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BRIEF COMMUNICATION

The role of tissue macrophages in the induction of proinflammatory cytokine production following intravenous injection of lipoplexes

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Recent studies have demonstrated that intravenous administration of a plasmid DNA–cationic liposome complex (lipoplex) induced significant proinflammatory cytokine production in blood and inhibited transgene expression in pulmonary endothelial cells. In this study, we examined the effects of gadolinium chloride ($GdCl_3$) pretreatment on the biodistribution and induction of proinflammatory cytokine production and transgene expression after intravenous injection of a lipoplex in mice. $GdCl_3$ is known to transiently deplete liver Kupffer cells and spleen macrophages after intravenous administration. Intravenous administration of a lipoplex triggers high levels of proinflammatory cytokine production, such as TNF-α, IFN-γ and IL-12 in serum and a large amount of ^{32}P-labeled lipoplex accumulates in the liver 1 h after intravenous administration. However, pretreatment with $GdCl_3$ dramatically reduces serum levels of these cytokines and liver accumulation of the lipoplex. RT-PCR analysis showed that mRNA expression of TNF-α greatly increases in the liver and spleen after lipoplex injection and that pretreatment with $GdCl_3$ reduces mRNA expression in these organs. Messenger RNA expression of TNF-α in the liver occurs in non-parenchymal cells (sinusoidal endothelial cells and/or Kupffer cells). Inhibition of cytokine production by pretreatment with $GdCl_3$ leads to recovery of transgene expression in the lung following the second injection of lipoplex, which was reduced following the first injection of lipoplex. Thus, the present study demonstrates that tissue macrophages involving liver Kupffer cells and spleen macrophages are closely involved in TNF-α production following i.v. administration of the lipoplex. It is also suggested that avoiding lipoplex uptake and subsequent cytokine production by these cells would be a useful method of maintaining a high level of gene expression in the lung after repeated injections.
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Several recent studies have shown that intravenous administration of plasmid DNA–cationic liposome complexes (ie lipoplexes) leads to systemic gene expression, particularly in the lung.[1–3] Therefore, increasing attention has focused on the development of efficient *in vivo* gene delivery systems. However, intravenous administration of cationic lipid vectors containing plasmid DNA has been shown to initiate potent cytokine responses.[4,5] High amounts of proinflammatory cytokines are produced in blood after lipoplex injection since bacterially derived plasmid DNA is recognized as foreign material by vertebrate cells. Unmethylated CpG sequences in plasmid DNA, occurring at a higher frequency in bacterial DNA, have been reported to have strong stimulatory effects on lymphocytes, NK cells, dendritic cells and macrophages to induce production of large quantities of proinflammatory cytokines, such as TNF-α, IFN-γ, and IL-12.[6–8] It appears that such cytokines activate the immune system and induce strong antitumor effects in mice.[4,5] On the other hand, Li *et al* have shown that such cytokines, which are secreted after intravenous injection of lipoplexes, can be toxic and cause short-term gene expression and refractory behavior on repeated dosing at frequent intervals.[9,10] This is a potential problem in gene therapy using plasmid DNA. However, it is not known which cells are responsible for the induction of cytokine production after lipoplex administration.

The liver and spleen are well known to play a central role in the removal of foreign particles and invasive microorganisms from the circulation, mainly via Kupffer cells and spleen macrophages.[11,12] Kupffer cells are large liver macrophages and their functions are activated by a variety of particles, viruses, lipopolysaccharides and TNF-α.[13] The phagocytosis of parasites by Kupffer cells is accompanied by the release of TNF-α. Spleen macrophages are also activated in addition to liver Kupffer cells. We and other groups have also reported that lipoplexes accumulate in the liver and spleen, as well as the

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lung after intravenous administration and liver Kupffer cells and spleen macrophages are directly involved in the uptake of lipoplexes.[14–17] These data suggest that these cells may play an important role in the production of proinflammatory cytokines. These cells are also involved in the uptake of adenovirus and cytokine production after intravenous administration of recombinant adenovirus vectors.[11,18,19] In this study, we evaluated the effects of pretreatment with $GdCl_3$ on the production of proinflammatory cytokines and gene expression in the lung after lipoplex injection. Several studies have reported that intravenous injection of $GdCl_3$, a rare earth metal salt, is able to not only block the phagocytosis of macrophages in liver and spleen, but also eliminate them and that a single injection of $GdCl_3$ blocks phagocytosis in more than 90% of Kupffer cells.[20,21] $GdCl_3$ has also been used to evaluate the function of these cells in many reports.[22,23] We report here that liver Kupffer cells and spleen macrophages play an important role in the induction of proinflammatory cytokine production after intravenous administration of a lipoplex and that prevention of lipoplex uptake by these cells eliminates the refractory behavior to repeated injection.

It is necessary for immune cells to endocytose DNA-containing CpG motifs in order to produce cytokines by CpG motif-mediated induction.[6,24] Therefore, we first examined the biodistribution of a ^{32}P-lipoplex after intravenous administration in saline-pretreated and $GdCl_3$-pretreated mice. Mice received intravenous injections of a $GdCl_3$ solution or saline 24 h before lipoplex administration. The lipoplex was rapidly removed from the bloodstream and only a few percent of the lipoplex remained in the blood 10 min after injection in both groups (data not shown). Rapid lung accumulation of the lipoplex was observed in both groups immediately after i.v. administration, as reported by other groups (Figure 1a).[25,26] The lung accumulation of the lipoplex gradually decreased in both groups, however, a significantly higher amount of lipoplex was left in the lung of $GdCl_3$-pretreated mice compared with saline-pretreated mice 1 h after injection. In addition, accumulation of lipoplex in the liver gradually increased in both groups (Figure 1b). However, there was reduced hepatic uptake of lipoplex in $GdCl_3$-pretreated mice. The hepatic uptake in $GdCl_3$-pretreated mice was two-fold lower than in saline-pretreated mice. Several reports have suggested that intravenously administered DNA–cationic molecule complexes are mainly taken up by liver Kupffer cells.[14–17] Kupffer cell depletion by $GdCl_3$ would lead to a reduction in lipoplex accumulation in the liver. We could not find any differences in the accumulation of lipoplex in the spleen (Figure 1c). Hardonk *et al*[20] reported that spleen macrophages were much less vulnerable to $GdCl_3$ than liver Kupffer cells. Only some of the red pulp macrophages of the spleen transiently disappeared and the macrophages in the white pulp were not affected. Lipoplex was also distributed in the white pulp of the spleen, as well as the red pulp after intravenous injection.[16] Some types of cells in the spleen, which are unaffected by $GdCl_3$ may be closely involved in the lipoplex uptake in $GdCl_3$-pretreated mice.

The total radioactivities recovered from the selected organs after lipoplex injection were lower (approximately 75% at 60 min) in the $GdCl_3$-pretreated mice than in the saline-pretreated mice (more than 90% at every time-point). A lower recovery of radioactivity in the $GdCl_3$-pretreated mice would be due to distribution to other organs and excretion into the urine, which we did not measure in the present study. The radioactivity would be derived from degradation products of ^{32}P-DNA. We speculated that faster DNA degradation would occur in the $GdCl_3$-pretreated mice compared with control animals. After intravenous injection of lipoplex, plasmid DNA would be dissociated from the cationic liposomes in the blood circulation by the interaction with biological components, such as blood constituents and proteoglycans.[25,27,28] Free DNA dissociated from cationic liposomes is susceptible to degradation by nucleases in the blood and the degradation products are excreted into the urine via the kidney and distributed throughout the body. Kawabata *et al*[29] have demonstrated these processes after i.v. injection of naked plasmid DNA labeled with ^{32}P. Lipoplex would be more susceptible to the interaction with these components in the $GdCl_3$-pretreated mice due to decreased lipolex uptake by the macrophages. In fact, the radioactivity in the kidney and heart of $GdCl_3$-pretreated mice was slightly higher than that of saline-pretreated mice (data not shown). On the other hand, the release of ^{32}P-DNA degradation products from the cells after endocytosis followed by lysosomal degradation will not significantly affect the biodistribution results in this study. Enzymatic degradation most probably occurs in the bloodstream. Even following intravenous injection of naked ^{32}P-DNA, the level of the radioactivity in the liver derived from DNA taken up by liver non-parenchymal cells was almost constant for 30 min.[29] In the case of lipoplex, intracellular stability of DNA should be enhanced. In addition, more than 20 min elapsed after injection before endocytosis of the lipoplex into the lung endothelial cells took place.[14,30] Wattiaux *et al*[31] have also examined the subcellular distribution of radiolabeled DNA complexed with cationic lipids in rat liver and suggested that DNA complexed with cationic lipid reached the lysosomal compartment more than 1 h later after injection.

Next, we compared the amounts of TNF-α, IFN-γ and IL-12 in serum induced after intravenous injection of the lipoplex in saline-pretreated and $GdCl_3$-pretreated mice. High amounts of these cytokines were secreted in serum after intravenous administration of lipoplex (Figure 2). It was also confirmed that negligible amounts of these cytokines were produced after i.v. administration of plasmid DNA or DOTMA/Chol liposomes alone to saline-pretreated mice (data not shown). The highest amount of TNF-α was observed 3 h after i.v. injection of lipoplex in saline-pretreated mice (2800 pg/ml). In the case of IFN-γ and IL-12, the peak was reached 6 h after injection (IFN-γ; 61 900 pg/ml, IL-12; 6800 pg/ml). The peak times for each cytokine were similar to those in previous reports.[4,5] In $GdCl_3$-pretreated mice, the amounts of these cytokines were significantly lower than those in the saline-pretreated mice. The amount of TNF-α, IFN-γ, and IL-12 in serum at the peak times was reduced 25-fold, three-fold, and 23-fold in $GdCl_3$-pretreated mice, respectively. These data indicate that blockade of phagocytosis by tissue macrophages, mainly liver Kupffer cells and spleen macrophages, would lead to a reduction in the production of these cytokines. $GdCl_3$ pretreatment was less effective in suppressing IFN-γ production, compared with TNF-α and IL-12. One possible explanation for this

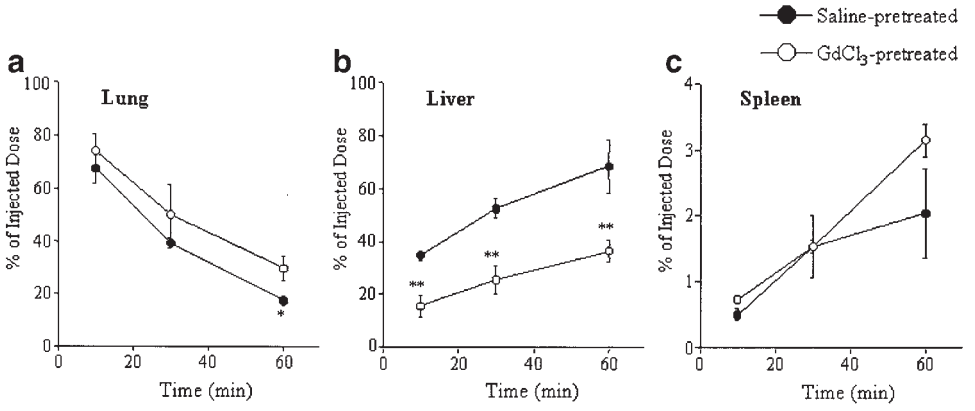

Figure 1 *Biodistribution of lipoplex containing ^{32}P-labeled plasmid DNA following i.v. administration to $GdCl_3$-pretreated (open circle) and saline-pretreated mice (closed circle). Plasmid DNA (pcDNA3) solution (2 mg/ml) was added to a DOTMA/Chol liposome suspension (1 mg DOTMA/ml) at a charge ratio of +2.24. pcDNA3 was obtained from Invitrogen (Carlsbad, CA, USA). The mixtures were incubated at room temperature for 30 min before use. DOTMA/Chol liposomes (DOTMA:Chol = 1:1, molar ratio) were prepared by allowing the lipids to dry as a thin film in a round-bottomed flask, and then the dried film was resuspended in 5% w/v glucose by vortexing. Plasmid DNA was isolated and purified using a QIAGEN plasmid Giga Kit with an EndoFree plasmid Maxi kit (Qiagen, Hilden, Germany). The lipopolysaccharide (LPS) concentration in the plasmid DNA preparation was measured using a LAL assay kit (Limus F Single Test Wako, Wako Pure Chemical, Osaka, Japan). The LPS concentration was negligible (less than 22 pg/μg DNA), which corresponds to the manufacturer's information. Plasmid DNA was radiolabeled with ^{32}P using a nick translation kit. Lipoplex containing ^{32}P-labeled plasmid DNA was injected intravenously into CDF1 mice at a dose of 25 μg DNA. Blood was collected from the vena cava under anesthesia at 10, 30 and 60 min, and mice were killed at each collection time-point. Tissues (heart, lung, spleen, liver and kidney) were isolated, washed with saline, blotted dry and weighed. Ten microliters of blood and a small amount of each tissue were digested with Soluene-350 following incubation overnight at 45°C. Then, 2-propanol, H_2O_2, HCl, and Clear-sol I were added to each tissue and blood sample. The samples were then stored overnight and the radioactivity of each tissue sample was corrected for blood contamination based on data from ^{111}In-labled BSA 10 min after intravenous injection. The results were expressed as the percentage of the injected dose per organ (n = 3). For transient tissue macrophage blockade, $GdCl_3$ was dissolved in saline, and 45 mg/kg body weight was injected via the tail vein at 24 h before lipoplex injection in a total volume of 150 μl. Control mice were injected with 150 μl saline.*

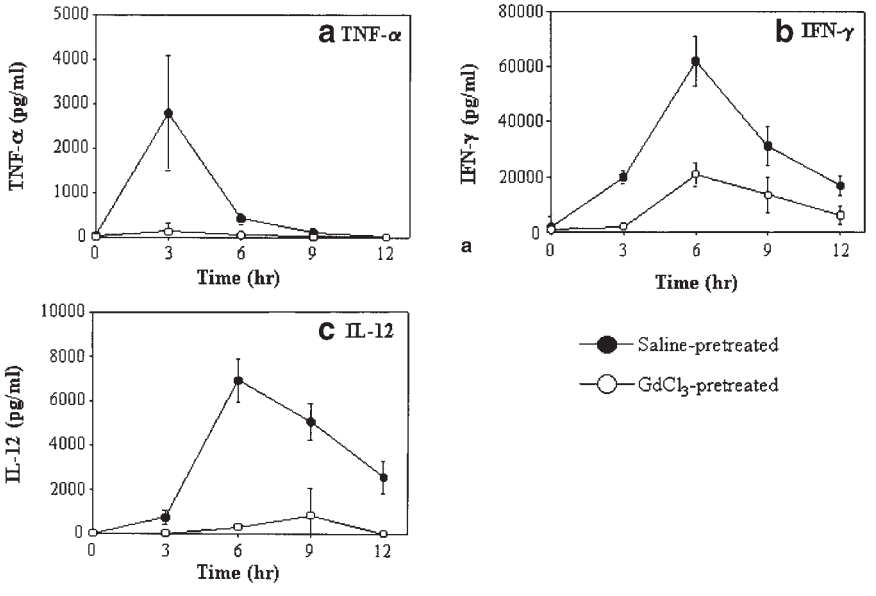

Figure 2 *Proinflammatory cytokine production following i.v. injection of lipoplex in $GdCl_3$-pretreated and saline-pretreated mice. (a) TNF-α; (b) IFN-γ; (c) IL-12. Amounts of TNF-α, IL-12 and IFN-γ in serum after i.v. injection of lipoplex were analyzed using an ELISA kit (Genzyme, Cambridge, MA, USA) as described previously.[4] For transient tissue macrophage blockade, $GdCl_3$ was intravenously injected as described in Figure 1.*

is that the lipoplex directly activates NK cells or T cells to secrete IFN-γ. CpG oligodeoxynucleotide has been shown to induce lymphocytes and NK cells to secrete IFN-γ directly.[7]

In order to further examine which organs are mainly involved in the production of these cytokines, mice were killed and several organs were collected 3 h after i.v. injection of the lipoplex, and the amounts of TNF-α in the cytoplasm fraction of each organ were examined as described in a previous report.[4] We have focused on TNF-α as a proinflammatory cytokine in the following experiments because it is secreted by macrophages after the phagocytosis of parasites and IFN-γ is secondarily secreted by NK cells following stimulation of IL-12 and TNF-α.[32] No TNF-α was observed in any organs or the serum of untreated mice (data not shown). Much higher

amounts of TNF-α were observed in the liver and spleen of saline-pretreated mice after i.v. administration of lipoplex (Figure 3, 30 000 pg/liver and 50 000 pg/spleen). Pretreatment with $GdCl_3$ significantly reduced the amount of TNF-α in the liver, spleen, and kidney. The amount of TNF-α in the liver, spleen and kidney of $GdCl_3$-pretreated mice was three-fold, 10-fold and three-fold lower, respectively, than that in saline-pretreated mice. These data suggest that the contribution of the spleen may be larger than that of the liver, since the spleen TNF-α level was higher. The potential for producing cytokines could be much higher in the spleen than in the liver based on a per cell basis. In fact, lipoplex uptake by the spleen was much less than that by the liver in absolute terms, but the uptake corrected by the tissue weight was comparable. Liver Kupffer cells, which would be mainly responsible for TNF-α production in the liver, represent about 3% of the entire liver mass and 15% in terms of cell numbers, although Kupffer cells exhibited efficient uptake of the lipoplex.[13] In addition to Kupffer cells, liver parencymal cells and sinusoidal endothelial cells significantly contribute to the hepatic uptake of lipoplexes,[14,15] but the contribution of these cells to cytokine production is much less than that of Kupffer cells.[33] In contrast, most spleen cells are immune-competent cells like macrophages, dendritic cells, etc, that generally have a greater potential to produce cytokines. Moreover, the spleen endothelial cells are not responsible for the lipoplex uptake,[14] suggesting that a large part of the lipoplex accumulated in the spleen would be taken up by the immune-competent cells and TNF-α production might occur efficiently. However, attention has to be paid to compare the TNF-α levels between organs and to discuss their contribution to the serum level. TNF-α is synthesized in a membrane-bound immature form in the cells and secreted into the blood in a mature soluble from upon cleavage by converting enzymes.[34] Although we determined the cytokine level in the cytoplasm in each organ by ELISA after appropriate treatment, it may be difficult to directly compare the TNF-α levels and their contribution since the determination conditions and secretion efficacy may vary between organs.

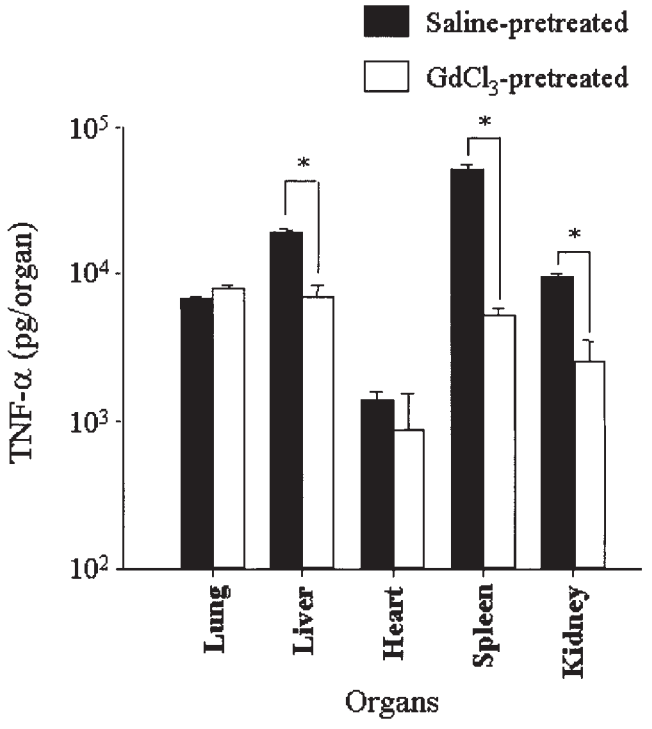

Figure 3 *Proinflammatory cytokines in the cytoplasmic fractions of various organs following i.v. administration of lipoplex. Mice were intravenously injected with lipoplex, as described in Figure 1. Three hours after injection, organs were collected, bled, washed five times in ice-cold saline, blotted dry and stored at −80°C until ELISA was carried out. At the time of ELISA, organs were slowly thawed on ice. The organs were then homogenized in 2 ml PBS(−) containing a cocktail of protease inhibitors (aprotinin-2.0 μg/ml, leupeptin-1.0 μg/ml, Pepstatin A-1.0 μg/ml, and PMSF-100.0 μg/ml) using a tissue-tearing homogenizer. The cytoplasmic fractions were isolated as the supernatant following centrifugation at 14 000 r.p.m. for 10 min at 4°C. These fractions were immediately analyzed for cytokines by ELISA. Cytokine levels associated with blood contamination of the organs were subtracted to give 'total cytokine' values. $GdCl_3$ was intravenously injected 24 h before lipoplex injection.*

The above data suggest that the liver and spleen are the major organs responsible for the production of TNF-α after i.v. administration of lipoplex. We next examined the mRNA expression of TNF-α in selected organs by reverse transcription PCR. Intravenous administration of lipoplex increased the mRNA expression of TNF-α in all organs of saline-pretreated mice (Figure 4a). We also found that there was an apparent decrease in the level of TNF-α mRNA expression in the liver and spleen of $GdCl_3$-pretreated mice. However, the mRNA expression in the heart, lung and kidney was not reduced by pretreatment with $GdCl_3$. We also found that the liver non-parenchymal cells (sinusoidal endothelial cells and/or Kupffer cell), but not parenchymal cells (hepatocytes), expressed mRNA of TNF-α (Figure 4b). It is well known that liver Kupffer cells rather than endothelial cells play an important role in the uptake of foreign particles and cytokine production,[33,35] suggesting that Kupffer cells play a major role in TNF-α production in the liver after intravenous administration of lipoplex. The reduced TNF-α mRNA expression level in the liver was correlated with the reduced hepatic uptake of lipoplex in this organ (Figure 1). The level of mRNA expression of TNF-α enhanced following lipoplex injection in the spleen also fell following $GdCl_3$ pretreatment, although $GdCl_3$ pretreatment did not reduce the lipoplex accumulation in the spleen (Figure 1). Although the reasons for this discrepancy are unclear, functional heterogeneity of spleen macrophages in terms of uptake and activation by the lipoplex may account for it, at least in part. For instance, lipoplex might accumulate in the white pulp of the spleen in the $GdCl_3$-pretreated group, in which macrophages would not be affected by $GdCl_3$ pretreatment. Dendritic cells in the spleen also might be closely involved in the cytokine production via a CpG motif-mediated mechanism.[36] Dendritic cells in the red pulp also have a high phagocytic activity, and lipoplexes accumulated in the red pulp. However, the effect of $GdCl_3$ on the functions of dendritic cells is unknown. Therefore, it can be speculated that $GdCl_3$ only had a minimal effect on the function of lipoplex uptake in the spleen, while it had a significant effect on the function of cytokine production of this organ following the lipoplex uptake. The details of this await further study. In the kidney, the mRNA expression level enhanced by the lipoplex was not reduced by $GdCl_3$ pretreatment. This does not correspond to the observation that the amount of TNF-α in the cytoplasmic fraction of the kidney following lipoplex

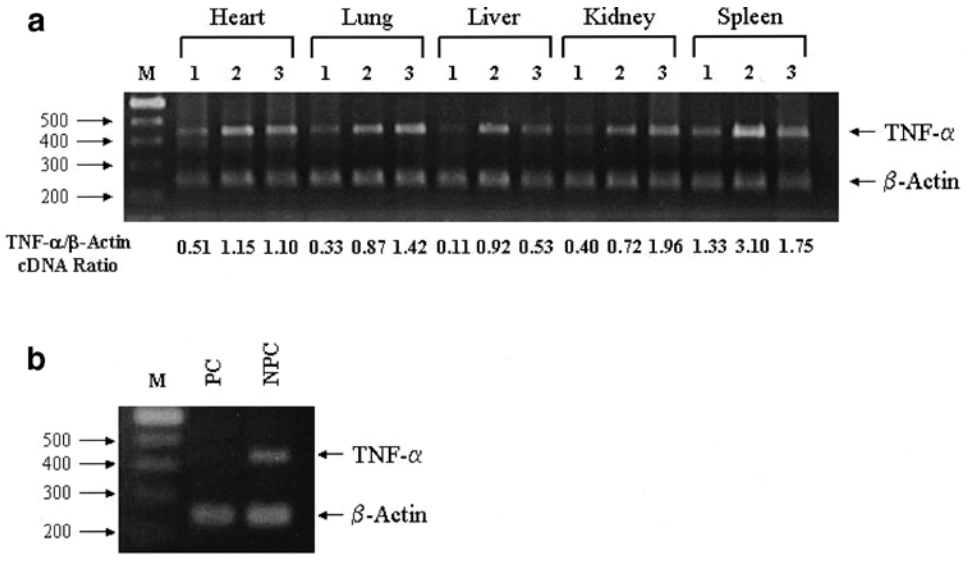

Figure 4 *Detection of TNF-α expression by RT-PCR. (a) mRNA expression of TNF-α in various organs following i.v. administration of lipoplex. Lane 1, control mice; lane 2, mice pretreated with saline 24 h before lipoplex injection; lane 3, mice pretreated with $GdCl_3$ 24 h before lipoplex injection. Mice were injected with lipoplex at a dose of 25 μg DNA per mouse then, 3 h after injection, the animals were bled from the inferior vena cava, while perfusing saline via the portal vein to remove blood from the organs. Total RNA from selected organs was extracted using Trizol (GIBCO BRL). RNA (2 μg) was reverse-transcribed to complementary DNA using oligo(dT) primers and the SUPERSCRIPT First-Strand Synthesis System for RT-PCR (GIBCO BRL). Complementary DNA products were coamplified by polymerase chain reaction (PCR) (30 cycles; 95°C for 1 min, 59°C for 1.5 min, and 72°C for 1 min). Primers for TNF-α (446-bp product) and β-actin (245-bp product) have been described elsewhere.[47] PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and photographed. Digitized photographs were assessed using image analysis software (Densito graph ver. 4.0, ATTO) and mRNA expression was evaluated by the band-intensity ratio of TNF-α to β-actin and presented as the percentage of β-actin. (b) mRNA expression of TNF-α in liver parenchymal (PC) and non-parenchymal (NPC) cells. Mice without $GdCl_3$ pretreatment were injected with lipoplex at a dose of 25 μg DNA per mouse. Collagenase perfusion was performed to separate PC and NPC cells 3 h after injection as described.[48]*

injection was reduced by pretreatment with $GdCl_3$ (Figure 3). Probably a significant amount of the TNF-α protein detected in the kidney is derived from other organs, such as the liver and spleen, upon stimulation with lipoplex. It is considered that TNF-α, which would be adsorbed on the cellular surface of the kidney and/or accumulate in the glomerulus, might be detected in this study. It is well known that TNF-α is rapidly eliminated from the bloodstream due to glomerular filtration and a marked renal accumulation of TNF-α occurs after intravenous administration to mice.[37,38] In addition, TNF-α excreted into the urine can also be detected by ELISA.[39]

After intravenous administration of lipoplex, the lung shows the highest amount of gene expression among various organs and the lung endothelial cells are the main contributor to transgene expression.[1–3] We and others have confirmed that the gene expression level in the lung is 1000–10 000 times higher than that in the liver and spleen (data not shown).[1–3] Therefore, intravenous administration of lipoplex may be a promising gene delivery method to treat pulmonary diseases. However, other groups have reported that such proinflammatory cytokines trigger damage and apoptosis of vascular endothelial cells including lung endothelial cells through various mechanisms.[40–42] Li *et al* have reported that the endothelial cells in the lung are apoptotic 12 h after cationic lipid-protamine-DNA complex injection.[9,10] TNF-α and IFN-γ could also inhibit gene transcription or decrease the stability of mRNA, resulting in inactivation of gene expression.[43,44] These would lead to short-term gene expression and refractory behavior on repeated dosing at frequent intervals. Suppression of the apoptosis by anti-mouse TNF-α and anti-mouse IFN-γ antibodies or dexamethasone leads to significantly higher levels of transgene expression in lung.[9,10] We believe that a reduction in serum cytokine levels would result in improved gene expression, and so we examined the transgene expression in the lung of $GdCl_3$-pretreated and saline-pretreated mice after intravenous administration of lipoplex. No significant difference was observed in the amount of gene expression in the lung after a single injection of lipoplex in $GdCl_3$-pretreated and saline-pretreated mice (Figure 5; control), although pretreatment with $GdCl_3$ slightly increased the lung accumulation of lipoplex (Figure 1) and reduced cytokine levels in serum and some organs (Figures 2 and 3). Next, we examined the gene expression level following repeated injection of lipoplex. Plasmid DNA encoding no reporter gene, pcDNA3, was used in the first injection in order to clearly show the effect of this injection on the gene expression following the second injection. In the second injection, plasmid DNA pCMV-Luc, which encoded firefly luciferase, was used. Preinjection of lipoplex containing pcDNA3 significantly inhibited the transgene expression after i.v. injection of lipoplex containing pCMV-Luc on days 3 and 5. This suggested that proinflammatory cytokines induced after preinjection of lipoplex triggers apoptosis of lung endothelial cells, inhibition of gene transcription and a decrease in mRNA stability, which inhibited the transgene expression as reported previously.[9,10] Although this inhibitory effect was observed in both groups, $GdCl_3$-pretreated mice showed significantly higher amounts of luciferase expression in the lung on days 3 and 5 compared with saline-pretreated mice. This suggests that pretreatment with $GdCl_3$ blocks cytokine production by the lipoplex and partly eliminates the refractory behavior to

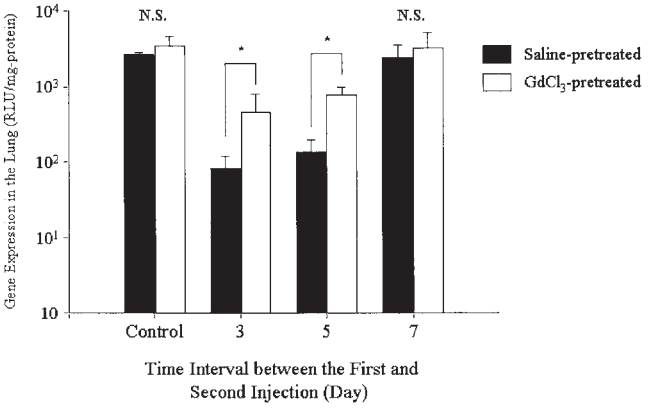

Figure 5 *Gene expression following repeated injection of lipoplex in $GdCl_3$-pretreated and saline-pretreated mice. Mice were pretreated with $GdCl_3$ solution or saline as described in Figure 1. Lipoplex containing pcDNA3 was intravenously injected 24 h after $GdCl_3$ pretreatment at a dose of 12.5 μg DNA per mouse. Mice received a second intravenous injection of lipoplex containing pCMV-Luc at a dose of 12.5 μg DNA per mouse at indicated times after the first injection of lipoplex containing pcDNA3. pCMV-Luc was constructed as described previously.[48] Gene expression in the lung was examined 6 h after the second injection of lipoplex containing pCMV-Luc. The amount of gene expression was evaluated as described previously.[48]*

repeated injections. However, the transgene expression in $GdCl_3$-pretreated mice was unable to completely return to control levels in spite of almost complete suppression of TNF-α and IL-12 production in serum. Also, we could not find any difference in the amount of transgene expression after a single injection of lipoplex in $GdCl_3$-pretreated mice and saline-pretreated mice (Figure 5; control). This is likely to be due to the fact that IFN-γ, which was not completely eliminated by $GdCl_3$, could be involved in the reduced gene expression. Li *et al* have reported that addition of IFN-γ to cultured mouse lung endothelial cells inhibited lipofection.[9] In addition, the toxicity of cationic lipids might be involved in this refractory behavior. A short time (6 h) exposure to the proinflammatory cytokines after injection would also partly explain how there was no increase in the amounts of transgene expression after a single injection of lipoplex in $GdCl_3$-pretreated mice. Luciferase activity in the lung was measured 6 h after injection in this study since this was the peak time for transgene expression in the lung after i.v. injection.[45] The short duration of gene expression and the refractory behavior to repeated injection are serious problems that have to be overcome for the development of *in vivo* gene therapy using nonviral vectors. Gene expression in the lung after intravenous administration of lipoplex gradually fell and had almost disappeared several days after injection.[46] Seven to 14 days were needed to show the same amount of gene expression in lung after the second injection of lipoplex compared with the first injection.[9,46] This is partly due to the cytotoxic effects of proinflammatory cytokines induced by the lipoplex. The data presented here suggest that prevention of lipoplex uptake by tissue macrophages, mainly liver Kupffer cells and spleen macrophages, would lead to a reduction in proinflammatory cytokine production and an improvement in transgene expression.

In summary, we have shown that $GdCl_3$ pretreatment results in the reduction of systemic proinflammatory cytokine production after intravenous administration of lipoplex and prevents the refractory behavior seen after repeated injections. Our results show that liver Kupffer cells and spleen macrophages play an important role in the induction of proinflammatory cytokine production. The present study also suggests that development of more efficient targeting vectors, which can avoid nonspecific uptake by these cells, will lead to a reduction in proinflammatory cytokine production and improvement in transgene expression. Our results provide useful basic information for the development of more efficient *in vivo* gene delivery vectors.

Acknowledgements

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