stimulating factor (GM-CSF, 20 ng/ml; PeproTech). After 7–8 days, the cells were collected and used as Flt3L-DCs or GM-CSF-DCs, respectively (Lutz *et al.*, 1999). Seven to 8 days after incubation, Flt3L-DCs were further sorted by flow cytometry (FACSAria; BD Biosciences Immunocytometry Systems, Mountain View, CA) after staining with antibodies against CD11c and B220. $CD11c^{+}B220^{+}$ Flt3L-DCs and $CD11c^{+}B220^{-}$ Flt3L-DCs were used as Flt3L-plasmacytoid DCs (Flt3L-pDCs) and Flt3L-conventional DCs (Flt3L-cDCs), respectively (Kato *et al.*, 2005). The purity of the Flt3L-pDCs and Flt3L-cDCs was greater than 91 and 86%, respectively, based on flow cytometric analysis (data not shown).

Stimulation of cells and measurement of cytokine production

DCs and macrophages (5×10^5 cells per well) seeded on 24-well plates were stimulated with Ad vector at 10,000 VP/cell (555 infectious titer units/cell) or with CpG-DNA (2.5 μM). After 48 hr of incubation, culture supernatants were collected and analyzed for cytokine levels by enzyme-linked immunosorbent assay (ELISA). Mouse IL-6 and IL-12 ELISA kits were purchased from R&D Systems. As a result of alamarBlue (BioSource, San Diego, CA) staining, we detected no cellular toxicity after infection with Ad vector at 10,000 VP/cell (data not shown).

Flow cytometric analysis of costimulatory molecule expression

After stimulation with Ad vector at 10,000 VP/cell for 48 hr, GM-CSF-DCs were collected, washed with ice-cold phosphate-buffered saline (PBS), and stained with FITC-labeled monoclonal anti-mouse CD11c and PE-labeled monoclonal anti-mouse CD86 in the presence of anti-CD16/32 to block nonspecific binding. Stained cells were washed, resuspended in 1% FBS–PBS, and analyzed by flow cytometry (FACSCalibur; BD Biosciences Immunocytometry Systems) followed by analysis with CellQuest software (BD Biosciences Immunocytometry Systems).

Reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from GM-CSF-DCs, Flt3L-cDCs, Flt3L-pDCs, and peritoneal macrophages with ISOGEN reagent (Wako) after infection with Ad vectors for 48 hr. Reverse transcription (RT) was performed with the SuperScript first-strand synthesis system for first-strand cDNA synthesis (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Polymerase chain reaction (PCR) was then done with the following primers: MyD88: forward, 5′-ATG TCT GCG GGA GAC CCC CGC GTG-3′; reverse, 5′-TCA GGG CAG GGA CAA AGC CTT GG-3′; TLR3: forward, 5′-TCA CTT GCT CAT TCT CCC TT-3′; reverse, 5′-GAC CTC TCC ATT CCT GGC-3′; TLR7: forward, 5′-GGT ATG CCG CCA AAT CTA AA-3′; reverse, 5′-TTG ACC TTT GTG TGC TCC TG-3′; TLR9: forward, 5′-ATG GAC GGG AAC TGC TAC TAC A-3′; reverse, 5′-GAC CTT GGA ACC AGG AAG AGT T-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5′-TTC ACC ACC ATG GAG AAG GC-3′; reverse, 5′-GGC ATG GAC TGT GGT CAT GA-3′. The expected sizes of the PCR products were as follows: MyD88, 891 bp; TLR3, 157 bp; TLR7, 456 bp; TLR9, 400 bp; GAPDH, 237 bp. The cycle conditions were as follows: 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C by 30 cycles (for GAPDH and TLR7), 37 cycles (for MyD88 and TLR9), or 38 cycles (for TLR3).

In vivo *innate immune response to adenoviral vectors*

Ad-L2 (3.0×10^{10} VP) was intravenously administered to wild-type, MyD88-deficient, and TLR9-deficient mice. Blood samples were recovered, at the indicated time points after injection, from the vena cava and subjected to measurement of IL-6 and IL-12 levels by ELISA. Six hours later, the heart, lung, liver, kidney, and spleen were isolated and homogenized as previously described (Xu *et al.*, 2001). Luciferase production in each organ was determined with a luciferase assay system (PicaGene 5500; Toyo Ink, Tokyo, Japan). Protein content was measured with a Bio-Rad assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin as a standard.

RESULTS

MyD88 and TLR9 are crucial for adenoviral vector-induced IL-6 and IL-12 production in GM-CSF-DCs and Flt3L-cDCs

To analyze the molecular mechanisms of innate immune responses caused by Ad vectors, it is appropriate to use primary DCs from secondary lymphoid tissues such as the spleen. However, it is difficult to prepare primary DCs because spleen contains a few number of DCs (Koizumi *et al.*, 2007). Therefore, we prepared GM-CSF-DCs and Flt3L-DCs from bone marrow. GM-CSF-DCs and Flt3L-DCs are frequently used for research concerning innate immunity (Kumar *et al.*, 2006).

To examine the role of MyD88 and TLR9 in the Ad vector-induced *in vitro* innate immune response, we prepared GM-CSF-DCs (Lutz *et al.*, 1999) from bone marrow cells of wild-type, MyD88-deficient, and TLR9-deficient mice, and examined IL-6 and IL-12 production after infection with Ad-L2. CpG-DNA, which is a ligand for TLR9, was used as a positive control. We have demonstrated that IL-6 was largely responsible for Ad vector-mediated tissue damage after systemic administration (Koizumi *et al.*, 2007). Therefore, we examined the effect of MyD88 or TLR9 on Ad vector-induced IL-6 and IL-12 production in GM-CSF-DCs. Ad-L2 elicited significant amounts of IL-6 and IL-12 in GM-CSF-DCs from wild-type mice, whereas their secretion in response to Ad vectors was largely decreased in MyD88-deficient or TLR9-deficient GM-CSF-DCs (Fig. 1A). Similar results were obtained when green fluorescent protein (GFP)-expressing Ad vector was used (data not shown). These results indicated that MyD88 and TLR9 are crucial for the Ad vector-elicited production of IL-6 and IL-12 in GM-CSF-DCs.

We next performed flow cytometric analysis to examine the surface expression of costimulatory molecules on GM-CSF-DCs after infection with Ad vectors. After infection with Ad-L2, expression of the maturation marker CD86 was significantly increased in wild-type DCs (Fig. 1B). MyD88-deficient and TLR9-deficient GM-CSF-DCs expressed CD86 in amounts al-

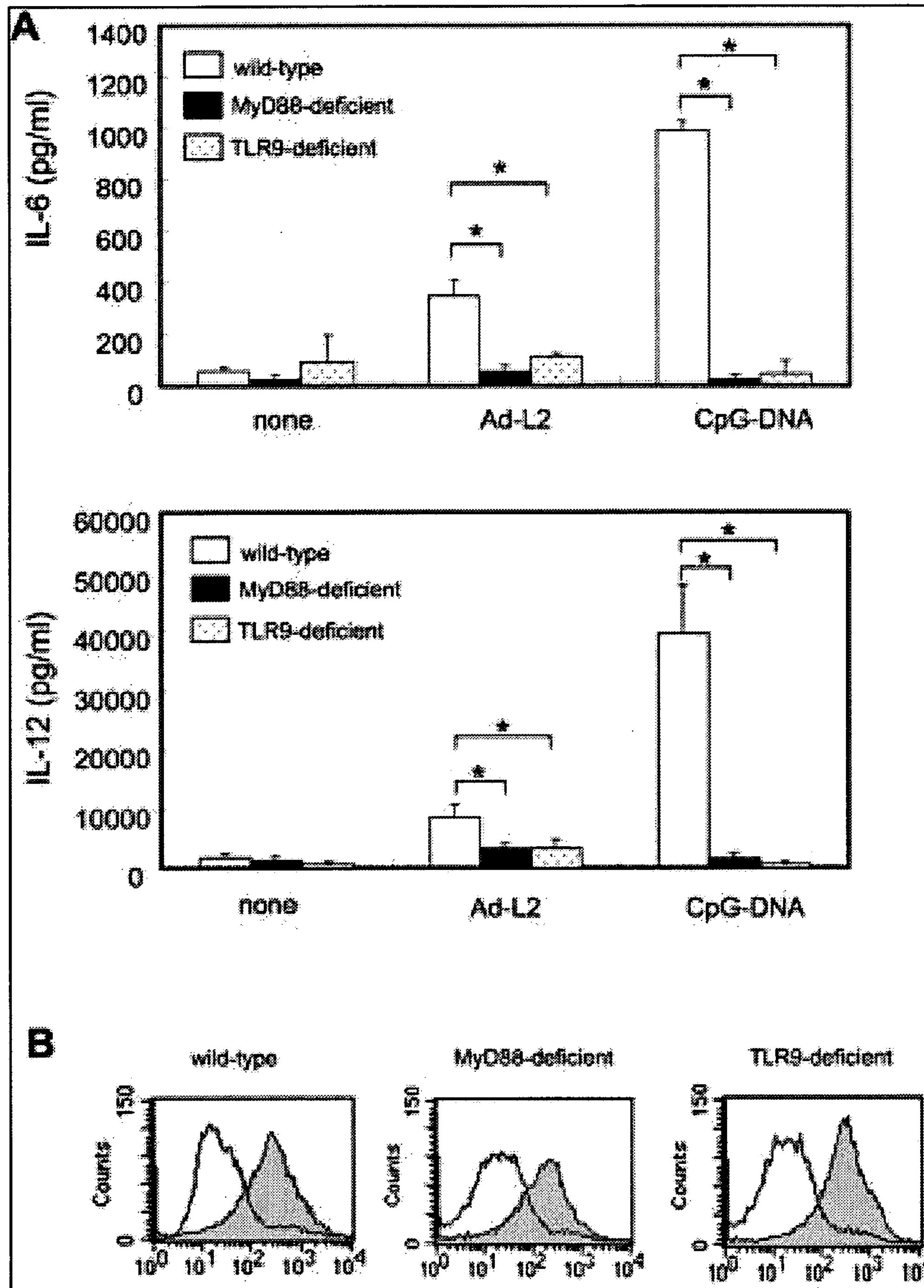

FIG. 1. MyD88- and TLR9-dependent activation of GM-CSF-DCs after Ad infection. (**A**) Ad vector-induced production of IL-6 (*top*) and IL-12 (*bottom*). GM-CSF-DCs from wild-type, MyD88-deficient, and TLR9-deficient mice were stimulated with Ad-L2 (10,000 VP/cell) or CpG-DNA (2.5 μM) for 48 hr, and production of IL-6 and IL-12 in the culture supernatants was measured by ELISA. All data represent means ± SD ($n = 3$). $*p < 0.01$. (**B**) Ad vector-induced maturation of GM-CSF-DCs. GM-CSF-DCs from wild-type, MyD88-deficient, and TLR9-deficient mice were stimulated with Ad-L2 (10,000 VP/cell). After 48 hr of incubation, the cells were collected, stained with FITC-labeled monoclonal anti-mouse CD11c antibody and PE-labeled monoclonal anti-mouse CD86 antibody, and subjected to flow cytometric analysis. Black lines represent cells without stimulus; gray areas represent cells infected with Ad-L2. Results are representative of two independent experiments.

most comparable to that of wild-type cells in response to Ad vectors (Fig. 1B). The surface expression of other maturation markers, such as CD40, CD80, and MHC class II, was also similar to that of CD86 (data not shown). These results indicate that Ad vector induces the maturation of GM-CSF-DCs via a MyD88/TLR9-independent pathway.

cDCs are professional antigen-presenting cells, whereas pDCs are known to be major IFN-producing cells after viral in-

fection (Colonna *et al.*, 2004; Wu and Dakic, 2004). Next, we examined Ad vector-induced IL-6 and IL-12 production in cDCs and pDCs. Flt3L-cDCs produced significant amounts of IL-6 and IL-12 after infection with Ad vectors (Fig. 2A). The Ad vector-mediated production of IL-6 and IL-12 in Flt3L-cDCs strictly depended on the presence of MyD88/TLR9, similarly to GM-CSF-DCs. In contrast, sorted Flt3L-pDCs produced negligible levels of IL-6 and IL-12 after stimulation with Ad vectors (Fig. 2B). These results indicated that MyD88 and TLR9 are essential for the Ad vector-elicited production of IL-6 and IL-12 in both GM-CSF-DCs and Flt3L-cDCs.

Adenoviral vectors elicit IL-6 production through MyD88- and TLR9-independent mechanisms in peritoneal macrophages

To investigate the role of MyD88 and TLR9 in the Ad vector-induced production of IL-6 in other types of antigen-presenting cells, IFN-γ-stimulated peritoneal macrophages were infected with Ad vectors at 10,000 VP/cell for 48 hr. MyD88- or TLR9-deficient macrophages produced IL-6 in amounts almost comparable to that of wild-type cells (Fig. 3). On the other hand, Ad vector-infected peritoneal macrophages from wild-type mice were able to produce only low amounts of IL-12 (data not shown). These data suggest that peritoneal macrophages produce IL-6 in response to Ad vectors through MyD88- and TLR9-independent pathways.

MyD88, TLR3, TLR7, and TLR9 mRNA levels in GM-CSF-DCs, Flt3L-cDCs, Flt3L-pDCs, and peritoneal macrophages

The preceding results indicated that MyD88 and TLR9 are crucial for IL-6 production in cDCs (GM-CSF-DCs and Flt3L-cDCs) in response to Ad vector; in contrast, peritoneal macrophages produce IL-6 via MyD88- and TLR9-independent path-

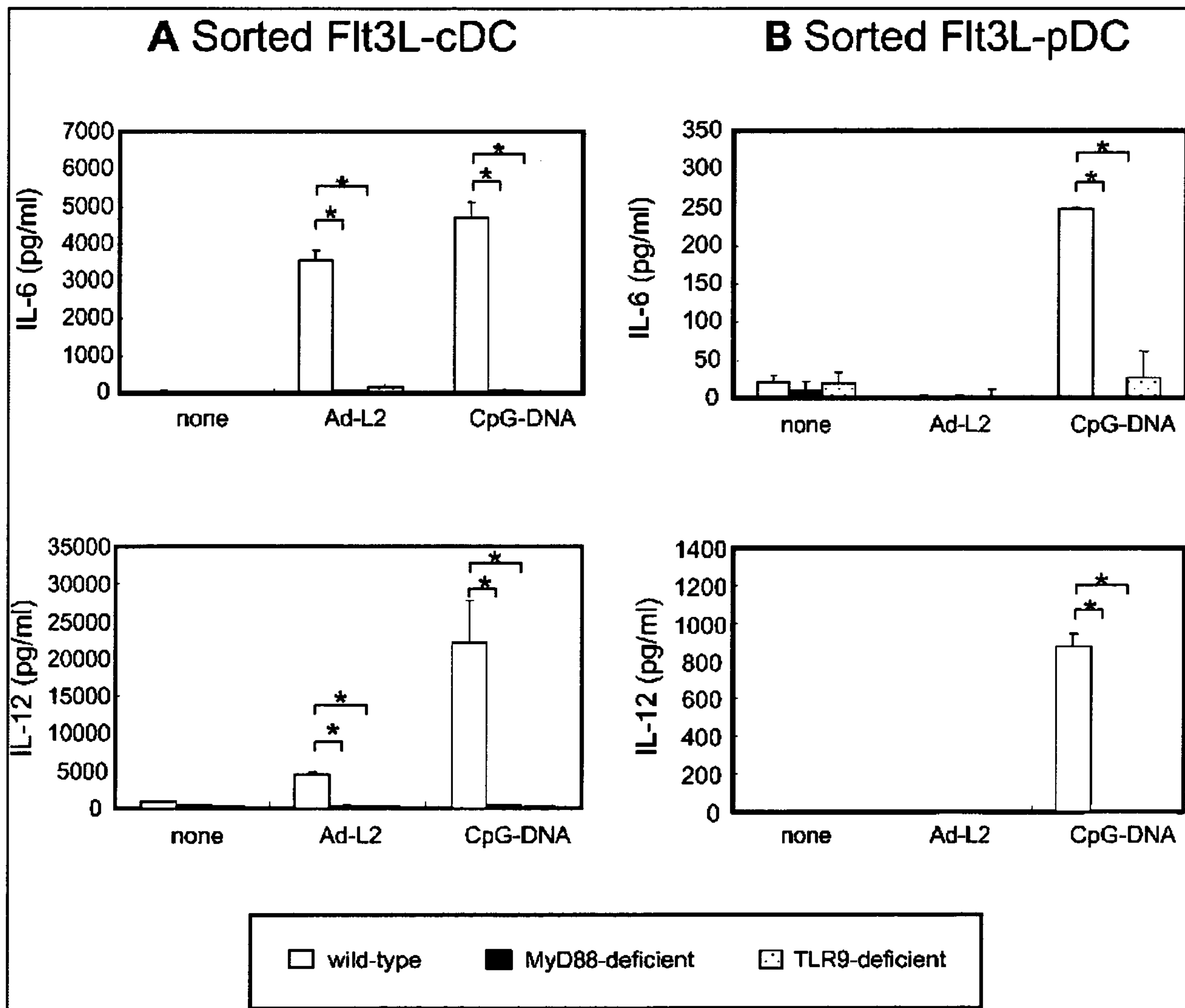

FIG. 2. Ad vector-infected MyD88- and TLR9-dependent production of IL-6 and IL-12 in Flt3L-DCs. cDCs (**A**) and pDCs (**B**) were isolated from Flt3L-stimulated bone marrow cells and purified by cell sorting. The cells were stimulated with Ad-L2 (10,000 VP/cell) or CpG-DNA (2.5 μM) for 48 hr, and then culture supernatants were harvested for measurement of IL-6 or IL-12 by ELISA. All data represent means ± SD ($n = 3$). *$p < 0.01$.

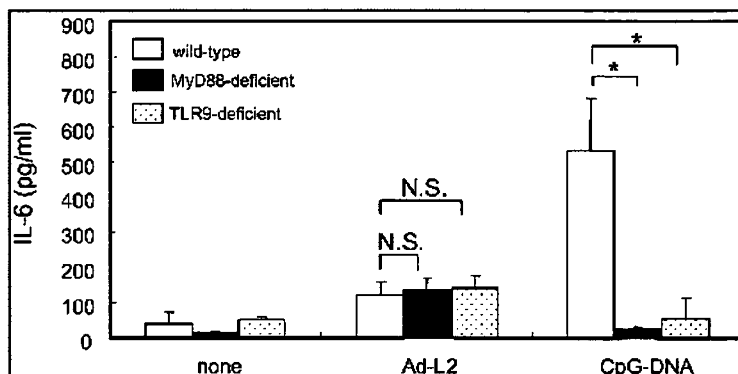

FIG. 3. MyD88- and TLR9-independent IL-6 production in peritoneal macrophages stimulated with Ad vectors. IFN-γ-activated peritoneal macrophages from wild-type, MyD88-deficient, and TLR9-deficient mice were incubated with Ad-L2 (10,000 VP/cell) or CpG-DNA (2.5 μM) for 48 hr, and the amounts of IL-6 in the culture supernatants were measured by ELISA. All data represent means ± SD (n = 3). N.S., not significantly different.

ways after stimulation with Ad vectors. These results raised doubt that the expression levels of molecules involved in Ad vector-mediated innate immunity might be different between cDCs and peritoneal macrophages. Therefore, RT-PCR analyses were performed to compare the expression of MyD88, TLR3, TLR7, and TLR9 mRNA levels in GM-CSF-DCs, Flt3L-cDCs, Flt3L-pDCs, and peritoneal macrophages (Fig. 4). GM-CSF-DCs, Flt3L-cDCs, and peritoneal macrophages, but not Flt3L-pDCs, expressed almost equal amounts of MyD88 mRNA. However, all four cell types expressed nearly equal amounts of TLR9 mRNA. These results indicated that MyD88 and TLR9 are expressed in peritoneal macrophages, but that macrophages use molecules other than MyD88 and TLR9 for Ad vector-induced IL-6 production.

Both MyD88- and TLR9-deficient mice produce levels of IL-6, IL-12, and luciferase comparable to those of wild-type mice after intravenous administration of adenoviral vectors

Next, to investigate the role of MyD88 and TLR9 in Ad vector-induced *in vivo* inflammatory cytokine production, Ad vectors were intravenously injected into wild-type, MyD88-deficient, and TLR9-deficient mice. Wild-type mice showed high levels of serum IL-6 and IL-12 six hours after injection of Ad vectors, as previously reported (Zhang *et al.*, 2001; Koizumi *et al.*, 2007). Surprisingly, MyD88- deficient mice and TLR9-deficient mice produced IL-6 and IL-12 in amounts almost comparable to that of wild-type mice (Fig. 5A). These results suggest that mechanisms other than the MyD88-dependent pathway are significantly involved in the *in vivo* IL-6 and IL-12 production induced by intravenously injected Ad vectors.

Finally, we examined luciferase expression in various organs after administration of Ad-L2 to mice. In a previous publication we examined luciferase expression 6 or 24 hr after administration of Ad vector. There was no difference in the amount of luciferase expression at these two time points (Sakurai *et al.*, 2007). Therefore, in this experiment it was measured 6 hr after administration of Ad vector. The levels of luciferase expression in the heart, lung, kidney, liver, and spleen of MyD88- or TLR9-deficient mice were similar to those in wild-type mice, indicating that the lack of MyD88 or TLR9 does not affect the *in vivo* transduction efficiencies of Ad vectors in these organs (Fig. 5B).

DISCUSSION

This study demonstrated that Ad vectors stimulate innate immunity via MyD88/TLR9-dependent and -independent pathways according to cell type, suggesting that the mechanisms of Ad vector-mediated innate immune response are more complicated than previously expected. Previous studies demonstrated that TLR9 plays an important role in inflammatory cytokine production in response to dsDNA viruses, including MCMV, HSV, and baculovirus (Lund *et al.*, 2003; Hochrein *et al.*, 2004;

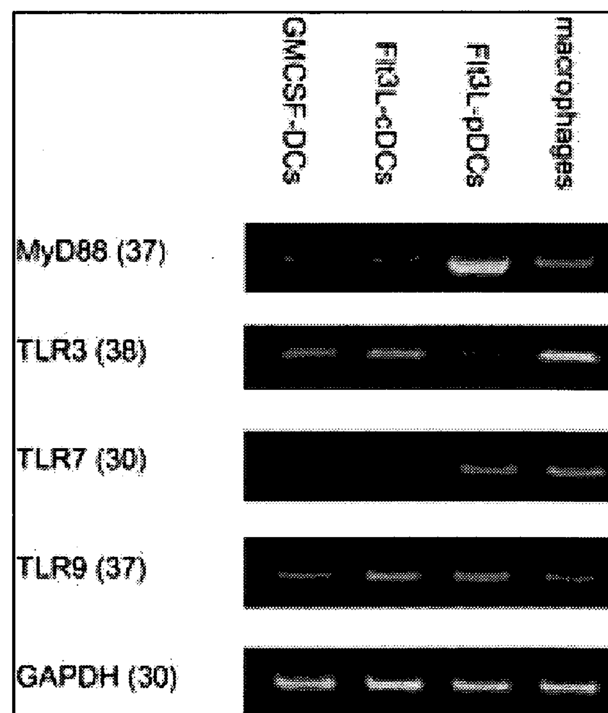

FIG. 4. MyD88, TLR3, TLR7, and TLR9 mRNA expression in immune cells. Total RNA samples were isolated from GM-CSF-DCs, Flt3L-cDCs, Flt3L-pDCs, and peritoneal macrophages. The expression of MyD88, TLR3, TLR7, and TLR9 mRNAs was tested by RT-PCR. From left to right: Lane 1, GM-CSF-DCs; lane 2, Flt3L-cDCs; lane 3, Flt3L-pDCs; lane 4, peritoneal macrophages. Numbers in parentheses indicate the number of cycles.

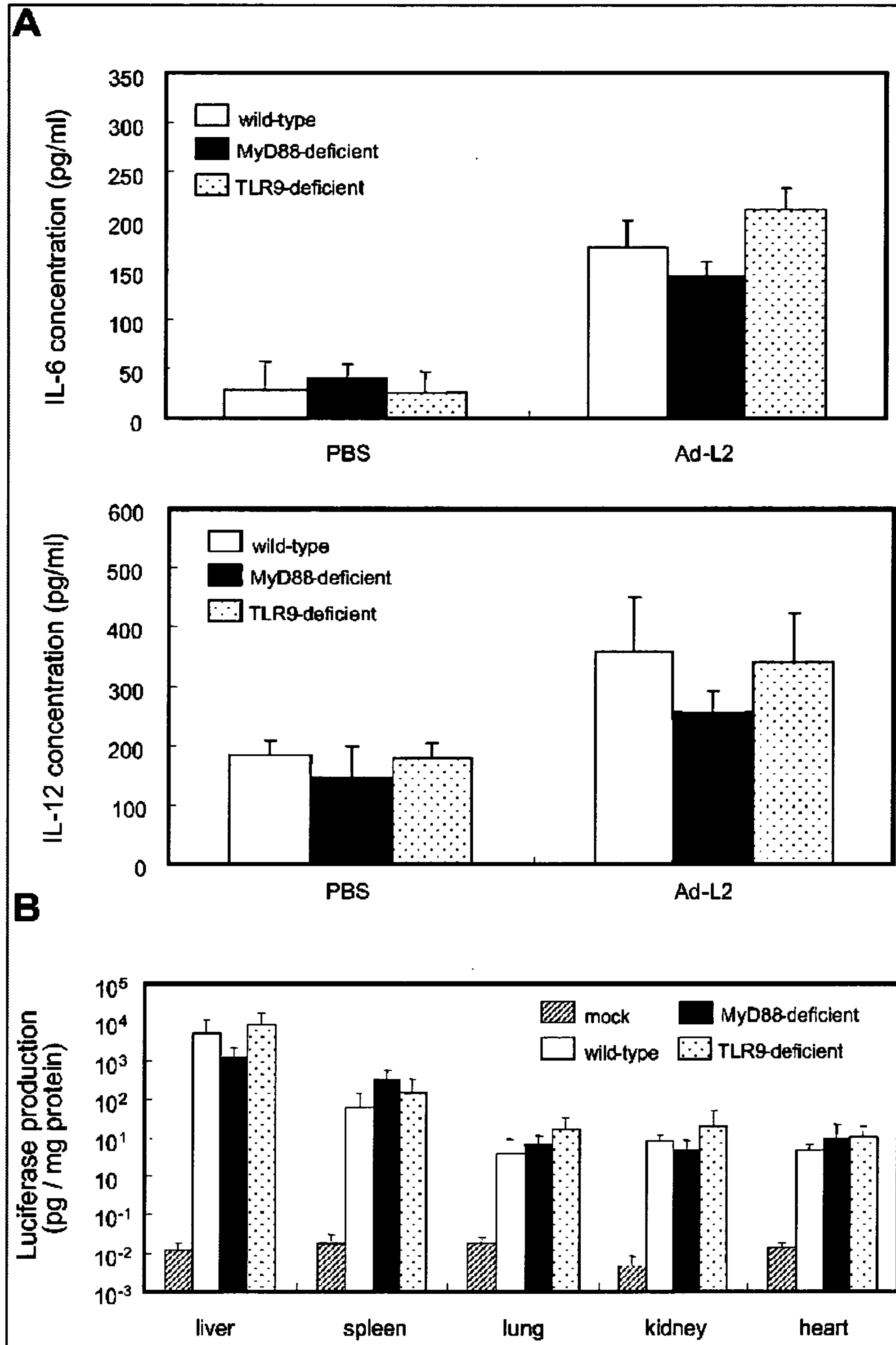

FIG. 5. (**A**) IL-6 (*top*) and IL-12 (*bottom*) concentration in serum and (**B**) luciferase production in organs after systemic administration of Ad-L2 to mice. Six hours postadministration, the blood and organs, including the heart, lung, liver, kidney, and spleen, were recovered. IL-6 and IL-12 levels in serum were measured by ELISA. Luciferase production in each organ was measured with a luciferase assay system. All data represent means ± SD (n = 4 or 5 mice).

Krug *et al.*, 2004a,b; Abe *et al.*, 2005). Adenovirus also possesses dsDNA as a genome, suggesting that adenovirus as well as these dsDNA viruses might stimulate inflammatory cytokine production through the same intracellular pathways. TLR9 was originally identified as a PRR for an unmethylated CpG motif (Hemmi *et al.*, 2000). The type 5 Ad genome, which is the most commonly used as a gene therapy vector, contains 151 unmethylated 5′-Pur-Pur-CpG-Pyr-Pyr-3′, which is reported to be

the most potent CpG motif for mice (Klinman *et al.*, 1996), and therefore, TLR9 would recognize the Ad genome after internalization of virions.

This study showed that GM-CSF-DCs and Flt3L-cDCs use both MyD88 and TLR9 for Ad vector-triggered IL-6 and IL-12 production (Figs. 1A and 2A); however, neither MyD88 nor TLR9 was crucial for Ad vector-induced IL-6 production in peritoneal macrophages (Fig. 3). Although RT-PCR analysis demonstrated that peritoneal macrophages express both MyD88 and TLR9 at levels similar to those of GM-CSF-DCs and Flt3L-cDCs (Fig. 4), peritoneal macrophages do not use MyD88 or TLR9 for IL-6 production after Ad vector infection. These results suggest that mechanisms other than the TLR pathway might be involved in the Ad vector-induced innate immune response in peritoneal macrophages. PRRs different from TLRs, such as RIG-I and melanoma differentiation-associated gene 5 (Mda5), were identified for RNA recognition (Yoneyama *et al.*, 2004; Kato *et al.*, 2005, 2006; Kawai *et al.*, 2005). Double-stranded B-form DNA (B-DNA) also promotes the production of type I IFNs by a TLR-independent pathway (Ishii *et al.*, 2006), although the receptor for B-DNA is currently unknown. Ad vectors contain dsDNA as their genome, suggesting that Ad as well as B-DNA might be recognized by the same receptor in peritoneal macrophages.

Our results showed that Ad vector-infected DCs matured through a MyD88-independent pathway (Fig. 1B). These results were consistent with data previously reported by Philpott *et al.* (2004). On the other hand, IL-6 and IL-12 production in DCs by Ad vectors was dependent on both MyD88 and TLR9. The reason for this contradiction is currently unknown. Further studies are clearly needed for detailed analysis of the mechanisms of DC maturation by Ad vectors.

During the preparation of this paper, Basner-Tschakarjan *et al.* reported that cDCs produce IL-6 in a TLR9-dependent manner, whereas pDCs from both wild-type and TLR9-deficient mice produce low or no IL-6 (Basner-Tschakarjan *et al.*, 2006). These results are consistent with our present study. Zhu *et al.* reported that the production of type I IFNs by cDCs is mediated by a TLR9-independent pathway, whereas that by pDCs is mediated by a TLR9-dependent pathway (Zhu *et al.*, 2007). The signaling pathway for IFN production is known to be different from that for inflammatory cytokine production (Kawai and Akira, 2006). Mechanisms of IFN production by Ad vectors need further examination. In this study, we focused on IL-6 and IL-12 as inflammatory cytokines, because IL-6 and IL-12 are the major cytokines produced by stimulation with Ad vectors. Notably, IL-6 plays a role in liver toxicity after systemic injection of Ad vectors (Koizumi *et al.*, 2007). Microarray analysis also showed that IL-6 and IL-12 are upregulated *in vivo* 3 hr after intravenous injection of Ad vectors, whereas type I IFN is not upregulated to the same extent (our unpublished observation).

We previously demonstrated that the spleen, but not the liver, is responsible for the Ad vector-triggered production of various types of cytokines and chemokines, and that cDCs, not pDCs, in the spleen play an important role in the systemic induction of IL-6 and IL-12 after systemic administration of Ad vectors (Koizumi *et al.*, 2007). This is consistent with this study, showing that Flt3L-cDCs produced a large amount of IL-6 and IL-12 by the MyD88/TLR9-dependent pathway after infection with Ad vectors; in contrast, Flt3L-pDCs did not exhibit IL-6 production (Fig. 2B). However, we did not find a decrease in IL-6 and IL-12 levels in the serum after Ad vector administration to MyD88- or TLR9-deficient mice (Fig. 5A). The efficiency of transduction by Ad vectors was similar between wild-type mice, MyD88-deficient mice, and TLR9-deficient mice. It currently remains unclear which pathways play key roles in IL-6 production in MyD88-deficient mice and TLR9-deficient mice. We speculated that some other kind of cell, different from cDCs, such as macrophages and endothelial cells, could still produce a sufficient amount of IL-6 and IL-12 even when the MyD88/TLR9 pathway was crippled. We and other groups demonstrated that systemically administered Ad vectors are taken up by tissue macrophages, including liver Kupffer cells (Zsengeller *et al.*, 2000; Koizumi *et al.*, 2003; Colonna *et al.*, 2004). Vascular endothelial cells are known to be activated by Ad vectors (Liu and Muruve, 2003; Schiedner *et al.*, 2003). In epithelial cells, Ad vectors induce the nuclear translocation of NF-κB, which promotes expression of the inflammatory chemokines IFN-γ-inducible protein 10 (IP-10) and RANTES (Borgland *et al.*, 2000). Moreover, nonimmune cell lines, such as HeLa, A549 respiratory epithelial cells, and the TGP61 mouse insulinoma cell line, showed similar chemokine activation patterns after Ad infection (Liu *et al.*, 2003). Therefore, these nonimmune cells might also be involved in the Ad vector-mediated activation of innate immunity.

In summary, this study provided important insights into the mechanisms of Ad vector-triggered innate immune responses. Further examination of the intracellular pathways of Ad vector-triggered innate immune responses would lead to the development of Ad vectors showing safer profiles and successful gene therapy.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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Fiber-Modified Adenovirus Vectors Decrease Liver Toxicity through Reduced IL-6 Production[1]

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Adenovirus (Ad) vectors are one of the most commonly used viral vectors in gene therapy clinical trials. However, they elicit a robust innate immune response and inflammatory responses. Improvement of the therapeutic index of Ad vector gene therapy requires elucidation of the mechanism of Ad vector-induced inflammation and cytokine/chemokine production as well as development of the safer vector. In the present study, we found that the fiber-modified Ad vector containing poly-lysine peptides in the fiber knob showed much lower serum IL-6 and aspartate aminotransferase levels (as a maker of liver toxicity) than the conventional Ad vector after i.v. administration, although the modified Ad vector showed higher transgene production in the liver than the conventional Ad vector. RT-PCR analysis showed that spleen, not liver, is the major site of cytokine, chemokine, and IFN expression. Splenic CD11c$^+$ cells were found to secret cytokines. The tissue distribution of Ad vector DNA showed that spleen distribution was much reduced in this modified Ad vector, reflecting reduced IL-6 levels in serum. Liver toxicity by the conventional Ad vector was reduced by anti-IL-6R Ab, suggesting that IL-6 signaling is involved in liver toxicity and that decreased liver toxicity of the modified Ad vector was due in part to the reduced IL-6 production. This study contributes to an understanding of the biological mechanism in innate immune host responses and liver toxicity toward systemically administered Ad vectors and will help in designing safer gene therapy methods that can reduce robust innate immunity and inflammatory responses. ***The Journal of Immunology,*** **2007, 178: 1767–1773.**

Recombinant adenovirus (Ad)[3] vectors are widely used for gene therapy experiments and clinical gene therapy trials. One of the limitations of Ad vector-mediated gene transfer is the immune response after systemic administration of the Ad vector (1, 2). The immune response to the Ad vector and Ad vector-transduced cells dramatically affects the kinetics of the Ad vector-delivered genes and the gene products. The potent immunogenic toxicities and consequent short-lived transgene expression of Ad vectors are undesirable properties if Ad vectors are to be more broadly applied. The immunogenic toxicities associated with the use of Ad vectors involve both innate and adaptive immune responses.

In the first generation Ad vector lacking the *E1* gene, leaky expression of viral genes from the vector stimulates an immune response against the Ad vector-transduced cells (3–5). The CTL response can be elicited against viral gene products and/or transgene products expressed by transduced cells. The molecular mechanism of this toxicity has been studied extensively, and the helper-dependent (gutted) Ad vector, which deletes all of the viral protein-coding sequences, has been developed to overcome this limitation (6–8). The humoral virus-neutralizing Ab responses against the Ad capsid itself are another limitation, preventing transgene expression upon the subsequent administration of vectors of the same serotype. Because hexons are mainly targeted by neutralizing Abs, hexon modification has been reported to allow for escape from neutralizing Abs (9). The Ad vectors belonging to types of the subgroup other than Ad type 5, including an Ad type 11- or 35-based vector, or to species other than human have also been developed (10–13).

Regarding the innate immune response, shortly after systemic injection of the Ad vector cytokines/chemokines are produced and an inflammatory response occurs in response to the Ad vector and Ad vector-transduced cells. It has been reported that activated Kupffer cells (and monocytes and resident macrophages) and dendritic cells (DC) release proinflammatory cytokines/chemokines such as IL-6, TNF-α, IP-10, and RANTES, causing the activation of an innate immune response (14, 15). NF-κB activation is likely to play a central role in inflammatory cytokine/chemokine production (16, 17). Although many papers regarding the innate immune response to the Ad vector have been published thus far, the biological mechanism has not been clearly elucidated. Even the cell types responsible for the innate immune response have not been identified. Understanding the mechanism of and identifying the cell types responsible for the innate immune response and liver inflammation are crucial to the construction of new vectors that are safer and efficiently transduce target tissue. Modification of the Ad vector with polyethylene glycol (PEG) reduces the innate immune response and also prolongs persistence in the blood and circumvents neutralization of the Ad vectors by Abs (18–21). We have previously reported that the mutant Ad vector ablating coxsackievirus and Ad receptor (CAR) (the first receptor) binding, α_v integrin (the secondary receptor) binding, and heparan sulfate glycosaminoglycan (HSG) (the third receptor) binding reduced (or blunted)

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[3] Abbreviations used in this paper: Ad, adenovirus; AST, aspartate aminotransferase; CAR, coxsackievirus and Ad receptor; DC, dendritic cell; HSG, heparan sulfate glycosaminoglycan; PEG, polyethylene glycol; VP, virus particle.

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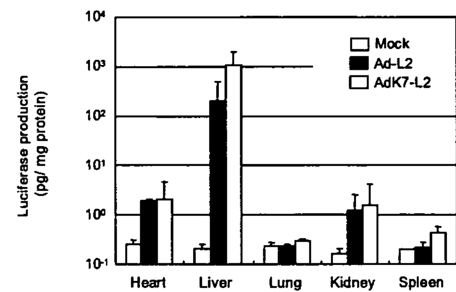
liver toxicity and IL-6 production (22). However, these two Ad vectors mediate significantly lower tissue transduction due to steric hindrance by PEG chains and a loss of binding activity to the receptor, respectively (20–22). An Ad vector showing efficient transduction and reduced innate immune response has not yet been developed.

In the present study, we elucidate the molecular mechanism of the innate immune response by the Ad vector and characterize the safer Ad vector, which reduces the innate immune response and liver toxicity. We found that the fiber-modified Ad vector containing a stretch of lysine residues (K7 (KKKKKKK) peptide) (23–25) that target heparan sulfates on the cellular surface greatly reduced IL-6 and liver toxicity after i.v. injection into mice compared with the conventional Ad vector. IL-6 and the other immune cytokines, chemokines, and IFNs were mainly produced from the spleen and especially from conventional DC ($CD11c^+B220^-$ cells), not the liver. The spleen distribution of the K7-modified Ad vector was reduced compared with the conventional Ad vector. The K7-modified Ad vector decreased the liver toxicity (aspartate aminotransferase (AST) levels), at least in part due to the reduced serum IL-6 levels. Importantly, this K7-modified Ad vector maintained high transduction efficiency in vivo and showed somewhat higher transgene production in the liver than a conventional Ad vector.

A



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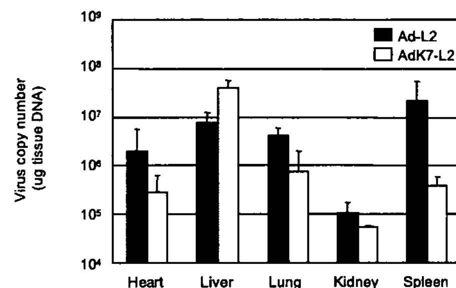


FIGURE 1. Luciferase production and biodistribution of viral DNA after the i.v. administration of Ad-L2 or AdK7-L2 into mice. Ad-L2 or AdK7-L2 (1.0×10^{10} VP) was i.v. injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested, and luciferase production (*A*) and Ad vector DNA (*B*) in each organ were measured by a luciferase assay system or the quantitative TaqMan PCR assay, respectively. All data represent the means ± SD of 4–6 mice.

Materials and Methods

Ad vector

Two luciferase-expressing Ad vectors, Ad-L2 and AdK7-L2, have been constructed previously (25, 26). The CMV promoter-driven luciferase gene derived from the pGL3-Control was inserted into the E1 deletion region of the Ad genome. Ad-L2 contains wild-type fiber, whereas AdK7-L2 contains the polylysine peptide KKKKKKK in the C-terminal of the fiber knob (25). Viruses (Ad-L2 and AdK7-L2) were prepared as described previously (25) and purified by $CsCl_2$ step gradient ultracentrifugation. Determination of virus particle titers was accomplished spectrophotometrically by the method of Maizel et al. (27).

Ad-mediated transduction in vivo

Ad-L2 or AdK7-L2 were i.v. administered to C57BL6 mice (1.0×10^{10} virus particles (VP)) (6-wk-old males obtained from Nippon SLC). Forty-eight hours later, the heart, lung, liver, kidney, and spleen were isolated and homogenated as previously described (28). Luciferase production was determined using a luciferase assay system (PicaGene 5500; Toyo Inki). Protein content was measured with a Bio-Rad assay kit using BSA as a standard.

The amounts of Ad genomic DNA in the each organ were quantified with the TaqMan fluorogenic detection system (ABI Prism 7700 sequence detector; PerkinElmer Applied Biosystems). Samples were prepared with DNA templates isolated from each organ (25 ng) by an automatic nucleic acid isolation system (NA-2000; Kurabo Industries). The amounts of Ad DNA were quantified with the TaqMan fluorogenic detection system (PerkinElmer Applied Biosystems) as described in our previous report (22).

To analyze the involvement of IL-6 signaling in liver toxicity in response to Ad vector administration, 100 μg per mouse of an anti-IL-6R Ab (clone D7715A7; BioLegend) that specifically blocks IL-6 signaling was i.p. administered to C57BL6 mice 1.5 h before Ad-L2 administration (3.0×10^{10} VP). Rabbit IgG (clone R3-34; BD Biosciences) was administered as a control. Serum samples and liver tissue were collected 48 h later, and AST levels in the serum and luciferase production in the liver were determined.

Liver serum enzymes and cytokine levels after systemic administration

Blood samples were collected by the inferior vena cave at the indicated times (3 or 48 h) after i.v. administration of Ad-L2 or AdK7-L2 (3.0×10^{10} and 1.0×10^{11} VP, respectively). IL-6 and IL-12 levels in serum samples collected at 3 h after Ad injection were measured by an ELISA kit (BioSource International). The levels of AST in serum samples collected at 24 and 48 h were measured with the Transaminase-CII kit (Wako Pure Chemical). Forty-eight hours after the Ad vector injection, the mice were killed and their livers were collected. The liver was washed, fixed in 10% formalin, and embedded in paraffin. After sectioning, the tissue was dewaxed in ethanol, rehydrated, and stained with H&E. This process was commissioned to the Applied Medical Research Laboratory (Osaka, Japan).

Cytokines and chemokines mRNA levels in tissue after systemic administration

Total tissue RNA samples were isolated by the reagent ISOGEN (Wako Pure Chemical) 3 h after the i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP). Reverse transcription was performed using the SuperScript first-strand synthesis system for first-strand cDNA synthesis (Invitrogen Life Technologies) according to the instructions of the manufacturer. IL-6 and IL-12 mRNA in the liver and spleen were quantified with the TaqMan fluorogenic detection system (PerkinElmer Applied Biosystems). Semiquantified RT-PCR analysis was also performed to determine mRNA levels of the cytokines, chemokines, and IFNs (total eight mRNA). The primer sequences and probes were as follows: IL-6 forward, 5′-GAG GAT ACC ACT CCC AAC AGA CC-3′; IL-6 reverse, 5′-AAG TGC ATC ATC GTT GTT CAT ACA-3′ (reverse); IL-6 probe, 5′-CAG AAT TGC CAT TGC ACA ACT CTT TTC TCA-3′; IL-12p40 forward, 5′-GGA AGC ACG GCA GCA GAA TA-3′; IL-12p40 reverse, 5′-AAC TTG AGG GAG AAG TAG GAA TGG-3′; IL-12p40 probe, 5′-CAT CAT CAA ACC AGA CCC GCC CAA-3′; TNF-α forward, 5′-CCT GTA GCC CAC GTC GTA GC-3′; TNF-α reverse, 5′-TTG ACC TCA GCG CTG AGT TG-3′; RANTES forward, 5′-ATG AAG ATC TCT GCA GCT GCC CTC ACC-3′; RANTES reverse, 5′-CTA GCT CAT CTC CAA ATA GTT GAT G-3′; MIP-2 forward, 5′-ACC TGC CGG CTC CTC AGT GCT GC-3′; MIP-2 reverse, 5′-GGC TTC AGG GTC AAG GCA AAC-3′; IFN-α forward, 5′-AGG CTC AAG CCA TCC CTG T-3′; IFN-α reverse, 5′-AGG CAC AGG GGC TGT CTT TCT TCT-3′; IFN-β forward, 5′-TTC CTG CTG TGC TTC TCC AC-3′; IFN-β reverse, 5′-GAT TCA CTA CCA GTC CCA GAG TC-3′; IFN-γ forward, 5′-GAG GAT ACC ACT CCC AAC AGA CC-3′; IFN-γ reverse, 5′-AAG TGC ATC ATC GTT GTT CAT ACA-3′; GAPDH forward, 5′-TTC ACC ACC ATG GAG AAG GC-3′; and GAPDH reverse, 5′-GGC ATG GAC TGT GGT CAT GA-3′. The expected sizes of the PCR products are as follows: IL-6, 193 bp; IL-12p40, 155 bp; TNF-α, 374 bp; RANTES, 252 bp; MIP-2, 221 bp; IFNα, 272 bp; IFNβ, 607 bp; IFN-γ, 306 bp; and GAPDH, 237 bp.

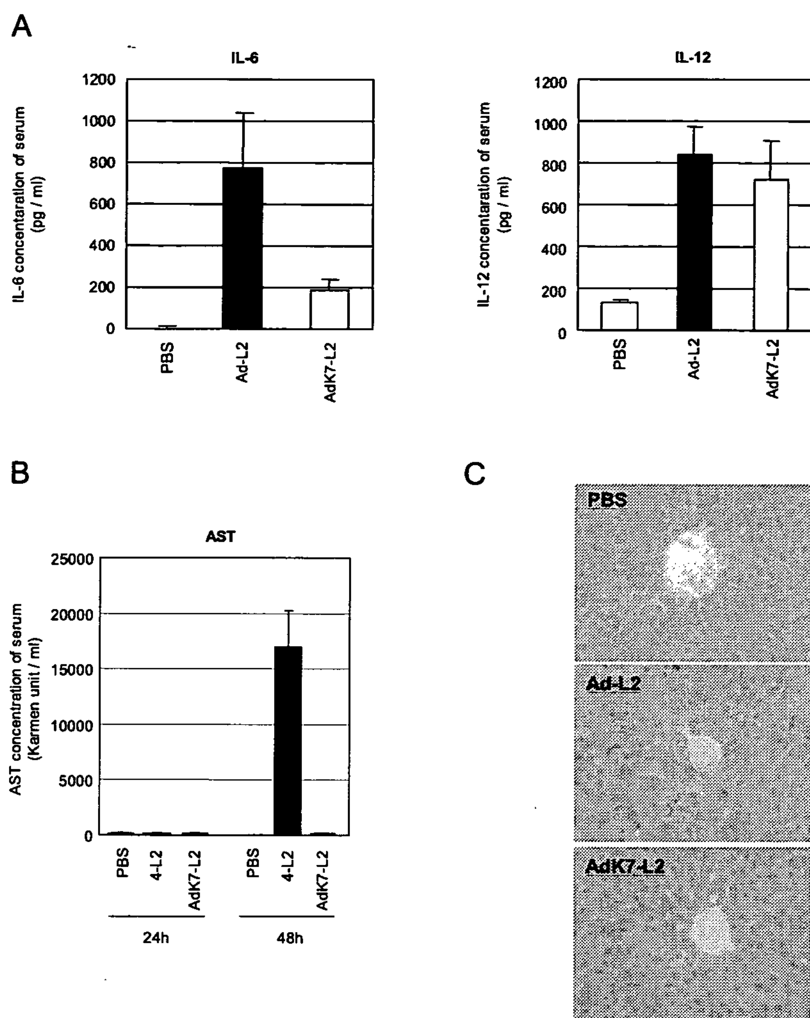


FIGURE 2. Cytokines and liver enzyme levels in serum after the systemic administration of Ad-L2 or AdK7-L2 into mice. Blood samples were collected by inferior vena cave at 3 h (*A*) or 24 and 48 h (*B*) after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP for *A* or 3.0×10^{10} VP for *B*). The livers were collected after 48 h following the injection (3.0×10^{10} VP) (*C*). *A*, IL-6 and IL-12 levels in the serum were measured by ELISA. *B*, AST levels in the serum were measured using a Transaminase-CII kit. *C*, Paraffin sections of the livers were prepared. Each section was stained with H&E. Data represent the means ± SD of four mice.

Cell sorting of splenic cells

Splenic conventional DC, plasmacytoid DC, and B cells, which were $CD11c^+B220^-$, $CD11c^+B220^+$, and $CD11c^-B220^+$ cells, respectively, were sorted by FACS Aria (BD Biosciences). Total RNA samples were isolated from each cell by the reagent ISOGEN, and RT-PCR analysis was then performed as described above.

Results

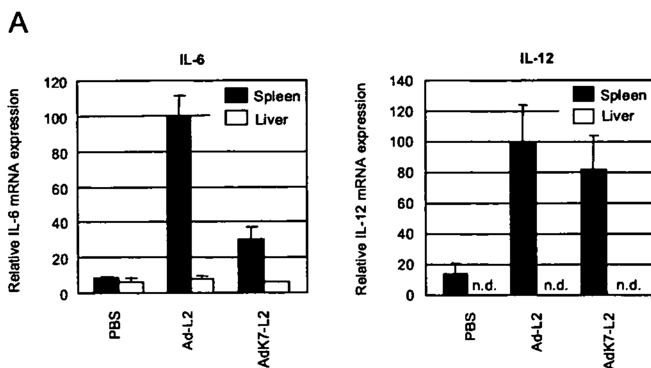
This study was undertaken to elucidate the biological mechanism in the innate immune host responses toward i.v. administered Ad vector. The relationship between the innate immune response and liver toxicity by systemic administration of the Ad vectors was also examined.

Gene transduction and Ad vector accumulation in vivo

In this study we used the conventional Ad vector (Ad-L2) and a fiber-modified Ad vector containing a polylysine (K7) peptide (AdK7-L2), both of which express luciferase under the control of the CMV promoter. First, we examined luciferase production in the organ and the biodistribution of viral DNA after i.v. administration of AdK7-L2 (1.0×10^{10} VP) into mice compared with Ad-L2 (see Fig. 3). The vector dose of 1.0×10^{10} VP was selected because this dose did not induce any apparent toxicity (IL-6 and AST production) with either Ad-L2 or AdK7-L2. When a higher dose (3.0×10^{10} or 1.0×10^{11} VP) was used, only Ad-L2 and not AdK7-L2 showed toxicity (described later), which does not reflect an exact comparison of the transduction efficiency. The Ad type 5-based vector delivers the foreign gene predominantly in the liver after i.v. injection into mice (29, 30). Interestingly, AdK7-L2 mediated ~6-fold higher liver transduction than Ad-L2 (Fig. 1*A*). In contrast, the luciferase production in the heart, lung, kidney, and spleen in response to AdK7-L2 was similar to that in response to Ad-L2. To examine the biodistribution of Ad-L2 and AdK7-L2 in mice, the amounts of Ad DNA in each organ 48 h after the injection of Ad vectors were measured with the TaqMan fluorogenic detection system. More AdK7-L2 DNA accumulated in the liver than Ad-L2 DNA (Fig. 1*B*), although the amounts of AdK7-L2 DNA in the heart, lung, kidney, and spleen were less than those of Ad-L2 DNA. In particular, the amounts of AdK7-L2 DNA in the spleen were ~56-fold less than those of Ad-L2 DNA. The data regarding luciferase production (Fig. 1*A*) and the amounts of Ad DNA in most organs (Fig. 1*B*) showed discrepancies. Luciferase production in the liver was >2 log order higher than that in other organs, while the amounts of Ad DNA in liver were not as striking among the organs compared with luciferase production. This difference is likely due to the difference in the amount of nonspecific viral uptake among the organs. Reduced spleen accumulation of AdK7-L2 DNA, compared with Ad-L2 DNA, was also observed at a dose of 1.0×10^{11} VP (data not shown).

Serum cytokines and AST levels

The systemic administration of Ad vectors results in the initiation of strong innate immune responses and inflammation in animals and humans (1), and this toxicity limits the utility of Ad vectors for gene therapy. To evaluate the innate immune response and liver toxicity of each Ad vector, we measured the levels of IL-6, IL-12, and AST in serum. Because IL-6 in the serum and hepatic toxicity



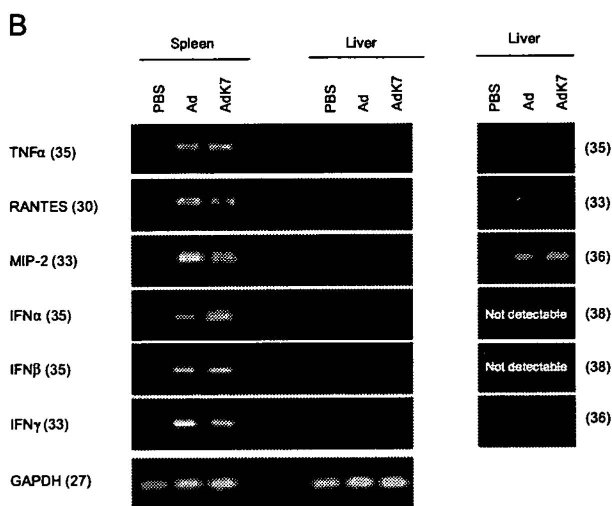


FIGURE 3. Cytokine, chemokine, and IFN mRNA levels in liver and spleen after the systemic administration of Ad-L2 or AdK7-L2 into mice. Total mRNA samples were isolated from liver and spleen at 3 h after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP). After the reverse transcriptase reaction, IL-6 and IL-12 cDNA were measured with the quantitative TaqMan PCR assay (*A*). The expression of TNF-α, RANTES, MIP-2, IFN-α, IFN-β, and IFN-γ was measured by semiquantitative RT-PCR assay (*B*). All data represent the means ± SD of four mice. Cycle number is given in parentheses.

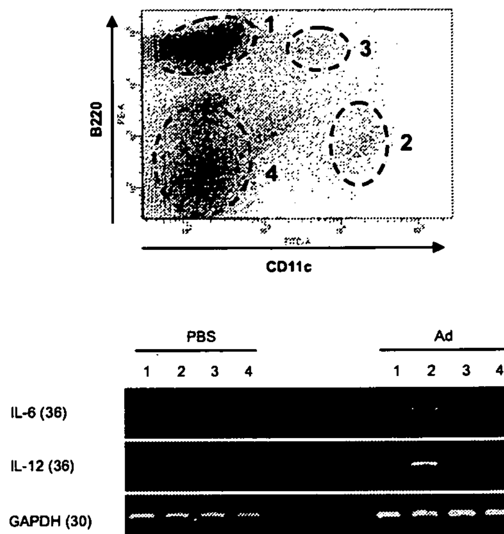


FIGURE 4. IL-6 and IL-12 mRNA levels in splenic CD11c-positive cells after the systemic administration of Ad-L2 into mice. Total mRNA samples were isolated from sorted splenic cells 3 h after i.v. administration of Ad-L2 (1.0×10^{11} VP). The expression levels of IL-6 and IL-12 mRNA were measured by RT-PCR assay. *Lane 1*, B cell ($B220^+CD11c^-$); *lane 2*, conventional DC ($B220^-CD11c^+$); *lane 3*, plasmacytoid DC ($B220^+$ $CD11c^+$); *lane 4*, other cells ($B220^-CD11c^-$). Cycle number is given in parentheses.

analysis was detected at a dose of $>1.0 \times 10^{11}$ or 3.0×10^{10} VP, respectively, these doses were used.

IL-6 levels in response to AdK7-L2 were one-fourth of those with Ad-L2 (Fig. 2*A*). In contrast, there was no difference in serum IL-12 levels between Ad-L2 and AdK7-L2. Thus, IL-6 and IL-12 appear to be produced by a different mechanism. TNF-α in the serum after the injection of Ad-L2 or AdK7-L2 could not be detected (data not shown). Ad-L2 led to high levels of serum AST at 48 h after injection, while AdK7-L2 did not induce AST (Fig. 2*B*). At 24 h, neither Ad-L2 nor AdK7-L2 induced AST. In histological analysis, degranulation or denucleation occurred in hepatocytes from Ad-L2, while AdK7-L2 did not induce hepatocyte toxicity (Fig. 2*C*). The results using AdK7-L2 were similar to those in the untreated mice (Fig. 2, *B* and *C*), suggesting that AdK7-L2 does not show any liver toxicity. These results suggest that AdK7-L2 shows less IL-6 production and almost no liver toxicity.

Cytokines mRNA levels in liver and spleen cells

Ad vectors induce the expression of various cytokines and chemokines in the innate immune responses by effector cells such as macrophages and DC (15, 17, 31–33). Liver and spleen are two major organs responsible for the location of immune cells. We attempted to determine which organ (liver or spleen) produces cytokines, chemokines, and IFNs (IL-6, IL-12, TNF-α, RANTES, MIP-2, IFNα, IFNβ, and IFN-γ) by quantitative real-time RT-PCR or semiquantitative RT-PCR analysis. IL-6 and IL-12 mRNA levels were not induced in the liver after i.v. administration of Ad vectors (Fig. 3*A*). This result was also checked by the result that specific IL-6 and IL-12 mRNA bands were not detected in the liver by RT-PCR analysis (data not shown). Expression of TNF-α, RANTES, MIP-2, IFN-α, IFN-β, and IFN-γ mRNA was also detected mainly in the spleen, not the liver (Fig. 3*B*). IL-6, MIP-2, and IFN-γ mRNA levels in the spleen in response to AdK7-L2 were lower than those in response to Ad-L2. In the liver, TNF-α, RANTES, MIP-2, and IFN-γ mRNA were detected by a high cycle number of PCR after Ad (Ad-L2 or AdK7-L2) injection, whereas IFN-α and IFN-β could be not detected (Fig. 3*B*).

We next identified the cell types responsible for the IL-6 and IL-12 expression in the spleen after i.v. administration of the Ad vector (Ad-L2). Spleen cells were sorted by FACS Aria based on the expression of CD11c and B220 in conventional DC ($CD11c^+B220^-$), plasmacytoid DC ($CD11c^+B220^+$), and B cells ($CD11c^-B220^+$ cells). IL-6 and IL-12 mRNA were mainly detected in the splenic conventional DC. Only a faint band of IL-12 mRNA was also detected in the splenic plasmacytoid DC ($CD11c^+B220^+$) (Fig. 4). These results suggest that splenic conventional DC are major effector cells of innate immune response (at least IL-6 and IL-12 production) against systemically administered Ad vectors.

Elimination of IL-6 signaling reduces liver toxicity

It has previously been shown that TNF-α is likely to be involved in host responses to Ad vectors in vitro and in vivo (34). Recently, Shayakhmetov et al. (35) have reported that IL-1 signaling, not TNF-α signaling, is involved in Ad vector-associated liver toxicity after i.v. administration. However, the mechanism of liver toxicity

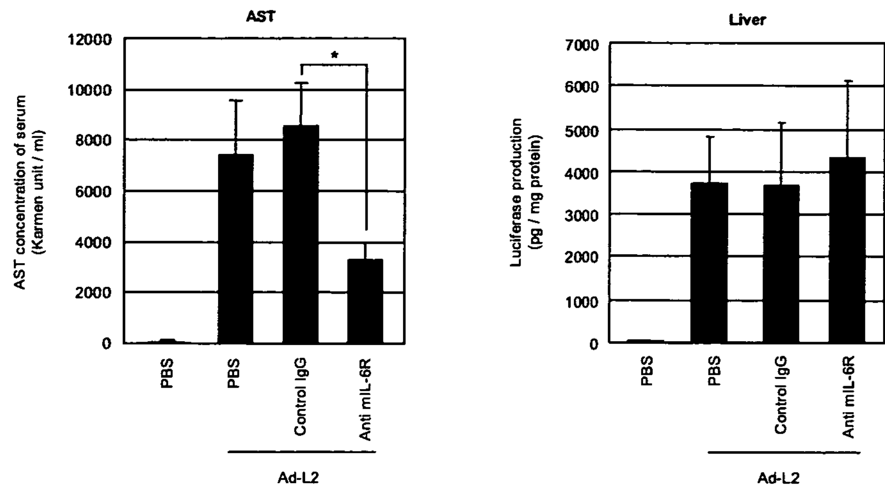


FIGURE 5. Effects of serum IL-6 on serum AST levels and liver luciferase production after the systemic administration of Ad-L2 into mice. C57BL/6 mice were i.p. administered 100 μg per mouse of anti-IL-6R Ab (clone D7715A7), which was specific for blocking IL-6 signaling, or rabbit IgG as a control (clone: R3-34). Ad-L2 or AdK7-L2 (3.0×10^{10} VP) was i.v. injected into the mice 1.5 h later. Blood samples and liver tissue were collected 48 h after the injection of Ad-L2. The AST levels in the serum were measured using a Transaminase-CII kit. Luciferase production in the liver was measured by a luciferase assay system. All data represent the means ± SD of three to four mice. *, $p < 0.01$.

after i.v. Ad administration is poorly understood. In the present study, although AdK7-L2 mediated higher luciferase expression and a higher accumulation of viral DNA in the liver than Ad-L2, it remains unclear why AdK7-L2 showed almost background levels of liver toxicity while Ad-L2 showed high toxicity. As reported previously, inflammatory cytokines, chemokines, and IFNs could be the mediators responsible for liver toxicity (2). IL-6 levels in the serum were the most strikingly different between AdK7-L2 and Ad-L2. Furthermore, IL-6 stimulated acute phase protein (serum amyloid A, fibrinogen, α_1-anti-trypsin, and α_1-acid glycoprotein) in rat and human hepatocytes (36, 37). Therefore, we next examined the effects of serum IL-6 on liver toxicity (Fig. 5). To do this, we used an anti-IL-6R Ab that inhibits the signal through the IL-6 receptor. The IL-6 receptor system consists of two functional molecules, an 80-kDa ligand-binding chain (IL-6R) and a 130-kDa nonligand-binding but signal-transducing chain (gp130). The anti-IL-6R Ab blocks the binding of IL-6 to the IL-6R (38, 39). The anti-IL-6R Ab or the control Ab was i.p. injected 1.5 h before the injection of Ad-L2. The AST levels in the serum and luciferase production in the liver were determined 48 h later. Administration of anti-IL-6R Ab significantly (~2-fold) reduced Ad vector-mediated AST levels in the serum compared with PBS or the control Ab (Fig. 5*A*). Importantly, anti-IL-6R Ab injection did not interfere with luciferase production in the liver (Fig. 5*B*). These results suggest that IL-6 signaling is involved in liver toxicity after i.v. administration of an Ad vector.

Discussion

In this study we found that the fiber-modified Ad vector containing the K7 peptide, which has high affinity with heparin sulfate, shows much lower serum IL-6 and liver toxicity than the conventional Ad vector. This improved characteristic is likely involved with the reduced biodistribution of the vector to the spleen compared with that of the conventional Ad vector. RT-PCR analysis showed that the spleen, not the liver, is the major site of cytokine, chemokine, and IFN (IL-6, IL-12, TNF-α, RANTES, MIP-2, IFN-α, IFN-β, and IFN-γ) production and that splenic conventional DC are the major effector cells of the innate immune response (at least IL-6 and IL-12 production) after i.v. administration of Ad vectors. We also showed that IL-6 signaling is involved in part with liver toxicity in response to Ad vectors. Importantly, this fiber-modified Ad vector containing the K7 peptide maintained higher transduction efficiency in all the organs examined, and the liver transduction was higher than that of the conventional Ad vector. Although there have been some reports that modified Ad vectors such as the pegylated Ad vector (18–21), the Ad vector containing the Ad type 35 fiber shaft and knob (40), and the triple mutant Ad vector with ablation of CAR, α_v integrin, and HSG binding (22) show decreased innate immune response and liver toxicity, these types of vector lose their transduction activity in vivo. To our knowledge, this is the first report of an Ad vector that maintains high transduction efficiency in vivo with reduced toxicity.

The fiber-modified Ad vector containing the K7 peptide has been developed to overcome the limitations imposed by the CAR dependence of Ad infection. Expanded and efficient gene transfer has been reported based on the use of mutant fiber proteins containing a stretch of lysine residues (23–25). However, there has been no report on the difference in gene transfer activity and toxicity in vivo between the conventional Ad vector and the fiber-modified Ad vector containing the K7 peptide. We have demonstrated that the fiber-modified Ad vector containing the K7 peptide mediates ~6-fold higher mouse liver transduction in response to i.v. administration than the conventional Ad vector (Fig. 1*A*). The amounts of fiber-modified Ad vector DNA in the liver after i.v. administration were also 5-fold higher than those with the conventional Ad vector (Fig. 1*B*). It has been reported that the interaction between the Ad type 5 fiber and the HSG of a hepatocyte is involved in the accumulation in the mouse liver and the cynomolgus monkey liver of systemically administered Ad vectors (41, 42). This fiber-modified Ad vector might mediate more efficient gene transduction through a much higher affinity for HSG. In contrast, the amounts of fiber-modified Ad vector DNA in the spleen after i.v. administration were 56-fold lower than those of the conventional Ad vector (Fig. 1*B*). Biodistribution of viral DNA reflects the total of receptor-mediated uptake and nonspecific uptake. Luciferase production in the cells mainly reflects receptor-mediated uptake. We previously reported that most Ad DNAs are taken up in the liver nonparenchymal cells, not parenchymal cells, after i.v. administration (22). In this study, the conventional Ad vector would also be taken up in the macrophages and DC by nonspecific uptake, resulting in significantly higher Ad DNA and lower luciferase production in the spleen. In contrast, the fiber-modified Ad vector would be taken up more in the liver via receptor-mediated uptake and nonspecific uptake, resulting in significantly lower Ad DNA in the other organs, especially the spleen. Even though the amount of AdK7-L2 uptake in the spleen, heart, lung, and kidney was less than that of Ad-L2 uptake, the amount of receptor-mediated uptake in these organs would be similar between Ad-L2 and AdK7-L2, suggesting that these vectors showed similar levels of luciferase production in the organs other than the liver.

The initiation of inflammatory innate immune responses occurs after the systemic administration of Ad vectors to animals and humans, and this toxicity limits the utility of Ad vectors for gene therapy. Increased cytokine/chemokine production after the injection of Ad vectors has been reported to be due to the introduction of input Ad vectors to Kupffer cells in the liver and DC (15, 17, 43–46). Detailed analysis of the organs responsible for the expression of cytokines, chemokines, and IFNs by RT-PCR suggests that their production can mainly be attributed to spleen cells (especially splenic conventional DC), not liver cells (Figs. 3 and 4), which is consistent with the recent report of Bart et al. (47). Therefore, interference with spleen distribution of the Ad vector should provide a useful method for safer gene therapy.

TLRs, which are crucial to the recognition of pathogen-associated molecular patterns, are expressed on various types of immune cells including macrophages, DC, B cells, splenic types of T cells, and even on nonimmune cells such as fibroblasts and epithelial cells (48). For example, HSV and CMV (dsDNA virus) activate inflammatory cytokines and type I IFN secretion by the stimulation of TLR9 (49–53). The innate immune receptor to the Ad has not yet been identified. It has not even been determined whether TLRs are involved in Ad-mediated innate immune response in vivo, although it has been reported that TLR signals are not involved in the DC maturation induced by the Ad vector (46). As shown in Fig. 3*B*, cytokine production against the Ad vector occurred mainly in conventional DC. It is noted that the TLR9-mediated innate immunity responses to DNA virus are cell type-specific and limited to plasmacytoid DC (50). The unidentified sensor receptor(s) for double-stranded Ad DNA or Ad capsid protein in conventional DC might play a critical role in the expression of inflammatory cytokines/chemokines and type I IFN. Although we have previously reported that large amounts of conventional Ad vector accumulate in nonparenchymal cells, including Kupffer cells and liver sinusoidal (endothelial) cells (22, 54), the expression of mRNA of cytokines, chemokines, and IFNs in the liver was weak after administration of the Ad vector (Fig. 3*B*). A lack of putative sensor receptor(s) against Ad or the inability of sensor receptor(s) to recognize Ad due to the specific cellular disposition of Ad in Kupffer cells might result in a reduced production of cytokines/chemokines/IFNs in the liver.

Another interesting finding is that the fiber-modified Ad vector containing the K7 peptide showed almost background levels of AST activity, which reflects liver toxicity (Fig. 2*B*). Histological analysis supported this finding (Fig. 2*C*). Because the K7-modified Ad vector showed higher transgene activity and a higher accumulation of viral DNA into the liver (Fig. 1), the transduction and distribution of the vector into the liver did not participate in liver toxicity. The cytokines/chemokines play a major causative role in liver damage associated with systemic Ad infusion as well as in the induction of an antiviral immune response (2). Ad-induced cytokines/chemokines recruit immune effector cells (neutrophils, monocyte/macrophages, and NK cells) to Ad-transduced cells (mainly liver), resulting in acute hepatic toxicity. Shayakhmetov et al. (35) have reported that hepatocytes and Kupffer cells trigger IL-1 transcription in liver tissue after i.v. administration of Ad vectors and that interference of IL-1-signaling reduces liver toxicity. We speculated that IL-6 could be the main mediator for hepatic toxicity because IL-6 is one of the main cytokines in the early stages of inflammation, IL-6 production by the fiber-modified Ad vector was much reduced (approximately a quarter) compared with that by the conventional Ad vector, and all of the cytokines/chemokines/IFNs we examined (including IL-6) were mainly produced by the spleen, not the liver. Treatment of the anti-IL-6R Ab decreased liver toxicity (Fig. 5), suggesting that IL-6 plays at least some role in liver toxicity induced by systemic injection of the Ad vector. Because the AST levels were only partially reduced by the treatment with the anti-IL-6R Ab, another mechanism such as IL-1 signaling, rapid Kupffer cell death (55, 56), activation of the liver endothelium (55), or other factors might be involved in the liver toxicity. Nevertheless, it is attractive that the K7-modified Ad vector did not show liver toxicity despite the higher transduction efficiency and higher accumulation of the vector into the liver (probably Kupffer cells).

Our present study provides new insight into the cellular biological mechanism related to the innate immune response and liver toxicity against the systemically administered Ad vector. Modification of vector tropism should contribute to safe gene therapy procedures.

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Disclosures

The authors have no financial conflict of interest.

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Comparison of gene expression efficiency and innate immune response induced by Ad vector and lipoplex

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Abstract

Vectors for gene expression are the essential tools for both gene therapy and basic research. There are two groups of gene therapy vectors, viral and non-viral vectors. At present, toxicity triggered by vectors is one of the major concerns for clinical trials. In general, non-viral vectors, such as plasmid DNA–cationic liposome complex (lipoplex), are thought to be safer than viral vectors, such as adenovirus (Ad) vector, although lipoplex is less efficient in term of gene expression than the Ad vector. However, there has been no study directly comparing the gene expression efficiency and safety of viral and non-viral vectors. Here, we present evidence that the Ad vector shows much more efficient gene expression and is safer than lipoplex, at least with respect to the innate immune response. After being systemically administered to mice, the Ad vector showed a transduction efficiency that was 2 to 5 log orders higher than that of lipoplex, depending on the organ. On the other hand, surprisingly, the administration of lipoplex produced a greater amount of inflammatory cytokines such as interleukin-6, interleukin-12, and tumor necrosis factor-α than did the administration of the Ad vector, whereas a comparable level of hepatotoxicity was induced by these vectors. The production of inflammatory cytokines induced by the injection of lipoplex was reduced when the CpG motifs were removed completely from plasmid DNA. Thus, care should be taken to ensure the innate immune response induced by gene therapy vectors, especially lipoplex.
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Keywords: Adenovirus vector; Lipoplex; Gene therapy; In vivo gene expression; Innate immunity

1. Introduction

Vectors for gene expression are requisite tools not only for gene therapy but also for basic research, such as the functional analysis of novel genes. The success of gene therapy is largely dependent on gene delivery vectors, which can be categorized into two groups, viral and non-viral vectors [1]. The viral vectors, such as the adenovirus (Ad) vector, have great advantages such as high-level gene expression in a broad range of tissue, but such vectors are thought to lack safety because they are based on a pathogenic virus [2,3]. Another problem is that some kinds of viral vectors have an oncogenic function. Retrovirus and lentivirus vectors, which can insert foreign genes into the host DNA in a random manner, sometimes leads to canceration [4]. On the other hand, non-viral vectors based on plasmid DNA are thought to be safe even though their transduction efficiency is low [2].

Viruses are highly developed agents specialized in infection and the transfer of genetic material to the cells of other organisms, because infecting the host cells is an essential stage in their life cycle. On the other hand, the immune systems of mammals have evolved to counterattack the efforts of viral pathogens [5]. One of the viral vectors, the Ad vector, has several advantages, including the ability to package relatively large-sized foreign DNA, the ease with which it can be produced, and broad cell tropism [5,6]. However, many studies have shown that systemic administration of Ad vectors immediately triggers the innate immune response to elicit an acute inflammation, such as occurs with the secretion of

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inflammatory cytokines and tissue injury [5,7,8]. Because of these problems with viral vectors, non-viral vectors have gained increasing attention recently [1]. Among the non-viral vectors, the lipoplex (complexes of cationic liposome/ plasmid DNA) is the most studied and represents the most promising approach for human clinical trials [2]. However, the utility of non-viral vectors is often limited because of their low level and narrow range of exogenous gene expression [9,10]. As in the case of viral vectors, the production of inflammatory cytokines and tissue damage have been reported to be induced by the systemic injection of lipoplex even though it contains no viral components [11–15]. It has been shown that the immunostimulatory CpG motifs present in plasmid DNA are responsible for a significant portion of this acute response [16]. Plasmid DNA and bacterial DNA contain a much higher frequency of unmethylated CpG motifs than does mammalian DNA [11,15–17]. Toll-like receptor (TLR) 9 has been identified as the receptor involved in the recognition of immunostimulatory CpG motifs [18]. TLR9 is a member of the family of TLRs, which play a critical role in innate immunity. Ten family members have been identified so far, and they appear to activate NF-κB, leading to the production of inflammatory cytokines [15,18,19].

Although both viral and non-viral vectors have pathogenic profiles, no study has directly compared the transduction efficiency, especially in vivo, and safety of these vectors. Thus, we performed a comparative study of the transduction efficiency and the level of production of inflammatory cytokines after systemic injection of the Ad vector or lipoplex into mice.

2. Materials and methods

2.1. Plasmids and adenovirus vectors

The plasmid vector, pCMVL1, and the Ad vector, Ad-L2, which were constructed to express luciferase under the transcriptional control of the cytomegalovirus (CMV) promoter and bovine growth hormone (BGH) polyadenylation signal, were described previously [20–22]. pCpG-mcs was purchased from Invivogen (San Diego, CA).

pCMVL1 and pCpG-mcs were amplified in DH5α and GT115, respectively, and isolated by using EndoFree Plasmid Mega Kit (QIAGEN, Valencia, CA). The concentration of lipopolysaccharide (LPS) in DNA solution was measured using the Limulus HS-F Single Test (Wako, Osaka, Japan). The amount of LPS in the DNA solution was <0.1 Endotoxin unit/ μg DNA, which is the amount endorsed by QIAGEN.

Ad-L2 was amplified in 293 cells; and purified by $CsCl_2$ gradient centrifugation; dialyzed with a solution containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol; and stored in aliquots at −70 °C. Virus particle titer was measured spectrophotometrically as described previously [23].

2.2. Preparation of DOTAP/Chol liposome and lipoplex

DOTAP/Chol liposome and lipoplex were prepared by a modification of the method used by Li et al. [12]. Briefly, an appropriate amount of DOTAP (AVANTI Polar Lipids, Alabastar, Al) was mixed with cholesterol in chloroform at the molar ratio of 1:1. The organic solvent was evaporated to make the dried lipid film. The dried films were then hydrated in 5% dextrose solution under a 37 °C water bath to make liposome solution. The liposome solution was sonicated for 1–2 min before the lipoplex was prepared. To prepare the lipoplex, plasmid DNA was diluted with 5% dextrose, and then liposome solution was added to achieve a 9.8:1.0 weight ratio of DOTAP: DNA. The theoretical charge ratio (+/−) of the complex was 4.6. The mixture was incubated at room temperature for 10 min before injection.

2.3. In vivo gene transfer and luciferase assay

Female C57BL/6 mice (5–6 weeks) were purchased from Nippon SLC (Hamamatsu, Japan). A final volume of 200 μl of Ad vectors (1×10^{10} or 5×10^{10} vector particle (VP)/mouse) or lipoplex (5 or 25 μg of plasmid DNA/mouse) was injected intravenously via tail vain of each mouse. An appropriate length of time after the injection, the mice were given anesthesic by diethylether and their hearts, lungs, kidneys, livers and spleens were collected. The organs were then homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris–HCl, pH 7.8). After being frozen and thawed, the homogenates were centrifuged at 15,000 ×g at 4 °C for 10 min, and the supernatants were collected. Luciferase activity in the supernatants was determined by using a luciferase assay system (PicaGene 5500; Toyo Inki, Tokyo, Japan). The protein content was measured with a Bio-Rad assay kit (Bio-Rad, Hercules, CA).

2.4. Cytokine measurement

An appropriate length of time after the injection, the mice were given anesthesic by diethylether and peripheral blood was collected via the inferior vena cava and fundus oculi. The peripheral blood was placed on ice for 2–3 h and then centrifuged at 15,000 rpm at 4 °C for 10 min to collect the blood serum. The cytokine concentration in serum was measured using an ELISA kit (IL-6 and IL-12p40; BD Biosciences, San Diego,CA, TNF-α; R & D Research Systems, Minneapolis, MN) according to the manufacturer's instructions. Alanine aminotransferase (ALT) activities of the blood serum were measured using the Transaminase CII-Test (Wako, Osaka, Japan).

2.5. Preparation of paraffin sections of liver

An appropriate length of time after the injection, the mice were given anesthesic by diethylether and their livers were collected. Each liver was washed, fixed in 10% formalin for 24 h at room temperature, and embedded in paraffin. After sectioning, the tissue was dewaxed in ethanol, rehydrated, and stained with hematoxylin and eosin. This process was commissioned to Applied Medical Research Laboratory (Osaka, Japan).

GENE DELIVERY

3. Results

3.1. Comparison of the transduction efficiency between Ad vector and lipoplex

To compare the transduction activity and safety of gene therapy vectors in vivo, we chose the Ad vector and DNA–liposome complex (lipoplex) as the viral vector and non-viral vector, respectively. We chose DOTAP/Chol as the liposome, because this has been used in many gene transduction studies in vivo, and its usability has been proven [2,10,24–26]. The mean diameter of the lipoplex, which was measured by Zetasizer (Malvern Instruments Ltd, United Kingdom) was about 350 nm (data not shown). Gel retardation assay showed that plasmid DNAs were indeed complexed with liposome (data not shown). To compare the in vivo transduction efficiency and the distribution of the gene expression from the Ad vector and lipoplex, we first analyzed the luciferase production in the organ after intravenous injection of the Ad vector (1×10^{10} or 5×10^{10} VP/mouse) or lipoplex (5 or 25 μg of plasmid DNA/mouse). The dose of the vectors injected in the present study was used in previous reports and was determined to be a limited dose that would not cause sudden death or raising of the hair [2,12]. High levels of luciferase production were obtained as a result of the injection of the Ad vector in all organs examined. On the other hand, lipoplex-mediated luciferase production was detected only in the heart and lung (Fig. 1). Luciferase production in lipoplex-injected mice was 10^{-5} to 10^{-2} lower than in Ad vector-injected mice in all organs. The luciferase production obtained from mice that were injected with lipoplex was approximately the same or slightly lower than has been shown in previous reports [2,25,27]. This difference might result from the differences in the mouse strains and liposome compositions used in the experiments. We also determined the duration of luciferase production after the intravenous injection of vectors (Fig. 2A and B). Luciferase production in all organs collected from mice that were injected with the Ad vector or lipoplex decreased in a time-dependent manner. It was striking that the livers collected from mice that were injected with high doses of the Ad vector maintained a high gene expression for 24 h (Fig. 2B). On the other hand, the gene expression of lipoplex decreased dramatically and reached levels similar to that shown in mock-treated mice in most organs after 9 h (Fig. 2A). Thus, we can conclude that the Ad vector can express a high level of foreign genes in a broad range of tissues.

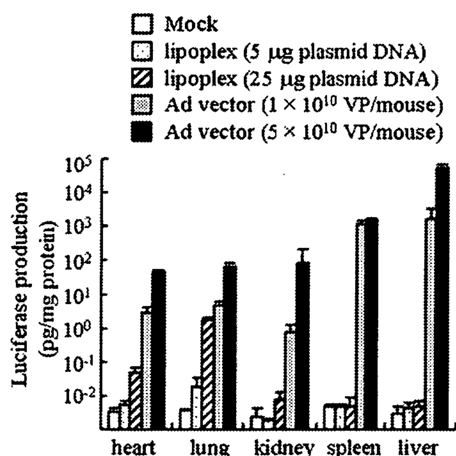

Fig. 1. Luciferase production in various organs after intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (1×10^{10} or 5×10^{10} VP/mouse; gray and black bar, respectively) or lipoplex (5 or 25 μg of plasmid DNA; dotted and slashed bar, respectively) was injected intravenously into each mouse. Organs were collected from the mice 6 h following the injection, and luciferase activity and protein concentration were assayed. The white bar indicates mock treatment. Data are expressed as means ± SD of 3–4 mice per group.

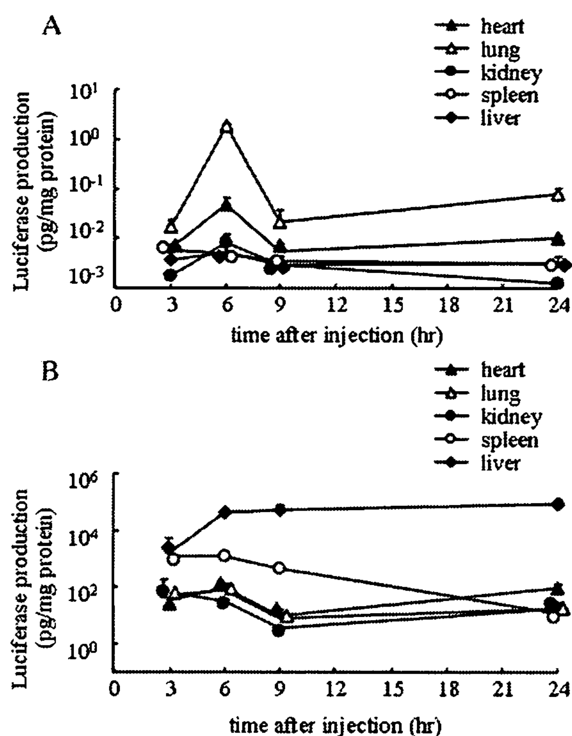

Fig. 2. Duration of the luciferase production. A final volume of 200 μl of lipoplex (25 μg of plasmid DNA; A) or Ad vectors (5×10^{10} VP/mouse; B) was injected intravenously into each mouse. After an appropriate length of time had passed following the injection, the heart (filled triangle), lung (opened triangle), spleen (opened circle), kidney (filled circle), and liver (filled diamond) were collected, and luciferase activity and protein concentration were measured. Data are expressed as means ± SD of 3–4 mice per group.

3.2. Vector-triggered cytokine production

There are many indices of the side effects caused by the intravenous injection of vectors, such as canceration, tissue damage, innate and adaptive immune response, etc. In the present study, innate immune response was examined as an indicator of the side effects induced by the administration of vectors, since the induction of innate immune response by the Ad vector is the primary limiting factor in the use of the Ad vector [5]. To investigate the level of immune response induced by these vectors, we measured the serum concentration of various inflammatory cytokines, including IL-6, IL-12 and TNF-α. At 6 h following the intravenous injection, the IL-6 concentration in serum from lipoplex-injected mice was 2- to 4-fold higher than that in serum from the Ad vector-injected mice (Fig. 3a). A similar profile was obtained for the production of

GENE DELIVERY

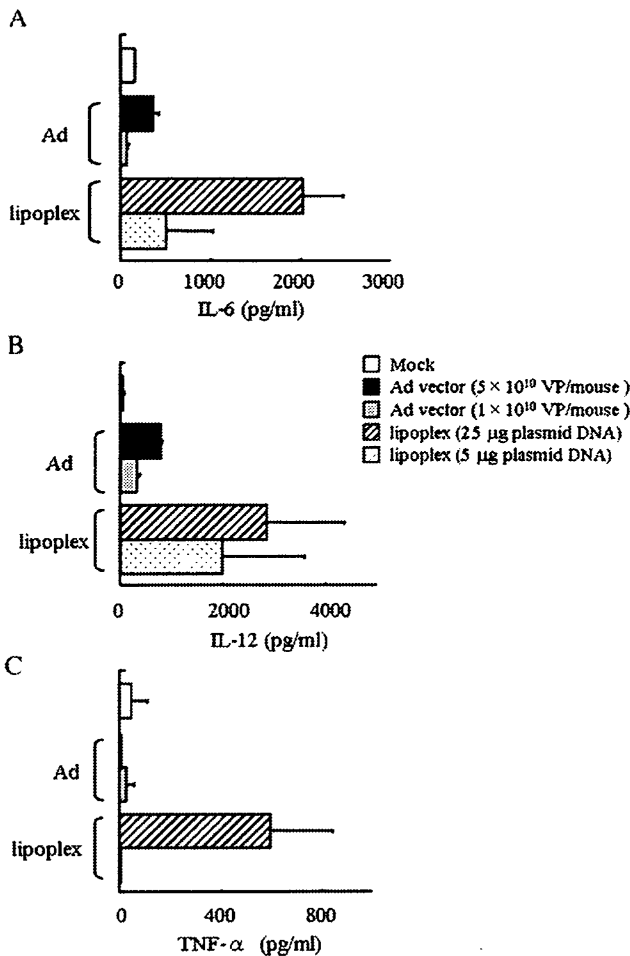

Fig. 3. Induction of various inflammatory cytokines by intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (1×10^{10} or 5×10^{10} VP/mouse; gray and black bar, respectively) or lipoplex (5 or 25 μg of plasmid DNA; slashed and dotted bar, respectively) was injected intravenously into each mouse. Six hours after the injection, peripheral blood was collected, and the concentrations of IL-6, IL-12, and TNF-α were measured by ELISA. The white bar indicates mock treatment. Data are expressed as means ± SD of 3–4 mice per group.

IL-12 induced by these vectors (Fig. 3b). The level of cytokine production induced by these vectors was dose-dependent and returned to the basal level after 24 h post-injection (Fig. 6 shows the data for lipoplex; data not shown for the Ad vector). When a higher dose of vectors was injected, the serum concentration of TNF-α from lipoplex-injected mice was 2-orders higher than that from Ad vector-injected mice and control mice (Mock) (Fig. 3c). The serum concentration of TNF-α from the Ad vector-injected mice was similar to that from control mice (Mock). These results suggest that lipoplex induces the innate immune response more strongly than the Ad vector.

3.3. Hepatotoxicity caused by vector injection

The majority of intravenously injected Ad vector is sequestered by the liver, which in turn causes an inflammatory response characterized by acute transaminitis and vascular damage [7]. Systemic administration of lipoplex also leads to toxic effects in the liver [13]. We investigated the hepatic damage induced by the intravenous administration of the Ad vector and lipoplex. The hepatotoxicity induced by the vectors was compared by measuring the activity of alanine aminotransferase (ALT) in serum and by investigating the histopathological changes. There were no significant differences in the ALT activity between the sera from the Ad vector-injected mice (5×10^{10} VP/mouse) and the lipoplex-injected mice (both 5 and 25 μg plasmid-DNA/mouse) after 24 h post-injection (Fig. 4). The serum ALT activity in mice injected with 1×10^{10} VP of the Ad vector was the same as in the controls (i.e., the mock-treated levels). In the case of hepatic histological changes, hepatocytes from lipoplex-injected mice (25 μg plasmid-DNA/mouse) started to granulate 3 h after the injection, even though the cells from the Ad vector-injected mice (5×10^{10} VP/mouse) seemed to be normal (Fig. 5B and E). Six hours after injection, hepatocytes from the Ad vector-injected mice started to granulate similarly to those from lipoplex-injected mice (Fig. 5C and F). At 48 h after the injection, degranulation or denucleation occurred in hepatocytes from both the Ad vector- and lipoplex-injected mice (Fig. 5D and G). These results showed that both the Ad vector and lipoplex caused hepatotoxicity, especially at high doses, and that the liver damage caused by lipoplex, such as granulation and degranulation, started earlier than in the case of the Ad vector.

3.4. Effect of unmethylated CpG motifs in plasmid DNA on cytokine production

It has been reported that bacterial DNA induces innate immune response because it has a much higher frequency of unmethylated CpG dinucleotides than mammalian DNA [18,28]. The injection of plasmid DNA, which contains fewer CpG motifs, reduces the induction of inflammatory cytokines [13,26]. We examined the production of cytokines induced by the intravenous injection of lipoplex containing plasmid DNA without CpG motifs (non-CpG lipoplex), which is completely lacking in CpG motifs. In this experiment, only the level of inflammatory cytokine was examined, because luciferase-coding cDNA without CpG motifs was not obtained. As expected, the production of IL-6 induced by non-CpG lipoplex

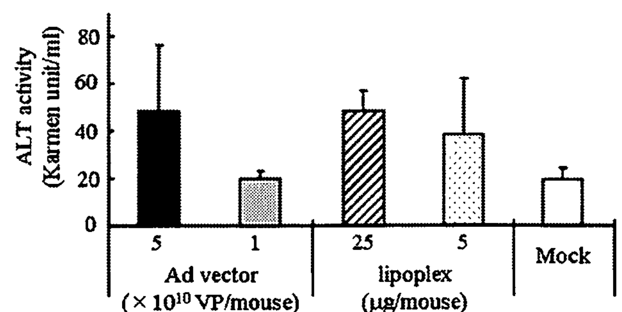

Fig. 4. Hepatotoxicity by intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (1×10^{10} or 5×10^{10} VP/mouse; gray and black bar, respectively) or lipoplex (5 or 25 μg of plasmid DNA; slashed and dotted bar, respectively) was injected intravenously into each mouse. Twenty-four hours after the injection, peripheral blood was collected, and the ALT activity was measured. The white bar indicates mock treatment. Data are expressed as means ± SD of 3–4 mice per group.

GENE DELIVERY

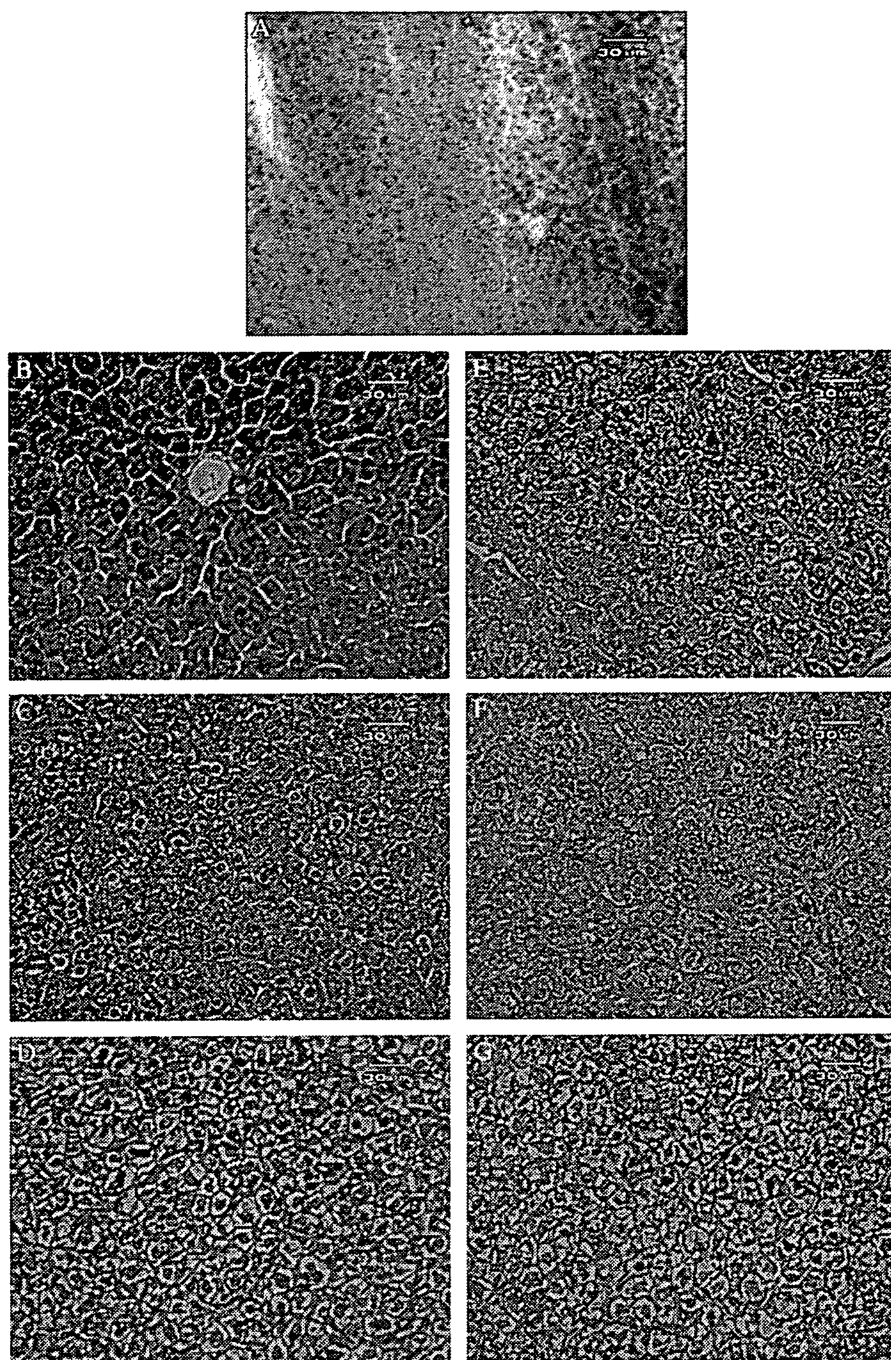

Fig. 5. Induction of acute liver failures by intravenous injection of the Ad vector or lipoplex. A final volume of 200 μl of Ad vectors (5×10^{10} VP/mouse; B–D), lipoplex (25 μg of plasmid DNA; E–G) or PBS (as Mock; A) was injected intravenously into each mouse. The livers were collected after 3 (B and E), 6 (C and F), and 48 (D and G) h following the injection, and paraffin sections were prepared. Each section was stained with hematoxylin and eosin.

was suppressed, but this complex still induced higher IL-6 production than the Ad vector (Figs. 3A and 6A). The injection of non-CpG lipoplex showed a different peak time of IL-6 production than in the case of CpG lipoplex injection. The level of producton of IL-12 and TNF-α induced by the injection of non-CpG lipoplex was lower than that induced by the injection of CpG lipoplex (Figs. 3B, C, 6B and C). The concentration of IL-12 in the serum from the non-CpG lipoplex-injected mice was lower than that in the serum from the Ad vector-injected mice. Unlike in the case of IL-6, non-CpG lipoplex showed the same profiles of IL-12 production as CpG-lipoplex. These results show that the removal of CpG motifs from the plasmid DNA in lipoplex could not completely suppress cytokine production, but there exists other mechanisms for suppressing immune response by lipoplex.

4. Discussion

A variety of viral and non-viral vectors have been developed for gene therapy [3,4,10]. At present, viral vectors dominate in