A DNA microarray based analysis of the host response to a non-viral gene carrier: a strategy for improving the immune response

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Short title: Microarray analysis of systemic delivery system

Abstract

The purpose of this study was to investigate the host response to systemically administered lipid nanoparticles (NPs) encapsulating pDNA in the spleen using a DNA microarray. As a model for NPs, we used a Multifunctional envelope-type nano device (MEND). Microarray analysis revealed that 1581 of the differentially expressed genes could be identified by PEG-unmodified NP using a 3-fold change relative to the control. As the result of PEGylation, the NP treatment resulted in the reduction in the expression of most of the genes. However, the expression of type I interferon (IFN) was specifically increased by PEGylation. Based on the microarray and a pathway analysis, we hypothesize that PEGylation inhibited the endosomal escape of NP, and extended the interaction of TLR9 with CpG-DNA accompanied by the production of type I IFN. This hypothesis was tested by introducing a pH-sensitive fusogenic peptide, GALA, which enhances the endosomal escape of PEGylated NP. As expected, type I IFN was reduced and IL-6 remained at the baseline. These findings indicate that a carrier design based on microarray analysis and the manipulation of intracellular trafficking constitutes a rational strategy for reducing the host immune response to NPs.

Introduction

The success of clinical gene therapy greatly depends on the development not only of efficient but also safe gene delivery systems [1]. Because of the ease of large-scale production and lack of a specific immune response unlike viral vectors, various types of non-viral gene delivery systems such as lipoplexes, polyplexes, and micelles have been developed, in attempts to improve the efficiency of in vivo gene expression [2-4]. However, innate immune responses are induced by the systemic administration of a lipoplex [5]. Unmethylated CpG motifs of plasmid DNA in a lipoplex have been reported to stimulate the innate immune response by interacting with host Toll-like receptor-9 (TLR9), expressed in endosomes, and to trigger the release of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-12 and type I interferon (IFN) [6]. It was reported that a lipoplex containing either methylated CpG or non-CpG pDNA reduced cytokine production, but the reduction was not complete [5,7,8]. Furthermore, cytokine production was not completely abolished in $TLR9^{-/-}$ mice after an i.v. administration of a lipoplex or in primary cultured macrophages from $TLR9^{-/-}$ mice after lipoplex treatment [7,9]. DNA-dependent activator of IFN-regulatory factor (DAI) has been identified as a cytosolic DNA sensor [10]. DAI, also known as Z-DNA binding protein-1 (ZBP1), recognizes dsDNA in a CpG-independent manner, which causes an TLR9-independent innate immune response [11]. These findings suggest that the immune reaction to a lipoplex is more complicated than previously thought. This appears to be true for viral

vectors [12] as well, and an understanding of host responses to the systemic administration of a lipoplex is necessary for the successful and efficacious development of in vivo gene delivery systems. However, examining the production of certain types of cytokines after i.v. administration is not sufficient to guarantee the safety of a gene delivery system.

To address and solve this issue, gene expression profiling represents a promising approaches to understanding the underlying mechanism of host responses [13-19]. Kay and co-workers reported that a DNA microarray-based comparison of the host response to adenoviral (Ad) and adeno-associated viral (AAV) vectors revealed that the host recognition of capsid and DNA of AAV is different from that of Ad [13]. This approach has been also applied to non-viral vectors in the form of toxicogenomics studies [16-19]. In the case of a polypropylenimine dendrimer based DNA complex, a microarray analysis revealed that gene expression in culture cells was altered by the generation of the dendrimer, and was dependent on the cell lines [16]. However, the response of a host to a systemically administrated non-viral gene vector has not been examined using this approach.

We recently developed a novel lipid nanoparticle (NP), a multifunctional envelope-type nano device (MEND), in which pDNA is condensed with a polycation, followed by encapsulation with a lipid envelope [20]. In the present study, an analysis of splenic expression profiles in mice was conducted after the intravenous injection of MENDs as an NP model, using a whole-genome DNA microarray. Since the spleen is the largest secondary lymphoid organ and contains tissue macrophages that are

associated with an immune response after an intravenous injection of a lipoplex [21]. It was hypothesized that modification with PEG would confer biocompatibility for non-viral vectors, resulting in an improved safety [22]. It would permit us to predict whether PEGylation would change the gene expression profile by NP administration for the better. However, since only a few studies of the effect of PEGylation on host response have appeared, detailed information on the influence of PEGylation is not available. Therefore, we attempted to elucidate the effect of the PEGylation of NP (PEG-NP) on the host response.

Results

Characterization of NPs

The average diameter and ζ-potential of the condensed pDNA/PEI complex particles were approximately 80 nm and -50 mV, respectively. The average diameters and ζ-potentials of the prepared NPs are summarized in Table 1. The PEG-unmodified NP (NP) was around 200 nm in diameter, and was highly positively charged due to the presence of a cationic lipid. PEG modification (PEG-NP) reduced the diameter of the NP and the positive charge was decreased, compared to an unmodified NP, as the result of the formation of a stable lamellar structure with a larger curvature and masking of the surface of the lipid envelope by the aqueous layer of the PEG moiety [23]. Modification of PEG-NP with chol-GALA (GALA-NP) slightly reduced the ζ-potential of the NP since GALA contains negatively charged glutamic acid residues, but it had no influence on the diameter.

Microarray data analysis

To understand what occurs in a host following the systemic administration of an NP and a PEG-NP, splenic gene expression profiles in mice were generated using whole genome oligonucleotide microarrays. The spleen is the largest secondary lymphoid organ and is associated with the immune response [21]. Mice were injected via the tail vein with Hepes-buffered glucose (HBG), NP or

PEG-NP. After 2 hr, the spleen was collected and RNA prepared from the tissues, microarrays were then hybridized, as described in the Materials and Methods section. Using a 3-fold change relative to the HBG treatment as a criterion for differential expression, 1581 genes were extracted from the administration of NP. A clustergram of these 1581 genes is shown in Figure 1. The down-regulated 402 genes resulting from the NP treatment, compared to HBG were classified in Cluster 1, and the other 1179 genes, which were up-regulated by the NP treatment, were classified into Clusters 2 to 5. In Clusters 1, 3 and 5 (55.8%), the variation in gene expression as the result of the PEG-NP treatment were reduced compared to the corresponding value for NP, suggesting that PEGylation reduces the biological stimulation of NP after systemic administration. On the other hand, the gene expression in Cluster 2 showed subtle alterations between NP and PEG-NP (42.7%). PEG-NP unexpectedly caused an increase in gene expression compared to NP, as shown in Cluster 4 (1.5%).

Gene Ontology (GO) analysis is used to identify the molecular pathways and describe the biological processes of the transcript profiling data. Based on the GO analysis, the GO terms of “Biological process” that were significantly overrepresented in each Cluster are shown in Table 2. The GO terms of the down-regulated genes are related to cell division such as “cytokinesis”, “mitosis”, “M phase of mitotic cell cycle”, differentiation and metabolism. On the other hand, the majority of GO terms for the up-regulated genes in Clusters 2 to 5 are mainly associated with “immune response”, “response to biotic stimulus”, “defense response” and related processes, which are generally

associated with the immune system. These observations indicate that the characteristics of the up-regulated genes and down-regulated genes resulting from the NPs treatment were completely different.

We further listed the top 25 genes in order of greatly altered expression level by the NP treatment compared with HBG in each Cluster, as shown in Table 3. The ratios of the gene expression level of PEG-NP/NP in Clusters 1, 3 and 5 were improved, and the ratios in Cluster 2 were comparable. However, the ratios for PEG-NP/NP in Cluster 4 were greatly enhanced. In Cluster 3, inflammatory cytokines such as IL-6 and IFN-γ are ranked higher with significantly lower levels of expression in the PEG-NP treatment compared to NP. As shown in Table 3, IFN-α subtypes and IFN-β, classified as type I IFN, are specifically located in Cluster 4.

Quantification of mRNA level in spleen and cytokine level in serum

To verify that mRNA levels are elevated in the spleen, the mRNA expression of IL-6, CD14, located in Cluster 3, and IFN-α and IFN-β, located in Cluster 4, the genes were further evaluated by quantitative RT-PCR. As shown in Figure 2, the semiquantitative RT-PCR results were in good agreement with the expression information from the microarray analysis, confirming that these genes are actually up-regulated after NP or PEG-NP administration. We next assessed the levels of IL-6 and IFN-α in serum at 2 and 6 hr after an i.v. injection of NP and PEG-NP. As shown in Figure

3a, NP induced the production of IL-6, and PEGylation markedly reduced the serum levels of IL-6. On the other hand, the serum level of IFN-α in the case of PEG-NP was equal or greater than that for NP. These observations were correlated with the amount of mRNA in the spleen, as evidenced by microarray analysis and quantitative PCR (Figure 2).

Pathway analysis and the effect of the acceleration of endosomal escape of PEGylated NP by GALA on type I IFN production

Based on the microarray analysis, PEGylation generally reduced the biological reaction to systemically administered NP. However, contrary to our expectations, PEGylation stimulated the production of type I IFN. To identify the mechanism underlying this, we performed a pathway analysis. According to the GO analysis and a subsequent quantitative determination of mRNA in the spleen, immune stimulation constituted a major biological reaction in the host after the systemic administration of NP. Since members of the TLR family are essential components in the CpG-mediated immune response, we focused on TLR pathway signaling using the Kyoto Encyclopedia of Genes and Genomes (KEGG) data base. As shown in Figure 4, IL-6, IL-1β and CD14, located in Cluster 3 (red) and IFN-α and IFN-β, located in Cluster 4 (blue) fall into Toll-like receptor signaling pathway.

As described above, PEGylation confers biocompatibility and safety for NPs. On the other hand, it

was reported that the modification of NP with PEG crucially inhibits the endosomal escape of NP [24,25], resulting in a reduced activity of the cargo. We assumed that endosomal trapping triggered the excessive interaction of the pDNA encapsulated in PEG-NP with TLR9 following destabilization and digestion of the PEG-NP in endosomes/lysosomes, which resulted in an enhanced type I IFN production. We previously demonstrated that a pH-sensitive fusogenic peptide, GALA promoted the endosomal escape of PEGylated NP, which resulted in enhanced gene expression and silencing activity [26-29]. To test the assumption, we examined the effect of the GALA modification of PEG-NP (GALA-NP) on the immune response. The physical properties of the prepared GALA-NP were nearly the same as those for PEG-NP (Table 1). As shown in Figure5, GALA modification successfully diminished serum IFN-α levels and IL-6 remained at a low level. The gene expression of IFN-α in the spleen was also reduced by GALA modification.

Discussion

In the present study, we applied a microarray analysis to understand the host response to pDNA encapsulated in lipid nanoparticles (NPs). For the microarray analysis, we used a MEND, in which pDNA is condensed with PEI, followed by encapsulation with a lipid envelope consisting of DOTAP, DOPE and cholesterol. The systemic administration of the PEI/pDNA complex alone induced severe hepatotoxicity, but the innate immune response was negligible, unlike NPs (Figure S1). These findings suggest that pDNA/PEI complex was successfully encapsulated by the lipid envelope of the MEND. These findings were also consistent with previous findings reported by Kawakami et al. in which a linear PEI polyplex showed negligible cytokine production and higher serum ALT levels after i.v. injection as compared with a DOTMA based lipoplex [30,31]. From this viewpoint, the MENDs can be thought of as a model of an NP.

The microarray analysis showed that, after the systemic administration of NPs, the up-regulated genes in the spleen were mainly related to the immune system and the down-regulated genes were associated with mitosis and differentiation, as shown in Table 2. These findings suggest that the characteristics between up- and down-regulated genes are completely different, presumably because the innate up-regulation of a gene related to immune system might turn out to down-regulate genes related to the maintenance of cell function such as cytokinesis, mitosis and cell differentiation. As we assumed, the variation in gene expression including IL-6 and IFN-γ in

Clusters 1, 3 and 5 (55.8%) showed a tendency for improvement (Figure 1 and 2). Serum inflammatory cytokines such as IL-6, TNF-α and IFN-γ were significantly decreased as the result of PEGylation (Figure 3 and Figure S1). Therefore, PEGylation appears to contribute to the prevention of inflammatory cytokine production. However, the variation of expression in Cluster 2 (42.7%) was equivalent to the level of NP (Figure 1). Unexpectedly, the expression of type I IFN in the spleen was conversely aggravated by PEGylation (Cluster 4; 1.5%)(Figure 1 and 2). As shown in Figure 3, the serum level of IFN-α in PEG-NP was equal to or greater than that for NP, in good agreement with the mRNA levels in the spleen. These results suggest that even though PEG is a well known biocompatible macromolecule, PEGylation is not an adequate solution to averting a host response to NPs.

The microarray analysis indicated that PEGylation altered the production of inflammatory cytokine such as IL-6 (better) and type I IFN such as IFN-α (unchanged or worse) in a different pattern. The question arises as to the cause of the production of inflammatory cytokines and type I IFN by NP and PEG-NP. PEG modification under these conditions did not alter the splenic accumulation of NP after systemic administration (Figure S2). Therefore, the change in cytokine production might be caused after NPs that had arrived in the spleen. The innate immune response to a lipoplex is partially, but not entirely, dependent on the CpG motif in pDNA via TLR9, which induces the production of type I IFN and inflammatory cytokines [6]. The plasmid DNA used in the present

study contains 425 CpG motifs.

Hartman et al. previously reported that a pathway analysis following a microarray of Ad revealed that the Myeloid differentiation primary response gene (88) (MyD88) in the TLR signaling pathway plays a major role in the immune response to Ad [14,15]. To elucidate the underlying mechanisms of the response to NP and PEGylated NP, we then focused on the Toll-like receptor signaling pathway using the KEGG database. As a result of the pathway analysis, IL-6, IL-1β, and CD14 in Cluster 3, and IFN-α and -β in Cluster 4 correspond to the Toll-like receptor signaling pathway (Figure 4). CD14 is a glycosylphosphatidylinositol-anchored cell surface protein that is expressed by phagocytic cells [32]. The recognition of Lipopolysaccharide (LPS) by cells is mediated by the LPS receptor complex, which consists of TLR4, MD2 and CD14 [6]. It was observed that CD14 expression by bone marrow granulocytes and odontoblasts was increased by treatment with an agonist for TLR4, such as LPS [33,34]. It was reported that diC14-amidine, a cationic lipid, is assumed to be an agonist for TLR4 due to the association of the acyl chains of diC14-amidine with the hydrophobic pocket in MD-2 [35]. Empty liposomes using the same lipid component in the envelope of NP showed neither inflammatory cytokine nor type I IFN production after systemic administration (data not shown). Therefore, it is very unlikely that the lipid components used in the present study have the potential to function as a TLR4 agonist. However, since CD14 expression was significantly altered, an NP that included pDNA would not be irrelevant to a TLR4 mediated immune response. Kedmi et al.

recently reported that the immune activation of DOTAP based cationic lipid nanoparticles containing siRNA might occur via TLR4, which provides support for our prediction [36].

PEGylation decreased the expression of CD14 as shown in Figure 2b, presumably because the cationic charge on the surface of the lipid envelope was masked by the PEG layer, which reduced the interaction of NP with biological milieu such as cellular membrane components. On the other hand, as describe above, PEGylation interrupts the intracellular trafficking of nanocarriers, especially in the case of endosomal escape [24,25]. It is quite likely that the exposure time of pDNA to TLR9 in endosomes/lysosomes is prolonged due to the trapping of PEG-NP, which would lead to excess stimulation of TLR9, followed by an enhanced expression of IFN-α and -β. The time difference in the production of IL-6 and IFN-α provides support for our prediction. Inflammatory cytokines such as IL-6 and TNF-α showed a peak response at 2 hr after i.v. administration, and the production dropped rapidly by 6 hr because the interaction of NPs with the cell surface had already occurred, which was followed by the immediate uptake of NPs via endocytosis. Although the initial production of IFN-α was slower than that of IL-6, the serum level of IFN-α increased over the 6 hr period after the i.v. injection of PEG-NP due to the prolonged interaction of CpG DNA with TLR9 in endosomes.

Based on our hypothesis, we examined the effect of accelerating the endosomal escape of PEG-NP with GALA on the type I IFN production. We previously reported on the successful

delivery of either an encapsulated aqueous phase marker, pDNA or siRNA into the cytosol by introducing GALA on the lipid envelope [26-28,37]. The acceleration in the endosomal escape of NP by GALA almost diminished IFN-α production, and IL-6 remained at low levels (Figure 5). The amount of GALA-NP in the spleen was comparable to that for NP and PEG-NP (Figure S2). As an alternate to the use of GALA, PEG detached systems which have the ability to promote the endosomal escape of NPs, are considered to be another potential strategy for reducing type I IFN production in response to intracellular environments with a low pH in endosomes/lysosomes, reducing environment generated by small thiolytical molecules, e.g. glutathione, and enzymes such as cathepsin B [38-41].

As anther type of DNA sensor, it was reported that DAI (ZBP1) has a role as a cytosolic dsDNA receptor in a CpG-independent manner [9]. In the present study, the expression of Zbp1 located in Cluster 2 was increased 10-fold by both NP and PEG-NP compared to the control. Although the amount of cytosolic pDNA escaping from endosomes would be increased by presence of GALA, no further immune response occurred. Therefore, the contribution of DAI in the immune response to NP would be miner, and GALA modification could reduce type I IFN production presumably because of the acceleration of endosomal escape. These results lead us to predict that the immune stimulation of NP mediated by TLR9 mainly results in the production of type I IFN in a CpG-dependent manner, whereas that mediated by TLR4 induces inflammatory cytokines in a

CpG-independent manner. Although TLR 1/2 and 6 on the cell surface are also linked to inflammatory cytokine production, the involvement with TLR1/2 and 6 are presently unclear. Of course, further studies will be required to completely understand the mechanisms and pathways for the immune response.

In summary, a microarray based analysis was performed, to explore the mechanism of host responses to systemically administrated NPs. As expected, PEGylation partially reduced the host response to NP. However, PEGylation also stimulated the response of type I IFN to NP. The pathway and mechanism analysis yielded insights into the causes of cytokine production and a strategy for the design of a carrier that can escape specific immune activation. The present study provides the first rational strategy for reducing immunological stimulation based on the genome wide microarray analysis of systemically administrated non-viral lipid nanoparticles.

Materials and Methods

Materials

Linear polyethyleneimine (PEI) (750kDa) was purchased from SIGMA-Aldrich (St. Louis, MO, USA). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), dioleoylphosphatidyl ethanolamine (DOPE), cholesterol and distearoyl-*sn*-glycero-3-phoshoethanolamine-N-[methoxy (polyethylene glycol)-2000] (PEG-DSPE) were obtained by Avanti Polar Lipid (Albaster, AL, USA). EndoFree Plasmid Giga Kit and RNeasy Mini Kit were purchased from QIAGEN (Hilden, Germany). RNase-free DNase I was purchased from TAKARA (Otsu, Japan). High Capacity RNA-to-cDNA Kit was obtained from Agilent Technologies (Palo Alto, CA, USA). Male ICR mice (5-6 weeks old) were purchased from CLEA (Tokyo, Japan). ELISA assay kits of Quantikine Immunoassay mouse IL-6 was purchased from R&D systems (Minneapolis, MN, USA). ELISA assay kits of Verikine Mouse Interferon Alpha ELISA kit was purchased from PBL Biomedical Laboratories (New Brunswick, NJ, USA).

Preparation of pDNA/PEI complex and NPs

pcDNA-3.1(+)-luc was prepared using an Endfree Plasmid Giga Kit, followed by purification with an Endotrap Blue to entirely eliminate traces of endotoxins. To formulate the pDNA/PEI complex, 200 μl of pDNA (0.1 mg/ml) was condenced with 100 μl of PEI (0.6 mM) in 10 mM hepes buffer (pH

7.4), at a nitrogen/phosphate (N/P) ratio of 1.5. NPs were prepared by the lipid hydration method as reported previously [42]. Briefly, a lipid film was prepared in a glass test tube by evaporating a chloroform solution of lipids, containing DOTAP, DOPE and cholesterol (300 nmol total lipids in 3:4:3 molar ratio). For modifying of NP with PEG-DSPE or chol-GALA, the lipid film was prepared by evaporation with the indicated amounts of PEG-DSPE or chol-GALA. The lipid film then was hydrated with the 300 μl of pDNA/PEI complex solution for 10 min at room temperature, followed by sonication for approximately 1 min in a bath-type sonicator (AU-25, AIWA). The average diameter and the ζ-potential of the condensed pDNA/PEI complex and NPs were determined using a Zetasizer Nano ZS ZEN3600 (MALVERN Instrument, Worchestershire, UK).

Animal experiments

Either the pDNA/PEI complex or NPs were administered to male ICR mice via the tail vein, at a dose of 25 μg of pDNA. Hepes-buffered glucose (HBG) treatment was used as a control. At the indicated times after injection, blood and spleen tissues were collected. Blood samples were stored for overnight at 4°C, followed by centrifugation (10000 rpm, 4 °C, 10 min) to obtain serum. Spleen samples were stored in RNAlater solution at -20 °C to avoid RNA degradation. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the “Guide for the Care and Use of Laboratory Animals”.

Determination of serum cytokine

IL-6 and IFN-α levels in serum were determined with ELISA kits according to the manufacturer's instructions.

Semi-quantitative RT-PCR

A spleen sample was homogenized and total cellular RNA was purified using an RNeasy mini kit. To exclude DNA contamination, the RNA sample was treated with RNA free DNase I. Approximately 2.0 μg of RNA from each sample was reverse transcribed using a High Capacity RNA-to cDNA kit by following manufacturer's instructions. PCR reactions were performed using the following primers: IL-6 (forward: 5'-TCCTCTGGTCTTCTGGAGTA-3' and primer: 5'-TCCTTAGCCACTCCTTCTGT-3'); CD14 (forward :5'-CTGATCTCAGCCCTCTGTCC-3' and reverse: 5'-GCTTCAGCCCAGTGAAAGAC-3'); IFN-α (forward: 5'-GCTGCATGGAATACAACCCT-3' and reverse: 5'-CTTCTGCTCTGACCACCTCC-3'); IFN-β (forward: 5'-GAGGAAAGATTGACGTGGGA-3' and reverse: 5'-ACCACCACTCATTCTGAGGC-3'); β-actin (forward: 5'-ACATGGAGAAGATGTGGCAC-3' and reverse: 5'-TCCATCACAATGCCTGTGGT-3'). β-actin was measured as an endogenous reference gene. The PCR thermocycling program was as follows: Denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 70 °C for 30 s

through 27-32 cycles. The PCR products were elctrophoresed through a 2.0% agarose gel and then stained using ethidium bromide and visualized under UV light.

DNA mircoarray experiments

Spleen samples were homogenized and total cellular RNA was purified using an RNeasy mini kit, as described above. Total RNA extracted from four mice spleen (125 ng each) were pooled into one sample (total 500 ng) for normalizing individual differences. The integrity of the pooled total RNA samples was evaluated using an Agilent 2100 Bioanalyzer (Agilent, Foster City, CA, USA). The pooled RNA was labeled with Cy-3 using the Low RNA Input Linear Amplification Kit PLUS, One-Color (Product No. 5188-5339), followed by purification using RNeasy mini kit to eliminate unlabeled Cy-3. Cy-3 labeled RNA sample was then hybridized to Agilent Whole Mouse Genome Microarray (Product No. G4122F) according to manufacturer's hybridization instruction. The microarray slides were analyzed using an Agilent Microarray scanner (Product No. G2565AA). Microarray expression data were obtained using the Agilent Feature Extraction software (Version A.6.1.1).

Data analysis

Microarray data were analyzed using GeneSpring software version 7.3 (Agilent, Foster City, CA,

USA). Genes were regarded as up-regulated when they had a ratio of ≥ 3 and as down-regulated when they had a ratio of ≤ 0.34 in the administration of NP compared with HBG treatment. To understand the differential gene expression pattern, a hierarchical clustering analysis was performed using a Pearson Correlation and an average linkage clustering algorithm. The Gene Ontology (GO) analysis was performed to assign biological meaning to the subset of gene clusters. Overrepresentation of genes with altered expression in the NP treatment compared with the HBG treatment within specific GO categories was determined using Fisher's exact probability test. Pathway analysis of Toll-like receptor signaling pathway was performed by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map

Statistical analysis

Comparisons between multiple treatments were made using one-way analysis of variance (ANOVA), followed by the Bonferroni test. Pair-wise comparisons between treatments were made using a student's t-test. A *P*-value of < 0.05 was considered significant.

Supplementary material

Figure S1. Serum levels of cytokines and ALT.

Figure S2. Accumulation of systemically administered NPs in the spleen.

Materials and Methods.

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Tables

Table 1 Physical properties of the prepared NPs

	NP	PEG-NP	GALA-NP
Diameter (nm)	**200±11**	**132±6**	**131±2**
ζ-potential (mV)	**58±12**	**13±2**	**6±2**

The data are expressed as the mean ± SD. (n=3).

Table 2 Statistically overrepresented GO terms (Biological Process) in each cluster

Cluster 1 (402 genes) p<0.0001

Category	Genes in Category	Genes in List in Category	p-Value
GO:35051: cardiac cell differentiation	8	4	9.80E-07
GO:7571: age-dependent general metabolic decline	3	3	1.33E-06
GO:1306: age-dependent response to oxidative stress	3	3	1.33E-06
GO:910: cytokinesis	187	11	7.85E-06
GO:7067: mitosis	242	12	1.72E-05
GO:87: M phase of mitotic cell cycle	245	12	1.94E-05
GO:45494: photoreceptor maintenance	8	3	7.14E-05
GO:51301: cell division	328	13	7.89E-05
GO:35050: embryonic heart tube development	22	4	9.06E-05

Cluster 2 (648 genes) $p<10^{-12}$

Category	Genes in Category	Genes in List in Category	p-Value
GO:6955: immune response	835	102	6.38E-61
GO:9607: response to biotic stimulus	1055	110	7.85E-59
GO:6952: defense response	1010	108	8.61E-59
GO:9613: response to pest, pathogen or parasite	450	48	1.49E-25
GO:43207: response to external biotic stimulus	505	48	2.35E-23
GO:9615: response to virus	44	19	4.77E-23
GO:50896: response to stimulus	3266	122	1.90E-20
GO:50874: organismal physiological process	2840	111	6.85E-20
GO:9605: response to external stimulus	778	51	1.11E-17
GO:8219: cell death	878	49	2.76E-14
GO:6915: apoptosis	817	47	3.23E-14
GO:16265: death	888	49	4.24E-14
GO:12501: programmed cell death	830	47	5.76E-14
GO:6954: inflammatory response	199	23	1.53E-13
GO:6950: response to stress	1156	55	4.42E-13

Cluster 3 (342 genes) $p<10^{-8}$

Category	Genes in Category	Genes in List in Category	p-Value
GO:9607: response to biotic stimulus	1055	45	1.36E-17
GO:6955: immune response	835	37	4.75E-15
GO:6952: defense response	1010	40	1.33E-14
GO:43207: response to external biotic stimulus	505	26	2.81E-12
GO:9605: response to external stimulus	778	31	1.51E-11
GO:9613: response to pest, pathogen or parasite	450	23	6.36E-11
GO:9611: response to wounding	365	20	3.56E-10
GO:6954: inflammatory response	199	14	7.44E-09
GO:45408: regulation of interleukin-6 biosynthesis	9	5	8.99E-09

Table 2 (continued)

Cluster 4 (23 genes) $p<10^{-8}$

Category	Genes in Category	Genes in List in Category	p-Value
GO:9607: response to biotic stimulus	1055	10	8.53E-11
GO:6952: defense response	1010	10	5.55E-11
GO:43207: response to external biotic stimulus	505	5	1.18E-05
GO:9605: response to external stimulus	778	5	9.33E-05
GO:9613: response to pest, pathogen or parasite	450	5	6.74E-06
GO:42830: defense response to pathogenic bacteria	10	2	1.74E-05
GO:42829: defense response to pathogen	10	2	1.74E-05
GO:9618: response to pathogenic bacteria	16	2	4.62E-05
GO:42828: response to pathogen	20	2	7.31E-05
GO:50896: response to stimulus	3266	11	3.21E-07
GO:9615: response to virus	44	3	2.82E-06

Cluster 5 (103 genes) $p<10^{-8}$

Category	Genes in Category	Genes in List in Category	p-Value
GO:16068: type I hypersensitivity	16	6	2.66E-12
GO:6955: immune response	835	17	6.47E-11
GO:6952: defense response	1010	18	1.38E-10
GO:9607: response to biotic stimulus	1055	18	2.80E-10
GO:43207: response to external biotic stimulus	505	13	9.64E-10
GO:9613: response to pest, pathogen or parasite	450	12	3.10E-09
GO:50874: organismal physiological process	2840	26	8.70E-09

"Genes in List in Category" represent the number of genes that were classified as the statistically overrepresented GO term.

Table 3 Genes that are differentially expressed in response to NP treatment in each cluster

Probe ID	Description	UniGene	Genbank	Raw Data			Ratio	
				HBG	NP	PEG-NP	NP/HBG	PEG-NP/ NP
Cluster 1								
A_52_P12289	G protein-coupled receptor 154 (Gpr154)	Mm.130824	NM_175678	6320	645	1479	0.10	2.29
A_51_P48016	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 8 (Edg	Mm.190619	NM_053190	732	104	170	0.14	1.63
A_51_P44289	RIKEN full-length clone:C130048D07	Mm.266843	AK048310	1205	172	491	0.14	2.86
A_51_P16453	Armadillo repeat gene deleted in velo-cardio-facial syndrome (Arvcf)	Mm.293599	NM_033474	699	118	171	0.17	1.45
A_51_P37067	Growth factor independent 1B (Gfi1b)	Mm.373385	NM_008114	20713	3503	5601	0.17	1.60
A_51_P47227	SRY-box containing gene 18 (Sox18)	Mm.264904	NM_009236	1598	273	506	0.17	1.85
A_52_P49586	V-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avia	Mm.330745	NM_010658	9821	1777	1203	0.18	0.68
A_52_P28960	Growth differentiation factor 6 (Gdf6)	Mm.302555	NM_013526	930	179	263	0.19	1.47
A_51_P22879	cDNA sequence BC020025	Mm.273254	NM_146030	1526	295	279	0.19	0.95
A_52_P56391	cDNA sequence BC019731	Mm.46582	NM_144914	1048	204	895	0.19	4.39
A_52_P11005	Duffy blood group (Dfy)	Mm.6393	NM_010045	2888	575	1181	0.20	2.05
A_51_P21749	Solute carrier family 2 (facilitated glucose transporter), member 4 (Slc2a4	Mm.10661	NM_009204	4426	888	1867	0.20	2.10
A_51_P34022	Sh3 domain YSC-like 1 (Sh3yl1)	Mm.218624	NM_013709	3304	684	1390	0.21	2.03
A_52_P44941	Vang, van gogh-like 1 (Drosophila)	Mm.331266	BC024687	3020	634	1262	0.21	1.99
A_51_P31161	Duffy blood group (Dfy)	Mm.6393	NM_010045	1361	291	628	0.21	2.16
A_52_P56539	Histocompatibility 2, Q region locus 2 (H2-Q2)	Mm.33263	NM_010392	1569	339	472	0.22	1.40
A_51_P38942	Fumarylacetoacetate hydrolase domain containing 1 (Fahd1)	Mm.347964	NM_023480	5281	1152	1789	0.22	1.55
A_52_P21947	Cell division cycle 6 homolog (S. cerevisiae) (Cdc6)	Mm.20912	NM_011799	2971	652	1098	0.22	1.68
A_51_P35452	RIKEN cDNA 2010011I20 gene	Mm.30013	NM_025912	513	113	205	0.22	1.82
A_52_P27652	Ankyrin repeat domain 9 (Ankrd9)	Mm.250989	NM_175207	801	177	247	0.22	1.39
A_52_P27652	Ankyrin repeat domain 9 (Ankrd9)	Mm.250989	NM_175207	2907	645	925	0.22	1.43
A_51_P51427	RIKEN full-length clone:2900072M03	Mm.104155	AK013768	1497	334	701	0.22	2.10
A_51_P39852	Fructosamine 3 kinase (Fn3k)	Mm.266448	NM_022014	16726	3751	7511	0.22	2.00
A_51_P17017	UDP-Gal:betaGal beta 1,3-galactosyltransferase polypeptide 7 (B7galt7)	Mm.192369	NM_146184	1235	280	263	0.23	0.94
A_51_P50184	DnaJ (Hsp40) homolog, subfamily B, member 3 (Dnajb3)	Mm.3075	NM_008299	2864	659	1100	0.23	1.67
Cluster 2								
A_52_P67640	Chemokine (C-X-C motif) ligand 11 (Cxcl11)	Mm.131723	NM_019494	14	13085	9038	938.67	0.69
A_51_P12363	Immune-responsive gene 1 (Irg1)	Mm.4662	L38281	71	29771	17509	417.14	0.59
A_51_P12362	RIKEN full-length clone:9830109K16	Mm.4662	AK036446	84	33973	20103	404.06	0.59
A_51_P28673	Chemokine (C-C motif) ligand 2 (Ccl2)	Mm.290320	NM_011333	362	106967	68653	295.57	0.64
A_51_P43665	Chemokine (C-C motif) ligand 7 (Ccl7)	Mm.341574	NM_013654	15	4271	1943	290.54	0.45
A_51_P33730	Serum amyloid A 3 (Saa3)	Mm.14277	NM_011315	9	2326	1290	268.34	0.55
A_51_P18448	Matrix metalloproteinase 13 (Mmp13)	Mm.5022	NM_008607	56	10257	6863	182.38	0.67
A_52_P61425	Myxovirus (influenza virus) resistance 1 (Mx1)	Mm.33996	NM_010846	29	3784	3860	128.80	1.02
A_52_P44643	Myxovirus (influenza virus) resistance 1 (Mx1)	Mm.33996	NM_010846	103	12169	12008	118.49	0.99
A_52_P24951	Chemokine (C-C motif) ligand 12 (Ccl12)	Mm.867	NM_011331	21	2451	2452	114.59	1.00
A_52_P20876	Chemokine (C-C motif) ligand 7 (Ccl7)	Mm.341574	NM_013654	18	1898	839	107.35	0.44
A_52_P55085	RIKEN full-length clone:D630022O22	Mm.30756	AK085407	14	1434	1248	106.14	0.87
A_51_P38581	Interleukin 12b (Il12b)	Mm.239707	NM_008352	32	2820	1833	89.07	0.65
A_51_P27960	Suppressor of cytokine signaling 1 (Socs1)	Mm.130	NM_009896	2821	245879	140246	87.16	0.57
A_51_P50957	Chemokine (C-C motif) ligand 4 (Ccl4)	Mm.244263	NM_013652	487	41709	47695	85.64	1.14
A_51_P51408	Myxovirus (influenza virus) resistance 2 (Mx2)	Mm.14157	NM_013606	735	61688	53591	83.94	0.87
A_52_P65305	Unknown	—	—	1589	118529	128723	74.59	1.09
A_52_P66368	RIKEN full-length clone:5031412D17	Mm.271850	AK077243	231	15706	17600	67.99	1.12
A_51_P35957	Interferon-induced protein with tetratricopeptide repeats 3 (Ifit3)	Mm.271850	NM_010501	1989	125337	135173	63.02	1.08
A_52_P45268	Activating transcription factor 3 (Atf3)	Mm.2706	NM_007498	289	17030	11720	58.89	0.69
A_51_P31578	Tumor necrosis factor alpha induced protein 6 (Tnfaip6)	Mm.3509	NM_009398	21	1113	583	53.59	0.52
A_52_P94784	RIKEN full-length clone:A530076D18	Mm.254989	AK041062	5	267	157	52.03	0.59
A_52_P10168	Tetratricopeptide repeat protein 6 (TPR repeat protein 6)	Mm.84118	XM_126988	3	166	254	49.52	1.53
A_52_P54238	Interferon-induced protein with tetratricopeptide repeats 2 (Ifit2)	Mm.2036	NM_008332	87	4310	5239	49.43	1.22
A_51_P32775	Interferon-induced protein with tetratricopeptide repeats 1 (Ifit1)	Mm.6718	NM_008331	1184	58059	57029	49.04	0.98
Cluster 3								
A_51_P21721	Interleukin 6 (Il6)	Mm.1019	NM_031168	26	25059	5883	949.92	0.23
A_51_P36318	Chemokine (C-X-C motif) ligand 1 (Cxcl1)	Mm.21013	NM_008176	27	24022	2221	880.57	0.09
A_51_P22097	Interferon gamma (Ifng)	Mm.240327	NM_008337	167	87420	10721	524.73	0.12
A_52_P68893	Interferon gamma (Ifng)	Mm.240327	NM_008337	287	110858	16703	386.53	0.15
A_51_P21746	Chemokine (C-X-C motif) ligand 2 (Cxcl2)	Mm.4979	NM_009140	7	2635	333	374.88	0.13
A_51_P45532	Selectin, endothelial cell (Sele)	Mm.5245	NM_011345	4	1218	317	323.25	0.26
A_52_P17705	Interleukin 22 (Il22)	Mm.103585	NM_016971	3	957	37	304.91	0.04
A_51_P23494	A disintegrin-like and metalloprotease (reprolysin type) with thrombospon	Mm.23156	NM_172845	27	5044	1633	190.12	0.32
A_51_P17107	Colony stimulating factor 2 (granulocyte-macrophage) (Csf2)	Mm.4922	NM_009969	14	1602	258	112.42	0.16
A_51_P42779	Actin binding LIM protein family, member 3 (Ablim3)	Mm.329478	NM_198649	12	1058	157	85.05	0.15
A_51_P25485	Prostaglandin-endoperoxide synthase 2 (Ptgs2)	Mm.292547	NM_011198	4	295	62	76.52	0.21
A_52_P22476	Prostaglandin-endoperoxide synthase 2 (Ptgs2)	Mm.292547	NM_011198	4	259	57	69.23	0.22
A_52_P29543	Chemokine (C-X-C motif) ligand 5 (Cxcl5)	Mm.4660	NM_009141	138	8986	1470	65.26	0.16
A_51_P16990	RIKEN full-length clone:A130019G07	Mm.297393	AK037442	3	193	85	56.61	0.44
A_51_P13851	Granzyme C (Gzmc)	Mm.14465	NM_010371	12	654	74	56.31	0.11
A_51_P18357	Serine (or cysteine) proteinase inhibitor, clade E, member 1 (Serpine1)	Mm.250422	NM_008871	101	3888	1815	38.34	0.47
A_52_P32920	Extracellular proteinase inhibitor (Expi)	Mm.1650	NM_007969	41	1430	164	35.03	0.11
A_51_P11116	Rho family GTPase 1 (Rnd1)	Mm.274010	NM_172612	1671	57998	17710	34.71	0.31
A_51_P33175	Small chemokine (C-C motif) ligand 11 (Ccl11)	Mm.4686	NM_011330	28	934	176	33.81	0.19
A_51_P16518	RIKEN cDNA 4933430F08 gene	Mm.159219	NM_028967	711	23794	10839	33.45	0.46
A_51_P43076	Interleukin 10 (Il10)	Mm.874	NM_010548	22	624	156	28.62	0.25
A_51_P17285	CD14 antigen (Cd14)	Mm.3460	NM_009841	1393	38202	11707	27.42	0.31
A_51_P47445	Suppressor of cytokine signaling 3 (Socs3)	Mm.3468	NM_007707	6010	158332	74355	26.34	0.47
A_52_P26161	Pentraxin related gene (Ptx3)	Mm.276776	NM_008987	3	85	26	25.18	0.31
A_52_P51038	RIKEN full-length clone:D230024E06	—	AK051959	3	78	32	23.57	0.41

Table 3 (continued)

Probe ID	Description	UniGene	Genbank	Raw Data			Ratio	
				HBG	NP	PEG-NP	NP/HBG	PEG-NP/ NP
Cluster 4								
A_51_P19293	Interferon alpha family, gene 6 (Ifna6)	Mm.377090	NM_008335	36	685	4873	19.16	7.12
A_51_P35538	Interferon alpha family, gene 13 (Ifna13)	Mm.246592	NM_177347	83	1195	7901	14.41	6.61
A_51_P43640	Interferon alpha family, gene 1 (Ifna1)	Mm.57127	NM_010502	16	2381	15577	147.70	6.54
A_51_P46543	Interferon alpha family, gene B (Ifnab)	Mm.377093	NM_008336	12	1926	12087	154.95	6.28
A_51_P38768	Interferon alpha family, gene B (Ifnab)	Mm.377093	NM_008336	72	1872	11539	26.12	6.16
A_52_P21117	Interferon alpha family, gene 12 (Ifna12)	Mm.246618	NM_177361	14	4494	27667	330.93	6.16
A_52_P48228	Mouse alpha-interferon (MuIFN-alpha), 3' cds & untranslated mRNA	Mm.14091	K01411	4	2324	14123	562.85	6.08
A_52_P93137	RIKEN full-length clone:C130037M17	Mm.1571	AK048149	3	14	78	4.16	5.74
A_51_P35582	Interferon alpha family, gene 4 (Ifna4)	Mm.377088	NM_010504	4	1038	5723	267.46	5.51
A_51_P36330	Interferon alpha family, gene 9 (Ifna9)	Mm.377092	NM_010507	138	845	4601	6.14	5.45
A_52_P13337	Unknown	—	—	331	1502	8085	4.54	5.38
A_52_P60284	Glycosylation dependent cell adhesion molecule 1 (Glycam1)	Mm.219621	NM_008134	4	40	212	11.04	5.32
A_51_P14418	Interferon beta 1, fibroblast (Ifnb1)	Mm.1245	NM_010510	4	5954	22857	1356.57	3.84
A_51_P32682	Melanoma antigen (Mela)	Mm.270157	NM_008581	229	4200	14885	18.36	3.54
A_51_P24351	Hypothetical protein	Mm.31376	XM_138397	5	23	68	5.05	2.99
A_52_P22324	Phospholamban (Pln)	Mm.34145	NM_023129	13	44	131	3.49	2.97
A_51_P42795	Olfactory receptor 869 (Olfr869)	Mm.334988	NM_146557	3	28	64	7.97	2.30
A_52_P63739	Antimicrobial peptide RYA3 (Rya3)	Mm.55392	NM_194357	3	27	60	8.17	2.25
A_51_P33896	Unknown]	—	—	7	42	93	5.84	2.19
A_52_P17769	Transcription factor 2 (Tcf2)	Mm.7226	NM_009330	11	63	107	5.66	1.71
A_52_P46110	G protein-coupled receptor 31, D17Leh66c region (Gpr31c)	Mm.335670	NM_0010138	8	36	60	4.32	1.66
A_51_P21514	RIKEN full-length clone:D430006K04	Mm.45514	AK084893	14	43	60	3.21	1.39
A_52_P36703	Tripartite motif protein 34 (Trim34)	Mm.263478	NM_030684	361	1157	1564	3.20	1.35
Cluster 5								
A_51_P46170	Major urinary protein 1 (Mup1)	Mm.237772	NM_031188	4	922	17	228.54	0.02
A_51_P31717	Colony stimulating factor 3 (granulocyte) (Csf3)	Mm.1238	NM_009971	4	760	19	184.20	0.03
A_51_P23262	Interleukin 22 (Il22)	Mm.103585	NM_016971	4	498	18	134.29	0.04
A_51_P16071	Albumin 1 (Alb1)	Mm.16773	NM_009654	4	290	6	72.18	0.02
A_51_P21133	UDP-Gal:betaGal beta 1,3-galactosyltransferase, polypeptide 6 (B3galt6)	Mm.347395	NM_080445	358	12044	707	33.68	0.06
A_51_P22921	RIKEN full-length clone:D130011C11	—	AK083791	3	109	3	32.24	0.03
A_52_P55414	Unknown	—	—	3	96	7	29.60	0.07
A_52_P11004	RIKEN full-length clone:C130095H06	Mm.374840	AK082018	6	161	8	26.76	0.05
A_52_P45399	Unknown	—	—	4	105	3	26.71	0.03
A_51_P25880	RIKEN full-length clone:2310020F24	Mm.121859	AK009417	33	784	58	23.62	0.07
A_52_P17068	Unknown	—	—	4	97	4	23.16	0.04
A_52_P23281	Gene model 1960, (NCBI) (Gm1960)	Mm.244289	NM_203320	6	121	11	21.53	0.09
A_52_P34825	Major urinary protein 1	Mm.237772	BC037152	4	84	9	20.99	0.11
A_51_P26688	Major urinary protein 4 (Mup4)	Mm.34335	NM_008648	27	554	43	20.50	0.08
A_52_P10438	RIKEN full-length, clone:A630095O09	Mm.360357	AK042484	3	64	3	20.23	0.04
A_51_P18031	RIKEN full-length clone:2610016E04	Mm.237772	AK011413	4	79	7	19.76	0.09
A_52_P13943	Unknown	—	—	4	78	9	18.90	0.12
A_52_P34286	Esterase 1 (Es1)	Mm.88078	NM_007954	3	63	3	18.04	0.05
A_52_P85152	RIKEN full-length clone:B230311D12	Mm.153019	AK045794	3	56	3	17.61	0.05
A_52_P13931	Unknown	—	—	9	154	12	17.47	0.07
A_52_P54550	RIKEN full-length clone:A430068J17	Mm.254835	AK079794	4	58	4	16.26	0.06
A_52_P14160	Hypothetical protein E030010A14	Mm.86388	NM_183160	31	488	64	15.89	0.13
A_52_P24191	RIKEN full-length clone:A230074D21	Mm.244393	AK038910	33	499	74	15.35	0.15
A_51_P12436	Defensin beta 9 (Defb9)	Mm.171224	NM_139219	4	53	5	15.02	0.10
A_51_P33726	Aldolase 2, B isoform (Aldob)	Mm.218862	NM_144903	5	65	5	14.23	0.08

Figure legends

Figure 1 Clustergram of genes that are differentially regulated by administration of NPs

1581 genes with an expression ratio of NP to HBG greater than 3 or less than 0.33 are represented. 402 genes were down-regulated after NP administration, classified in Cluster 1. The remaining 1179 genes were up-regulated, classified in Clusters 2-5. Red, yellow and blue represent relative gene expression among HBG, NP and PEG-NP.

Figure 2 Transcriptional levels obtained by microarray were in agreement with mRNA quantities by qRT-PCR

Bars represent transcriptional levels of (a) IL-6, (b) CD14, (c) IFN-α, and (d) IFN-β obtained by microarray analysis. Gene expressions were confirmed by semiquantitative RT-PCR as shown in electrophoretic images. qRT-PCR results were in good agreement with the microarray analysis.

Figure 3 Serum levels of (a) IL-6 and (b) IFN-α are correlated with the transcriptional levels in spleen

Each sample (25 μg pDNA/mouse) was intravenously injected at a normal pressure. At 2 and 6 hr after the i.v. injection, (a) serum IL-6, (b) IFN-α were evaluated by ELISA. (a) NP (closed squares) enhanced IL-6 production, and PEGylation (open circles) effectively reduced it. (b) On the other hand, PEGylation

(open circles) stimulated IFN-α compared to NP (closed squares). Neither IL-6 nor IFN-α were detected in the HBG treatment. These values are in good agreement with the microarray and RT-PCR results. Data are presented as the mean ± SD (n=4). ***P*<0.01.

Figure 4 Differentially expressed genes in the Toll-like receptor signaling pathway from KEGG PATHWAYS

Red and blue columns represent differentially expressed genes located in Cluster 3 and Cluster 4, respectively.

Figure 5 Modification of GALA suppressed IFN-α production of PEG-NP

Each sample (25 μg pDNA/mouse) was intravenously injected at a normal pressure. At the indicated time after i.v. injection, serum (a) IL-6 and (b) IFN-α were evaluated by ELISA. The gene expression of IL-6 and IFN-α in the spleen was observed by semiquantitative RT-PCR. Closed squares, open circles and closed triangles represent NP, PEG-NP and GALA-NP, respectively. Even though serum IL-6 levels remained at the level in PEG-NP, GALA-NP caused negligible IFN-α production unlike PEG-NP. The gene expression of IFN-α in the spleen was also decreased by GALA modification.

Supplementary Materials

Figure S1 Serum levels of (a) IL-6 and (b) IFN-γ (c) TNF-α and (d) ALT

Each sample (25 μg pDNA/mouse) was intravenously injected at a normal pressure. At 2, 6 or 24 hr after i.v. injection, cytokines or ALT value in serum were evaluated. Closed squares, open circles and gray diamonds represent NP, PEG-NP and PEI/pDNA complex, respectively. NP induced inflammatory cytokine productions, on the other hand, PEGylation inhibits that. PEI/pDNA complex alone presented no cytokine in serum. However, PEI/pDNA induced severe hepatotoxicity.

Figure S2 Accumulation of NPs in the spleen

NPs were labeled with [^{3}H]CHE. Each sample (25 μg pDNA/mouse) was intravenously injected at a normal pressure. At 2 hr after i.v. injection, spleen was collected and the radioactivity in the spleen was measured. Tumor accumulation is represented as the % injected dose (ID) per tissue. Comparisons between multiple treatments were made using one-way analysis of variance (ANOVA). N.D.: Not significant differences. The modification of PEG and GALA didn't alter the accumulation of NPs in spleen. Therefore, the different pattern of cytokine and interferon production presumably resulted from the alternation of intracellular fate of NP by modification of PEG and GALA.