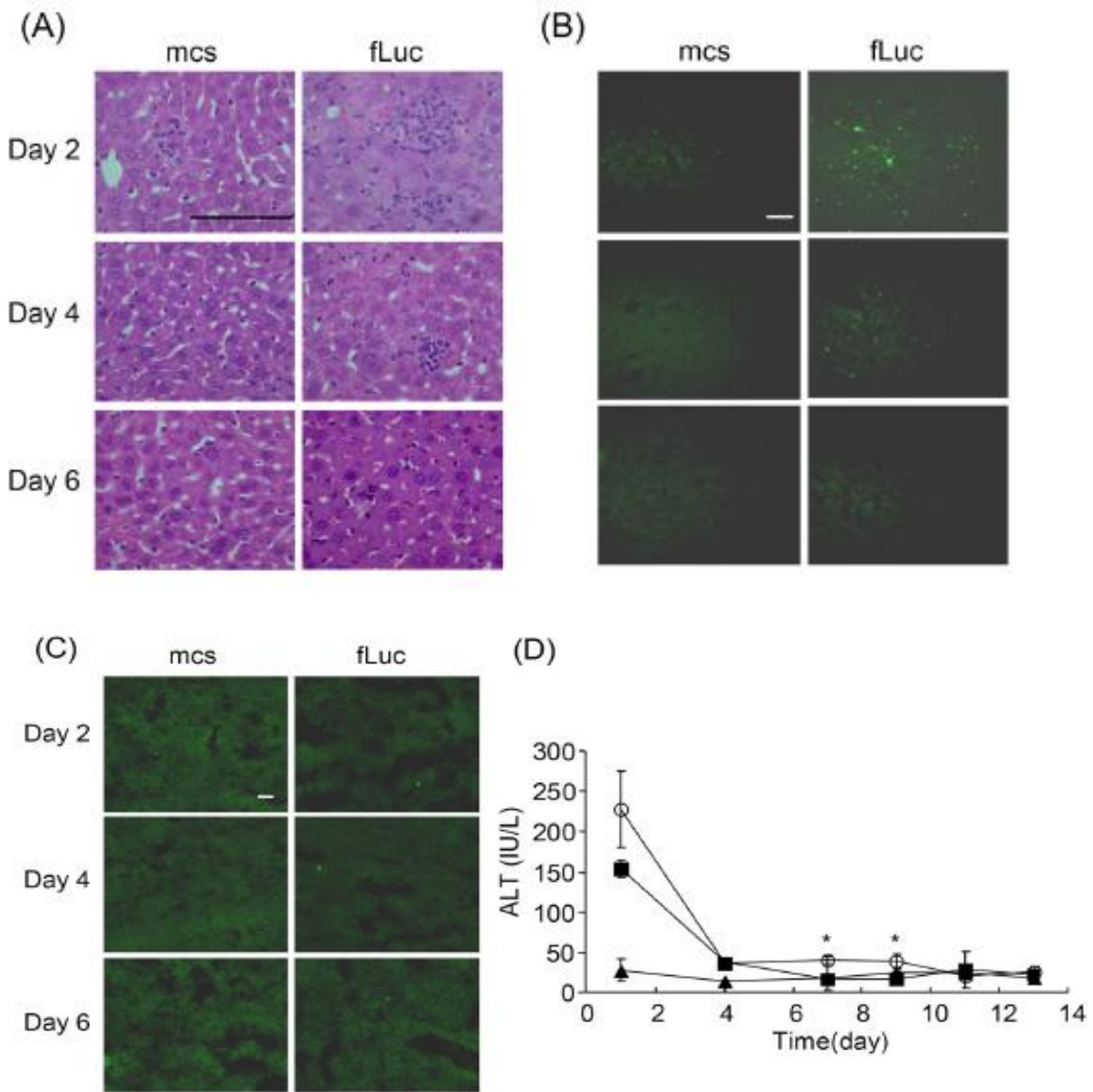
(A) The mice initially received hydrodynamic injection of 10  $\mu$ g of pROSA-gLuc with 10  $\mu$ g of pCpG-fLuc. Fourteen days after the first injection, the mice received co-injection of 10  $\mu$ g of pROSA-gLuc with 10  $\mu$ g of pCpG-fLuc (open circles) or a sequential injection of 10  $\mu$ g of pROSA-gLuc followed by 10  $\mu$ g of pCpG-fLuc after a 12-h interval (closed circles). The results are expressed as the mean  $\pm$  SD of five mice. \* $p$ <0.05 compared to the simultaneous injection group. (B) fLuc and gLuc activities in the liver 21 days after simultaneous (open column) or sequential (closed column) injections of pROSA-gLuc with pCpG-fLuc. The results are expressed as the mean  $\pm$  SD of five mice. \*  $p$ <0.05 compared to the simultaneous injection group. (C) Mice initially received hydrodynamic injection of 1  $\mu$ g of pROSA-gLuc.

Seven days after the first injection, the mice received saline injection (open circle) or co-administration of 10  $\mu$ g of pROSA-gLuc with 10  $\mu$ g of pCpG-mcs (closed square) or pCpG-fLuc (closed triangle). The results are expressed as the mean  $\pm$  SD of five mice.

### **I-3-g Inflammatory cells, including CD8<sup>+</sup> cells, were detected in the liver after pCpG-fLuc administration**

Mice were co-administered pROSA-gLuc with pCpG-fLuc or pCpG-mcs twice with an interval of 2 weeks. The livers were collected from the mice 2, 4 or 6 days after the second injection, and liver sections were prepared to evaluate the effect of the fLuc-specific immune response. HE staining of the liver sections (Figure 8A) revealed a large number of infiltrating cells in the liver 2 days after the second administration of pCpG-fLuc. The number of infiltrating cells declined on day 4 and returned to a level comparable to that found in the pCpG-mcs group by day 6. The liver sections were stained with CD4- or CD8- specific antibodies to characterize the type of infiltrating cells (Figures 8B and 8C). A slight difference was observed in the number of CD4<sup>+</sup> cells between the pCpG-mcs and pCpG-fLuc groups. By contrast, the number of CD8<sup>+</sup> cells was higher in the pCpG-fLuc group than in the pCpG-mcs group 2 days after the second administration; the number decreased on day 4 and returned to a level comparable with that found in the pCpG-mcs group by day 6. Because it was hypothesized that the removal of transgene-expressing hepatocytes by the infiltrating cells was related to hepatic injury, the time-course of serum ALT level was measured.

As previously reported, an increase in serum ALT level was detected immediately after hydrodynamic injection, irrespective of the types of plasmid DNAs, indicating a transient increase in the permeability of cellular membrane by the injection. At 7 and 9 days after injection, the serum ALT level of mice receiving pCpG-fLuc was significantly higher than that of mice receiving pCpG-mcs (Figures 8D). This result also implies that hepatocytes expressing fLuc are damaged by the fLuc specific immune response.

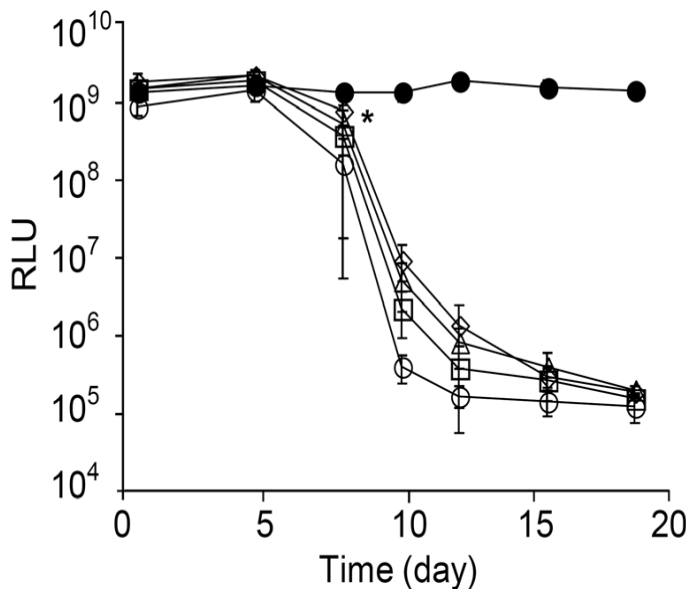


**Figure 8. Histological analysis of the liver after repeated hydrodynamic delivery of fLuc-expressing plasmids.**

(A–C) Mice received hydrodynamic co-injections of 10 µg of pROSA-gLuc with 10 µg of pCpG-mcs or pCpG-fLuc twice with an interval of 2 weeks. Livers were collected from the mice 2, 4 or 6 days after the second injection. The liver sections were subjected to HE staining (A) or immunofluorescence staining using CD8- (B) or CD4-specific antibodies (C). Scale bar=100 µm. (D) Time-course of ALT levels in the serum in untreated mice (closed triangle) or mice receiving hydrodynamic injection of 10 µg of pCpG-fLuc (open circle) or pCpG-mcs (closed square). The results are expressed as the mean ± SD of five mice. \*p<0.05 compared to the pCpG-mcs injected group.

### I-3-h Spleen removal and macrophage depletion did not change the profile of serum gLuc activity after co-administration of pROSA-gLuc with pCpG-fLuc

Although hepatocytes are the chief cell type expressing transgenes after hydrodynamic injections of plasmid DNA, transgene expression also occurs in nonparenchymal cells in the liver including Kupffer cells and in other organs such as the spleen [32]. Kupffer cells and splenic macrophages are known to function as APCs; therefore, the fLuc-specific immune response induced after hydrodynamic injection of pCpG-fLuc might be a result of fLuc expression in these cells. To test this hypothesis, the mice were given hydrodynamic injections of pROSA-gLuc with pCpG-fLuc or pCpG-mcs after spleen removal, macrophage depletion, or a combination of spleen removal and macrophage depletion (Figure 9). Neither treatment was found to affect the time course of serum gLuc activity after administration of pROSA-gLuc with pCpG-mcs (data not shown). However, the serum gLuc activity after co-administration of pROSA-gLuc with pCpG-fLuc decreased in splenectomized or macrophage-depleted mice. Moreover, even in mice subjected to both spleen removal and macrophage depletion, serum gLuc activity was observed to decrease 1 week after administration of pROSA-gLuc with pCpG-fLuc. On the other hand, there was a tendency that the degree of reduction in gLuc activity was slightly smaller in mice receiving splenectomy or macrophage depletion than untreated mice, and the treatments of splenectomy and macrophage depletion appeared to have an additive effect.



**Figure 9. Time course of serum gLuc activity with hydrodynamic injection after spleen removal and macrophage depletion.**

Mice were left untreated (circle) or subjected to macrophage depletion (triangle), spleen removal (square) or both treatments (diamond). The mice were then co-administered 10  $\mu$ g of pROSA-gLuc with 10  $\mu$ g of pCpG-mcs (closed symbols) or pCpG-fLuc (open symbols). The results are expressed as the mean  $\pm$  SD of five mice. \* $p < 0.05$  compared to untreated mice.

## I-4 Discussion

In the present study, single pROSA-gLuc administration was found to result in serum gLuc activity that was stable for more than 1 year, implying that sustained gLuc expression did not induce a gLuc-specific immune response such as anti-gLuc antibody production. On the other hand, sustained fLuc expression induced a fLuc-specific immune response, suggesting that the antigenicity of fLuc protein is higher than that of the gLuc protein.

Co-administration of high doses of pCpG-fLuc with pROSA-gLuc resulted in a reduction in serum gLuc activity, which is likely caused by the removal of transgene-expressing cells as a consequence of the fLuc-specific immune response induced by sustained fLuc expression. On the other hand, co-administration of high doses of pCMV-fLuc, which results in a high but transient transgene expression, induced neither a detectable fLuc-specific immune response, nor a reduction in the serum gLuc activity. This suggests that the duration of fLuc expression would be an important factor for eliciting the fLuc-specific immune response, which in turn eliminates the fLuc-expressing cells. The results obtained in the present study indicate that long-term expression of a transgene has a higher risk of eliciting an immune response than short-term expression.

Aubert *et al.* [33] demonstrated that a cytotoxic immune response was the chief mechanism responsible for the removal of transgene-expressing cells after retroviral-mediated  $\beta$ -galactosidase gene transfer in the liver. In addition, an *ex vivo* study suggested that a cytotoxic immune response resulted in the removal of transgene (green fluorescent protein)-expressing cells in concert with the helper T cells after an epidermal gene transfer [34]. In the present study, hydrodynamic injection of pCpG-fLuc induced a cellular immune response specific to fLuc. In addition, CD8<sup>+</sup> cell infiltration was observed in the liver of themice that received pCpG-fLuc administration. These results suggest that fLuc-specific cytotoxic T lymphocytes play an important role in eliminating the fLuc-expressing cells, which is in agreement with previous studies [33]. In addition, hydrodynamic injection of pCpG-fLuc and pROSA-gLuc resulted in the increase in serum ALT levels at approximately 1 week after the administration, when the decline in gLuc activity occurred, also suggesting that hepatocytes expressing both fLuc and gLuc were damaged. Furthermore, fLuc-specific antibodies may be involved in the removal of the fLuc-expressing cells.

A reduction in serum gLuc activity was observed only when pROSA-gLuc was co-administered with pCpG-fLuc. On the other hand, gLuc expression from preadministered pROSA-gLuc was scarcely affected by the second injection of pROSA-gLuc with pCpG-fLuc. From these results, it is likely that the decrease in serum gLuc activity after the simultaneous injection of pROSA-gLuc and pCpG-fLuc is caused by the elimination of cells expressing both gLuc and fLuc, as a consequence of the fLuc-specific immune response. This fact suggests that co-administration of pROSA-gLuc with a vector expressing a

gene of interest could be a useful method for monitoring the effect of an immune response to the product of interest in transgene-expressing cells. However, there is a possibility that transgene expression level from a plasmid vector may be affected by the simultaneously delivered plasmid vector via the interference in transgene expression process [35]. Detailed molecular studies would be required to exclude this possibility.

When the transgene product is a secretory protein, the transgene-expressing cells secrete the products so that they may be taken up by APCs to elicit an immune response [36–39]. fLuc is a nonsecretory protein and is scarcely released from the fLuc-expressing cells. Therefore, a fLuc-specific immune response induced after pCpG-fLuc administration is likely a result of the direct gene delivery of plasmid DNA into APCs. By using a target sequence for miRNA-142-3p, a miRNA highly expressed in APCs, Brown *et al.* [40] demonstrated that transgene expression in APCs, resulting in sustained transgene expression, is the chief reason for the induction of an immune response to the transgene products. Hydrodynamic injection also delivers plasmid DNA into Kupffer cells in the liver and splenic cells, both of which function as APCs; I therefore evaluated the role of Kupffer and splenic cells in the induction of the immune response. I found that, even in mice that had been subjected to both spleen removal and macrophage depletion, serum gLuc activity declined after hydrodynamic administration of pROSA-gLuc with pCpG-fLuc, which implied that the fLuc-specific immune response could eliminate fLuc-expressing cells in the absence of macrophages in the liver (Kupffer cells) or spleen (splenic macrophages), or in fact any splenic cells. It is known that the liver has some dendritic cells; gene transfer to the dendritic cells might occur after hydrodynamic injection of plasmid DNA [41] and these dendritic cells might function as APCs. In addition, hepatocytes might also function as APCs after hydrodynamic injection because hepatocytes have been previously reported to exhibit antigen presenting ability [42–44]. Further studies are required for determining which types of cells functioned as APCs in the induction of the fLuc-specific immune response after hydrodynamic injection of pCpG-fLuc.

Although induction of an immune response to a transgene product is a serious problem in gene therapy, eliciting the immune response is, in turn, desirable for DNA vaccination. My results demonstrate that single pCpG-fLuc administration could induce a strong cytotoxic immune response against fLuc; therefore, hydrodynamic administration of the recombinant pCpG vector encoding an antigenic protein might prove to be potent as a DNA vaccine because it also shows sustained transgene expression. On the other hand, in the development of DNA vaccine by hydrodynamic gene delivery, the administration of adjuvant is desirable to induce stronger immune response because the degree of immune activation is limited even after hydrodynamic delivery of CpG-rich plasmid DNA [45].

In conclusion, the present study demonstrates that high levels of sustained expression of a transgenic antigen induce an immune response and that the cells expressing the transgene product are eliminated,



probably as a result of the transgene product-specific immune response.

## Chapter II

### Importance of transgene expression level in each cell on the induction of transgene expression-specific immune response

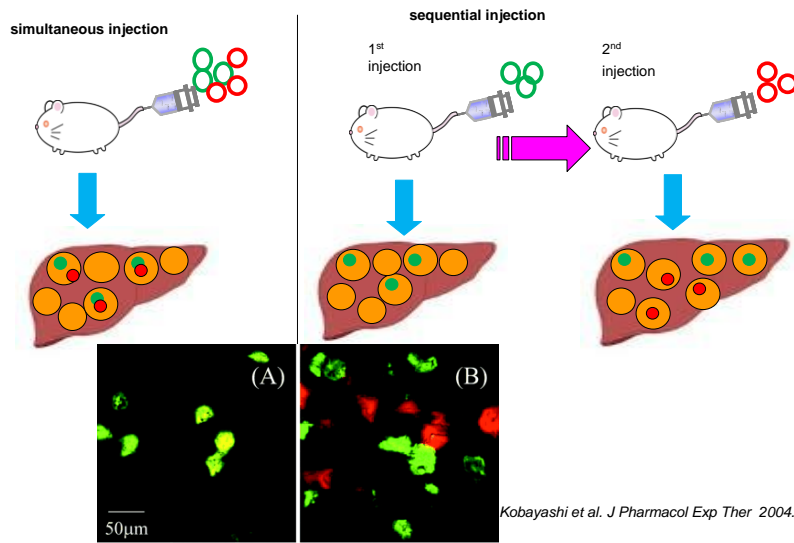
#### II-1 Introduction

Induction of transgene product-specific immune response is a serious concern in gene therapy because it can result in serious adverse effects and reduction in the therapeutic effect by affecting the transgene expression profile [46, 47]. It has been demonstrated that several factors such as the antigenicity of the protein and transgene expression profile are important for the induction of the immune response [48]. In Chapter I, I demonstrated that hydrodynamic injection of long-term expression plasmid vector encoding transgene product with antigenicity induced transgene-specific immune response in a dose-dependent manner [49]. There are several literatures reporting about the importance of transgene expression level in the induction of the immune response [50, 51]. However, in these previous studies, transgene expression level was evaluated by measuring the amount of transgene product in organ or in the blood, so that it was not clear whether total amount of transgene product or the level of transgene expression per cell is important for the induction of immune response.

In Chapter I, I found that hardly affected immune response was induced by hydrodynamic gene transfer in mice that received spleen removal or macrophage depletion [49]. In addition to my result, it has been shown that reduction in transgene expression level in dendritic cells (DCs) by utilization of DC-specific micro RNA hardly affected immune response induced after hydrodynamic gene transfer [25]. From these facts, it was hypothesized that the major transgene-expressing cells after hydrodynamic gene transfer, hepatocytes, are the chief APCs in the induction of transgene-specific immune response after hydrodynamic gene transfer. It has been reported that hepatocytes might work as APCs in some situations [52-54]. If hepatocytes work as APCs after hydrodynamic gene transfer, transgene expression level per hepatocyte is important factor for the induction of transgene-specific immune response as enough amount of antigenic protein in APCs is required for the antigen presentation.

In Chapter II, I investigated the impact of transgene expression level in each cell on the induction of transgene-specific immune response and investigated whether hepatocytes work as APCs after hydrodynamic gene transfer. I chose *Cypridina* luciferase (cLuc) as a model antigen protein because of its characteristics as secretory reporter protein [55-57]. As simultaneous hydrodynamic injection of two types of plasmid DNAs results in the overlap in the cells expressing different transgenes while sequential injection of the vectors results in the expression in the different cells [28], I utilized this experimental procedure to obtain equal amount of total transgene expression level with different level of transgene

expression per cell (Figure 10). In addition, ovalbumin (OVA) was also used as a model antigen to investigate whether hepatocytes work as APCs.



**Figure 10. Experimental image for the evaluation of immune response for transgene expression per cell.**

## II-2 Material and Methods

### Plasmid DNA

pCpG-mcs was obtained as described in Chapter I. Plasmid vectors encoding cLuc, pCpG-cLuc and pCMV-cLuc, were constructed by cLuc cDNA fragment ( obtained from pCMV-*Cypridina* Luc vector, Thermo Fisher Scientific, Waltham, MA, USA) into pCpG-mcs and pcDNA3.1 (Life Technologies, Carlsbad, CA, USA), respectively. pROSA-cLuc, a plasmid expressing cLuc, pROSA-gLuc, a plasmid expressing *Gaussia* luciferase (gLuc), was constructed as described in Chapter I. pCpG-OVA and pCMV-OVA were constructed as described previously [58,59].

### Mice and plasmid DNA administration

Four-week-old female ICR mice (approximately 20 g body weight), six-week-old female BALB/c mice (approximately 20 g body weight) were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were brought under deliberation and approved for the Ethics Committee for Animal Experiments at the Graduate School of Pharmaceutical Sciences, Kyoto University. Administration of plasmid DNA to mice was performed by the hydrodynamics-based procedure in which plasmid DNA dissolved in 8 % vol/ body weight of saline were injected into the tail vein of mice over less than 5 s.

## **Cell culture**

CD8 OVA1.3 T cells, T hybridoma cells against SIINFELK-Kb [60], were a generous gift from Dr. C. V. Harding (Case Western Reserve University, Cleveland, OH). Hepatocytes were isolated from mice according to a standard two-step perfusion protocol [62, 63]. CD8OVA1.3 T cells were cultured in Dulbecco's modified Eagle medium (Nissui Co.Ltd, Tokyo, Japan) supplemented with 10 % heat-inactive fetal bovine serum (Equithedh-Bio, Kerrville, TX), 0.5 mM monothioglycerol, 2 mM L-glutamine, antibiotics and nonessential amino acid (all from Life Technologies, Carlsbad, CA). CD8 OVA1.3 T cell and primary hepatocytes were co-cultured in RPMI 1640 medium (Nissui Pharmaceuticals, Tokyo, Japan) supplemented as described for Dulbecco's modified Eagle medium.

## **Luciferase Assay**

At indicated time points, blood was collected from the tail vein of mice. To measure luciferase activities in the liver, the liver was harvest and homogenized in 10 ml/g liver of lysis buffer (0.1 M Tris (pH 7.8), 0.05 % TritonX-100, 2 mM EDTA), and the homogenates were centrifuged at  $12000 \times g$  for 10 min at 4 °C. Then, the supernatant was mixed with Cypridina luciferase assay buffer (Thermo Fisher Scientific, Rockford, USA) or luciferase assay buffer (PicageneDual, Toyo Ink, Tokyo, Japan) to measure cLuc activity and gLuc activity, respectively. The chemiluminescence produced was measured in a luminometer (Lumat LB 9507; EG and G Berthold, Bad Wildbad, Germany).

## **Detection of antibody**

Serum samples were obtained as described above. The amount of cLuc-specific and OVA-specific antibodies was measured by enzyme linked immunosorbent assay (ELISA) as described previously [49].

## **Interferon (IFN)- $\gamma$ production**

Indicated days after gene transfer, spleens were isolated from the immunized mice and single cell suspensions were prepared. Cells were stimulated by addition of 0.1 mg/ml cLuc or 0.5 mg/ml OVA for 4 days. IFN- $\gamma$  levels in the supernatant were measured by ELISA using antibodies for capture and detection in accordance with the Manufacturer's protocol (Ready-SET-Go! Mouse IFN- $\gamma$  ELISA, eBioscience, San Diego, CA, USA).

## **PKH26 cell labeling**

PKH26 was purchased from Sigma-Aldrich, and used to label cells as described in manufacturer's protocol. PKH26-labeled cells were seupended in Hanks' balanced salt solution and intravenously administrated into mice.

### **Histochemical analysis of the liver**

Livers were collected from euthanized mice and embedded in Tissue-Tek OCT embedding compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen. Frozen liver sections (10  $\mu\text{m}$  thick) were made using a cryostat (Jung Frigocut 2800E; Leica Microsystems AG, Wetzlar, Germany) by the routine procedure. The sections were fixed with 4% paraformaldehyde in PBS.

### **Antigen presentation assay**

Hepatocytes were purified as described above and co-incubated with CD8 OVA1.3 T cells in RPMI 1640 medium as described above. After 24 h incubation, culture supernatants were collected and Interleukin-2 (IL-2) levels in the supernatants was measured by ELISA (BD OptiEIA Mouse IL-2, BD Biosciences, San Diego, CA) as an indicator of CD8 OVA 1.3 T cell stimulation [62-64].

### **Statistical analysis**

Differences were statistically evaluated by Student's t-test. The level of statistical significance was set at  $P < 0.05$ .

## **II-3 Results and Discussion**

Expression profile of cLuc after administration of different doses of pCpG-cLuc, pROSA-cLuc or pCMV-cLuc was evaluated. After hydrodynamic delivery of pCpG-cLuc and pROSA-cLuc, serum cLuc activity was stable at early time points (data not shown). After the administration of high doses of pCpG-cLuc or pROSA-cLuc, cLuc activity in the serum declined approximately 10 days after the administration, which suggests the removal of transgene-expressing cells by cLuc-specific immune response. On the other hand, administration of pCMV-cLuc resulted in high serum cLuc activity at early time points followed by immediate decline (data not shown).

Next, pROSA-gLuc was co-administrated with different doses of pROSA-cLuc to further investigate the effect on transgene-expressing cells. gLuc activity in the serum was stable after co-administration of pROSA-gLuc with 1 and 10  $\mu\text{g}$  pROSA-cLuc while reduction in serum gLuc activity was observed 9 days after the co-administration of pROSA-gLuc with 20 and 30  $\mu\text{g}$  of pROSA-cLuc (data not shown). In addition to serum gLuc activity, serum cLuc activity started to decline at 9 days after the co-administration of pROSA-gLuc with 20 and 30  $\mu\text{g}$  of pROSA-cLuc, which implies the removal of cells expressing both cLuc and gLuc by cLuc-specific immune induced by the administration of high doses of pROSA-cLuc.

To investigate the importance of transgene expression level per cell on the induction of

transgene-specific immune response mice received single injection of high dose (30  $\mu\text{g}$ ) of pROSA-cLuc or thrice injection of low dose (10  $\mu\text{g}$ ) of pROSA-cLuc. After single administration of high dose of pROSA-cLuc, serum cLuc greatly decreased at day 10 while serum cLuc activity was stable for 3 weeks in mice that received thrice injection of low dose of pROSA-cLuc despite the fact that cLuc level in the serum was comparable between these two groups at early time points (data not shown). From this result, it was implied that high level of transgene expression per cell is necessary for the induction of immune response. To evaluate cLuc-specific humoral immune response, the amount of cLuc-specific antibody in the serum was measured on day 14. cLuc-specific antibody was detected in the both groups, although more cLuc-specific antibody was detected in single administration of high dose of pROSA-cLuc group than that in 3 times injection of low dose of pROSA-cLuc group (data not shown). Next, to evaluate cLuc-specific cellular immune response, splenocytes were collected from the gene-delivered mice and stimulated with cLuc. Significantly higher amounts of IFN- $\gamma$  was produced from splenocytes of mice that received single administration of high dose of pROSA-cLuc than that from the splenocytes of mice that received thrice injection of low dose of pROSA-cLuc (data not shown). On the other hand, IFN- $\gamma$  was hardly produced from splenocyte of mice that received thrice injection of low dose of pROSA-cLuc, which suggests that cellular immune response was hardly induced in this group. These results imply that cLuc-specific cellular immune response was more important in the reduction in cLuc activity, which is supposed to be caused by the removal of cLuc-expressing cells, than humoral immune response. In addition to my results, importance of cellular immune response is in the removal of transgene-expressing cells was also reported by Ian *et al* [65]. In addition, these results also suggest that high level of transgene expression per cell is required for the induction of transgene-specific cellular immune response after hydrodynamic gene transfer.

As it was found that transgene expression level per cell is important for the induction of immune response, I hypothesized that transgene product in hepatocytes were presented by hepatocytes to stimulate T cells. To investigate the hypothesis, I selected OVA as model antigen and CD8 OVA 1.3 T cell, CD8-T cell expressing a TCR specific for the OVA epitope was used as model T cell. First, it was confirmed that hydrodynamic injection of pCpG-OVA induced OVA-specific humoral and cellular immune response by the procedure described above (data not shown). Then, to investigate whether antigen presentation, which is mediated by direct contact of APCs with T cells, occurs in the liver after hydrodynamic gene transfer, PHK26-labeled CD8 OVA 1.3 T cells were intravenously administered to mice that had received hydrodynamic gene transfer. As a result, more CD8 OVA1.3 T cells were detected in the liver of mice receiving pCpG-OVA than that in the mice receiving pCpG-mcs or pCpG-cLuc (data not shown).

To investigate whether antigens are presented by hepatocytes, mice received pCpG-OVA administration and hepatocytes were isolated from the mice. The isolated hepatocytes were cocultured with CD8

OVA1.3 T cells and IL-2 secretion from CD8 OVA1.3 T cells was measured. As a result, amount of IL-2 released from CD8 OVA1.3 T cells cocultured with hepatocytes collected from mice receiving pCpG-OVA was significantly higher than that from CD8 OVA1.3 T cells cocultured with hepatocytes collected from mice receiving pCpG-cLuc or pCpG-mcs (data not shown), which suggests that OVA-specific epitope was presented by hepatocytes collected from mice receiving pCpG-OVA administration.

In conclusion, it was found that the level of transgene expression per cell is important in the induction of transgene-specific immune response after hydrodynamic gene transfer. Moreover, it was also demonstrated that hepatocytes may work as APC after hydrodynamic gene transfer.

## Summary

In this thesis, the effect of transgene expression profile and types of transgene expressing cells on the induction of transgene-specific immune response was investigated. The main findings obtained in each chapter are as follows.

### **I. Elucidation of the effect of the duration of transgene expression on the induction of transgene-specific immune response**

A high level of sustained fLuc expression in the liver triggered antigen-specific immune responses while short-term expression of the fLuc elicits little, if any, immune response. Moreover, fLuc-specific immune response induced by the sustained transgen fLuc expression removed the cells expressing fLuc in turn. When gLuc-expressing vector was coadministered with sustained fLuc-expressing vector, sudden decrease in gLuc expression at approximately 1 week after the coadministration occurred, which indicates that cells expressing both fLuc and gLuc were recognized and attacked by fLuc-specific immune response. By histological analysis of the liver sections of mice, CD8<sup>+</sup> cell infiltration was observed, implying that the transgene-expressing hepatocytes were removed by the infiltrating cells. In addition, spleen removal and macrophage depletion did not change the profile of serum gLuc activity after co-administration of pROSA-gLuc with pCpG-fLuc, suggesting that fLuc-specific immune response induced by sustained fLuc expression is not dependent on the transgene expression in these types of cells.

### **II. The importance of transgene expression level in each cell on the induction of transgene expression-specific immune response**

In Chapter II, the importance of transgene expression level in each cell on the induction of transgene expression-specific immune response was investigated. By using pROSA-cLuc, it was found that the transgene expression level per cells, not the total amount of transgene, is important in the induction of the transgene-specific immune response after hydrodynamic gene transfer. In addition, it was also suggested that higher level of transgene-expression level per cells was required for the induction of transgene-specific cellular immune response than that was required for the induction of transgene-specific humoral immune response.

After pCpG-OVA hydrodynamic gene transfer, CD8 OVA1.3 T cells accumulated in the liver while nearly no CD8 OVA1.3 T cells were found after pCpG-cLuc and pCpG-mcs injection, which suggests that CD8 OVA1.3 T cells received antigen presentation in the liver via direct contact. IL-2 secretion from CD8 OVA1.3 T cells co-cultured with hepatocytes collected from mice receiving pCpG-OVA implies that hepatocytes expressing high level of an antigenic transgene product work as APCs to prime



transgene-specific immune response after hydrodynamic gene transfer.

In conclusion, I demonstrated that a high level of sustained expression of antigenic transgene induces an immune response that removes the cells expressing the transgene product and that the transgene expression level per cells, not the total amount of transgene product, is important in the induction of transgene-specific immune response after hydrodynamic gene transfer. In addition, transgene expression in the spleen and macrophages are not important in the induction of transgene-specific immune response after hydrodynamic gene transfer, while high level of transgene expression per cell is important in the immune induction. Moreover, it was demonstrated that hepatocytes with high level of transgene expression may prime transgene-specific immune response after hydrodynamic gene transfer. These results provide useful information to treat with the immune response in gene therapy.

## **Acknowledgements**

The author wishes to express sincerely her wholehearted and utmost gratitude to Professor Yoshinobu Takakura for providing me the opportunity to study in Kyoto University, and his considerable understanding, warm support, continuous encouragement, and thoughtful guidance throughout this study.

The author wishes to express her deepest appreciation to Associate Professor Makiya Nishikawa for his logical thinking guidance and direction, valuable discussions, perceptive and supports during the whole this study.

The author would like to express sincere thanks to Dr. Yuki Takahashi for his logical thinking guidance and direction, valuable discussions, perceptive and supports during the whole this study.

The author is greatly indebted to Dr. Mitsuru Ando, Mr. Norifumi Ebisuura and all members of Department of Biopharmaceutics and Drug metabolism and of Drug Delivery Research, Graduate School of Pharmaceutics Sciences, Kyoto University, for their experimental assistance.

Finally, the author would like to express her deepest gratitude to her family and friends for their love, encouragements, supports and understanding throughout the course of this study.

## List of Publications

Removal of transgene-expressing cells by transgene-specific immune response induced by sustained transgene expression

Yalei Yin, Yuki Takahashi, Norifumi Ebisuura, Makiya Nishikawa, Yoshinobu Takakura

*Journal of Gene Medicine*, 2014; 16: 97–106.

Induction of transgene-specific immune response dependent on the transgene expression level per cell after hydrodynamic gene transfer

Yalei Yin, Yuki Takahashi, Makiya Nishikawa, Yoshinobu Takakura

*Manuscript in preparation*

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