E. 結論

1) 各種 Ad ベクターを全身投与したマウス肝臓、脾臓における遺伝子発現変動を DNA microarray により網羅的に解析した結果、従来型 Ad ベクター投与群ではトリプル改変型 Ad ベクター投与群と比較して3-5倍の遺伝子が発現変動した。また、K7 型 Ad ベクター投与による発現変動遺伝子は従来型 Ad ベクター投与群とほぼ同程度であった。従来型 Ad ベクター投与群における炎症性サイトカイン/ケモカイン遺伝子の変動は主に脾臓で起こっており、一部肝臓でも変動がみられたことから、Ad ベクター投与により炎症性サイトカイン/ケモカインが主に脾臓で産生され、肝臓では二次的な炎症反応が惹起されていることが示唆された。さらに、従来型 Ad ベクター投与により遺伝子発現が上昇もしくは低下し、かつトリプル改変型 Ad ベクターで変動しなかった遺伝子群を自然免疫応答関与候補遺伝子群として抽出したところ、肝臓、脾臓においてそれぞれ 101 遺伝子、22 遺伝子が抽出された。このうち機能が未知な遺伝子はそれぞれ 33 遺伝子、2 遺伝子であり、両臓器において共通に抽出されてきた遺伝子は 1 遺伝子（ankyrin repeat domain 2 (stretch responsive muscle); Ankrd2）であった。今後、これらの遺伝子の機能を解析することにより、Ad ベクターによる自然免疫応答のメカニズムがより詳細に明らかになるものと考えられる。

2) Ad ベクター臨床応用での問題点の一つである自然免疫誘導と肝障害メカニズムの解析を行った。その結果、Ad ベクター投与後の炎症性サイトカインは主に脾臓で産生され、特に脾臓中のコンベンショナル樹状細胞が関与していること、さらに Ad ベクター投与後に産生される IL-6 が肝障害に関与していることを明らかとした。また、K7 型 Ad ベクターは脾臓への集積が低く、自然免疫および肝障害誘導能は極めて低いことが明らかとなった。

3) MyD88 欠損および TLR9 欠損マウスを用いて誘導した DC に Ad ベクターを作用させた結果、MyD88/TLR9 一部依存的に IL-6 産生が行われていることが明らかとなった。一方、マクロファージでは MyD88/TLR9 非依存的経路を介し IL-6 産生が行われていることを明らかにした。このことから、細胞種によって Ad ベクターによる炎症性サイトカイン産生経路が異なることが示唆された。

4) 炎症性サイトカイン産生に重要な転写因子である NF-κB に対する DNA デコイをフコースもしくはマンノース修飾カチオン性リポソームを用いて肝臓や脾臓のマクロファージに選択的に送達することにより、Ad ベクター投与による炎症性サイトカイン（IL-6、IL-12）産生ならびに肝障害を有意に抑制することが可能であった。特にマンノース修飾リポソームを用いた場合には、最も優れた抑制効果が観察された。本研究結果より、NF-κB デコイを用いることにより、Ad ベクター投与後の自然免疫誘導を抑制できる可能性が示唆された。

F. 健康危険情報

該当事項なし

G. 研究発表

1. 論文発表

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3) Sakurai H., Sakurai F., Kawabata K., Sasaki T., Koizumi N., Huang H., Tashiro K., Kurachi S., Nakagawa S., Mizuguchi H. Comparison of gene expression efficiency and innate immune response induced by Ad vector and lipoplex, *J. Control. Release.*, 117, 430-437 (2007)

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2) 黄海瑛、櫻井文教、川端健二、小泉直也、樋口ゆり子、川上茂、橋田充、水口裕之；アデノウイルスベクター投与後初期に起こる副作用軽減— NF-κB デコイの細胞選択的デリバリーによる試み —；第 2 回創剤フォーラム若手発表討論会（京都）；2006 年 10 月 13-14 日

3) 黄海瑛、櫻井文教、川端健二、小泉直也、樋口ゆり子、川上茂、山下富儀、橋田充、水口裕之；NF-κB デコイによるアデノウイルスベクター生体投与後の自然免疫誘導抑制；第 21 回日本 DDS 学会（東京）；2006 年 7 月 7-8 日

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5) Haruna Sakurai, Fuminori Sakurai, Kenji Kawabata, Tomomi Sasaki, Naoya Koizumi, Kaiei Kou, Shinnnosuke Kurachi, Shinsaku Nakagawa, Hiroyuki Mizuguchi ; COMPARISON OF GENE EXPRESSION EFFICIENCY AND INNATE IMMUNE RESPONSE INDUCED BY ADENOVIRUS VECTOR AND LIPOPLEX; The first FIP-APSTJ

joint workshop on gene delivery (Sapporo) ; 2006 年 7 月 10-12 日 Best Presentation Award

H. 知的財産権の出願・登録状況

1. 特許取得

該当事項なし

2. 実用新案登録

該当事項なし

3. その他

該当事項なし

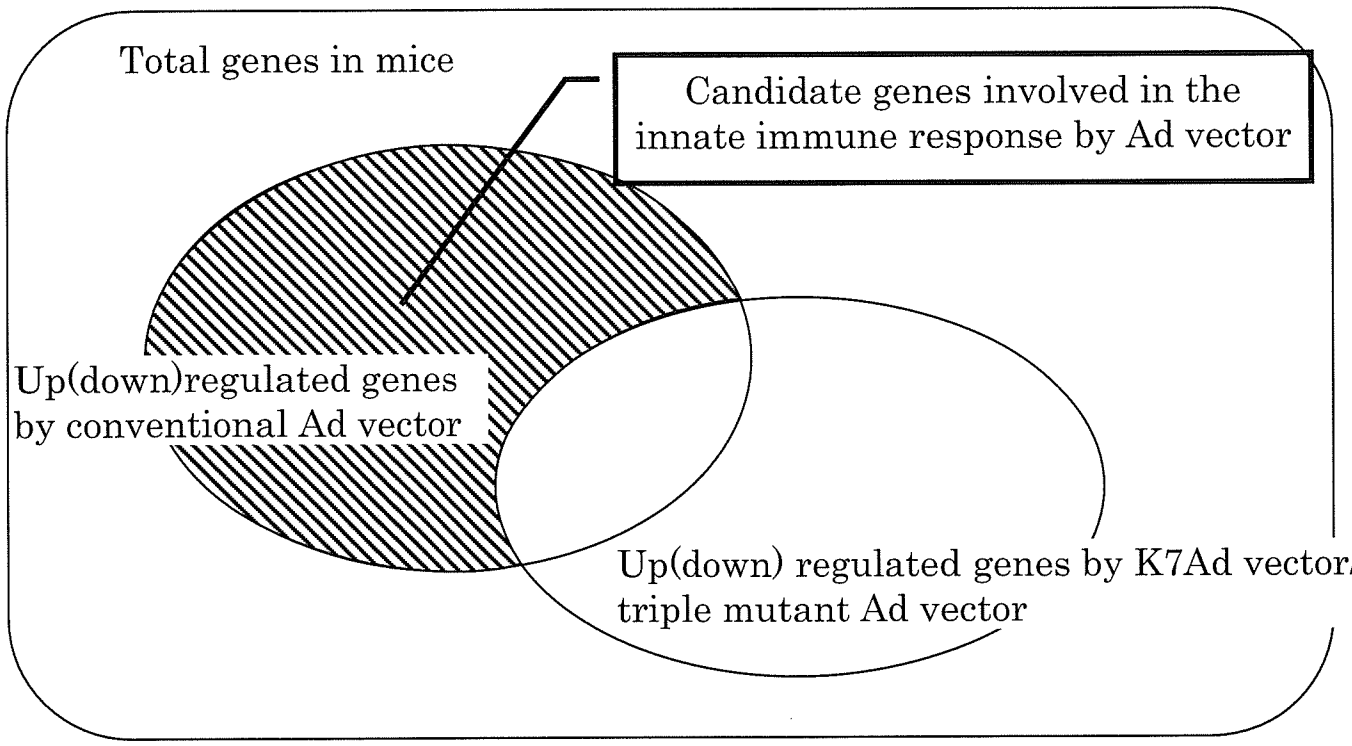

Fig.1 Selection of the candidate genes involved in the innate immune response induced by the systemic administration of Ad vector

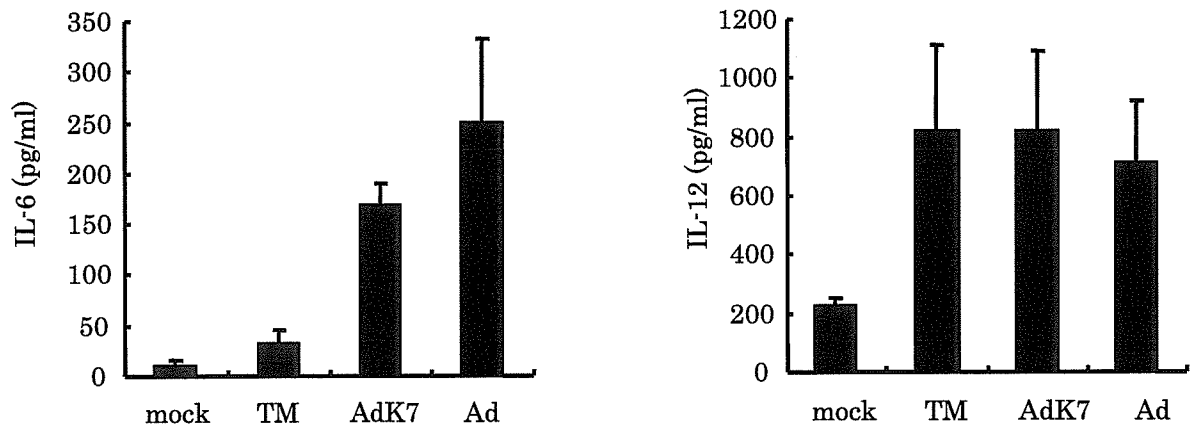

Fig.2 Cytokine production induced by systemic administration of Ad vectors. A final volume of 200 μl of each Ad vector ($1x10^{11}$ VP/mouse) was injected intravenously into each mouse. After 3 hours, peritoneal blood was collected, and the serum concentration of IL-6 and IL-12 was measured by ELISA. The TM, AdK7 and Ad indicates triple mutant Ad vector, AdK7 vector and conventional Ad vector, respectively. Data are expressed as means±S.D. of 3 mice per group.

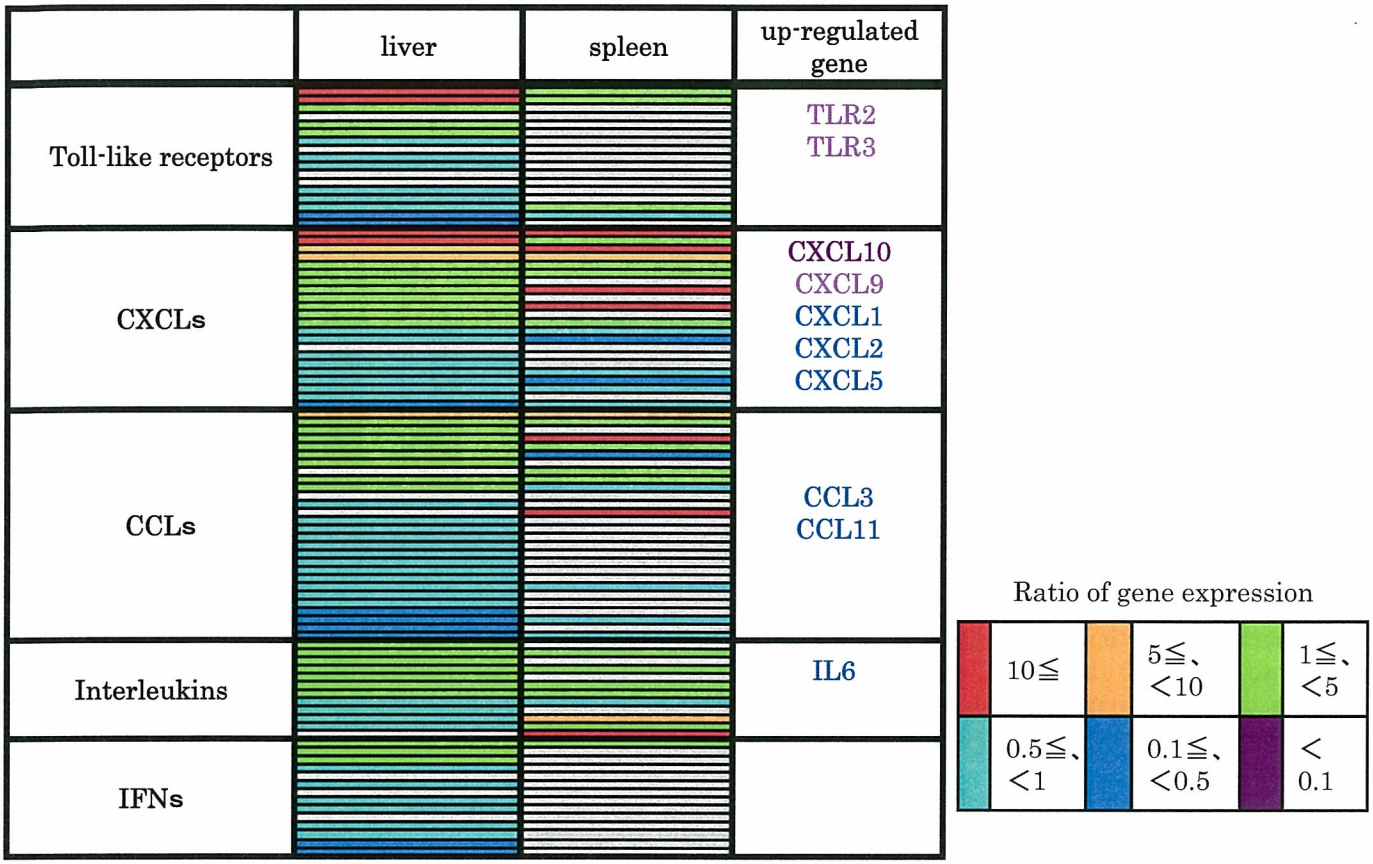

Fig.3 Expression patern of genes involved in innate immune response after the systemic administration of conventional Ad vectors. Final volume of 200 μl of conventional Ad vector ($1x10^{11}$ VP/mouse) was injected intravenously into each mouse. After 3 hours following injection, liver and spleen were collected and analysed by DNA microarray. Horizontal rows represent particular genes (colored according to the ratio of gene expression in conventional Ad vector-injected mice/ gene expression in Mock mice). If the copy number of particular gene is less than 1, its column is colored in grey. The column of "Up-regulated gene" indicates the name of genes up-regulated over 10-fold only in liver (pink), in spleen (blue), or in both tissue (purple).

A

	liver	spleen
10≦	40	29
10>A≧5	83	48
5>A≧1	9365	2337
1>A≧0.5	21436	8425
0.5>A≧0.1	3272	1116
<0.1	36	1
total	34232	11956

B

	liver	spleen
10≦	45	20
10>A≧5	134	38
5>A≧1	11256	1635
1>A≧0.5	21182	8335
0.5>A≧0.1	2340	1820
<0.1	28	5
total	34985	11873

C

	liver	spleen
10≦	7	4
10>A≧5	34	12
5>A≧1	7182	3789
1>A≧0.5	22420	8040
0.5>A≧0.1	4219	103
<0.1	17	0
total	33879	11948

Fig.4 The level of gene expression profile by various kinds of Ad vector. Final volume of 200 μl of conventional Ad vector ($1x10^{11}$ VP/mouse) was injected intravenously into each mouse. After 3 hours following injection, liver and spleen were collected and analysed by DNA microarray. Horizontal rows represent the particular range of changes induced by systemic administration of conventional Ad vector (A), AdK7 vector (B) and triple mutant Ad vector (C). The genes which the copy number is less than 1in both mock and Ad vector-treated mice are omitted.

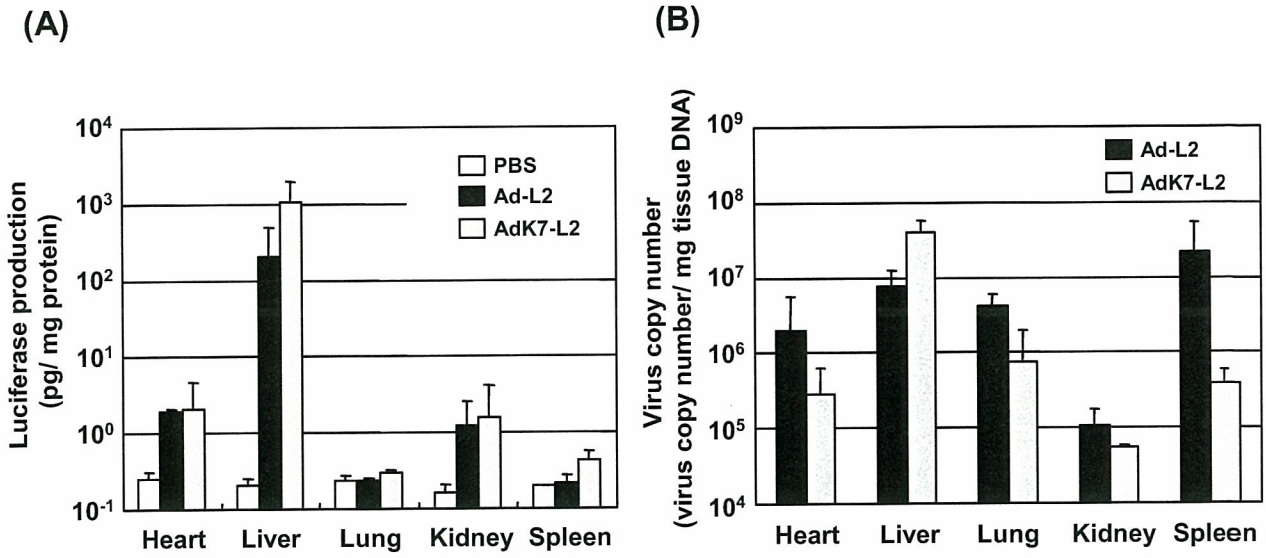

Fig.5 Luciferase production and biodistribution of viral DNA after the intravenous administration of Ad-L2 or AdK7-L2 into mice. Ad-L2 or AdK7-L2 ($1.0x10^{10}$ VP) was intravenously injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested, and luciferase 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 ± S.D. of 4-6 mice.

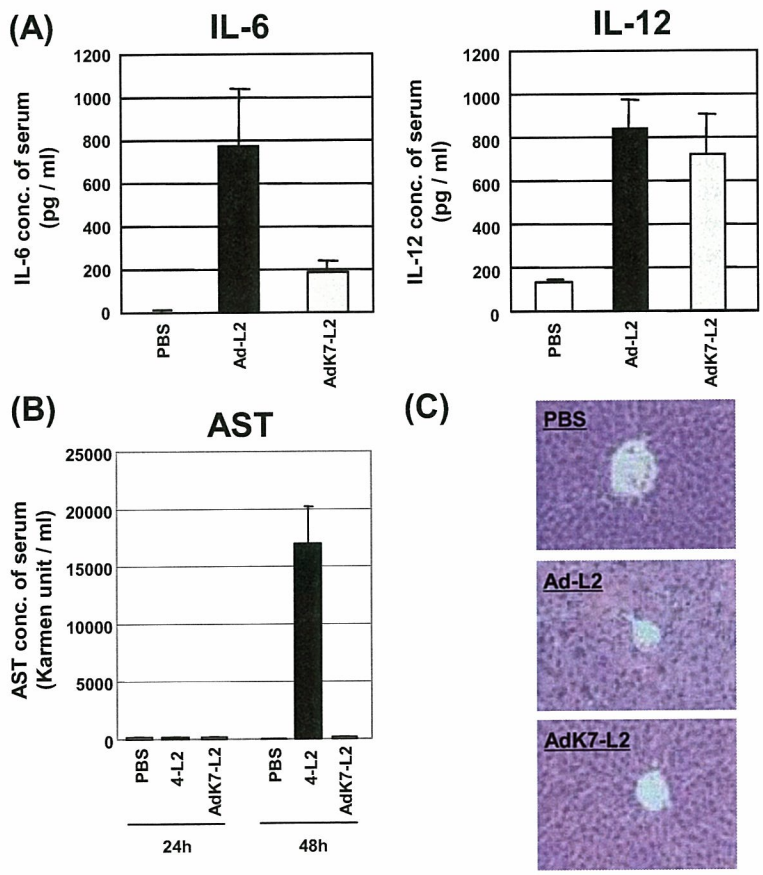

Fig.6 Cytokines and liver enzyme levels in serum after the systemic administration of Ad-L2 or AdK7-L2 into mice. Blood samples were collected by inferior vena cave at (A) 3 or (B) 24 and 48 hours after intravenous administration of Ad-L2 or AdK7-L2 ((A) $1.0x10^{11}$ VP or (B) $3.0x10^{10}$ VP). The livers were collected after 48 hours following the injection (C) ($3.0x10^{10}$ VP). (A) IL-6 and IL-12 levels in the serum were measured by ELISA. (B) Aspartate aminotransferase (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 hematoxylin and eosin. Data represent the means ± S.D. of four mice.

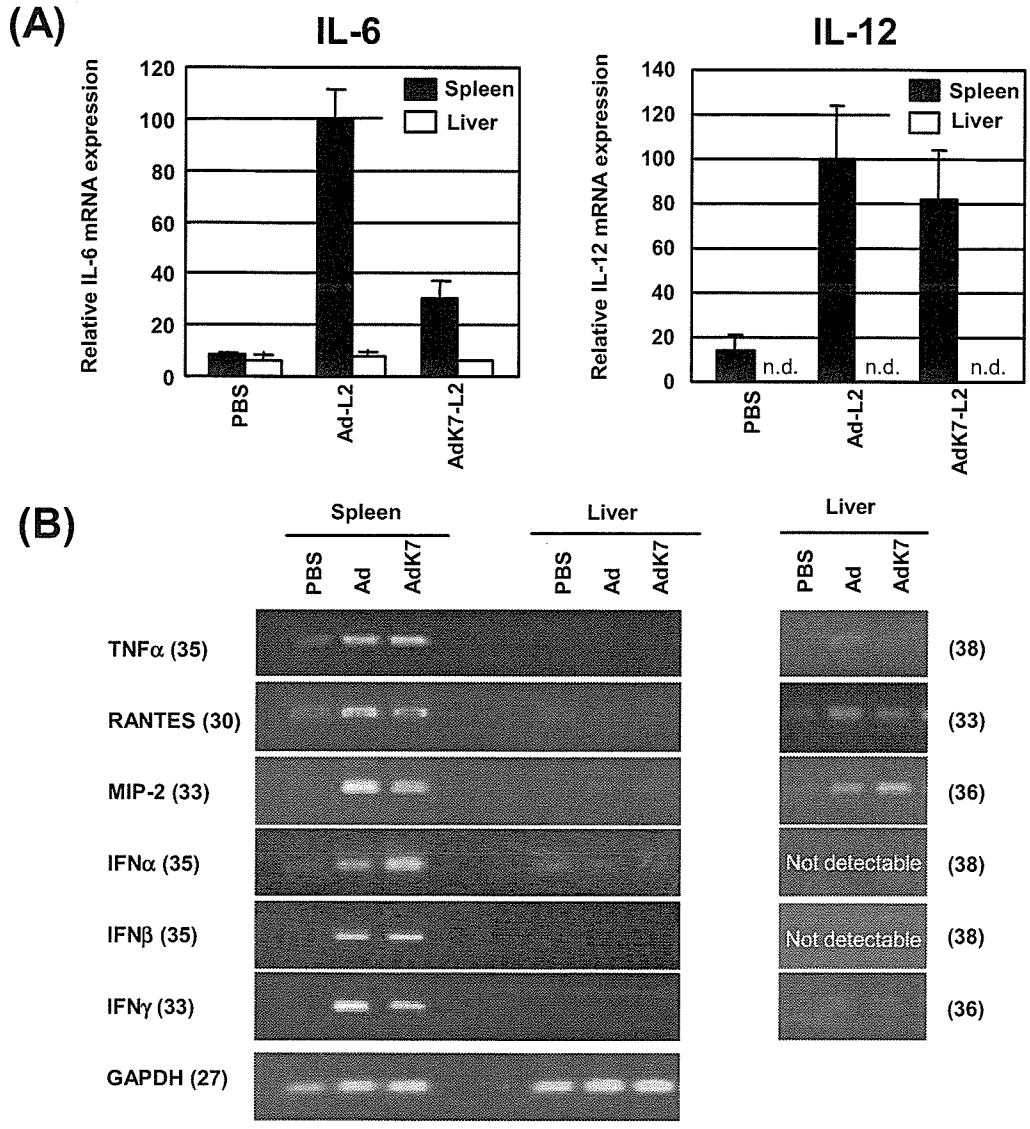

Fig.7 Cytokine, chemokine, and interferon mRNA levels in liver and spleen after the systemic administration of Ad-L2 or AdK7-L2 into mice. Total mRNA samples were isolated from liver and spleen at 3 hours after intravenous administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP). After the RT reaction IL-6 and IL-12 cDNA were measured with the quantitative TaqMan PCR assay (A). The expression of TNFα, RANTES, MIP-2, IFNα, IFNβ, and IFNγ was measured by semi-quantitative RT-PCR assay (B). All data represent the means ± S.D. of four mice. ( ); cycle number

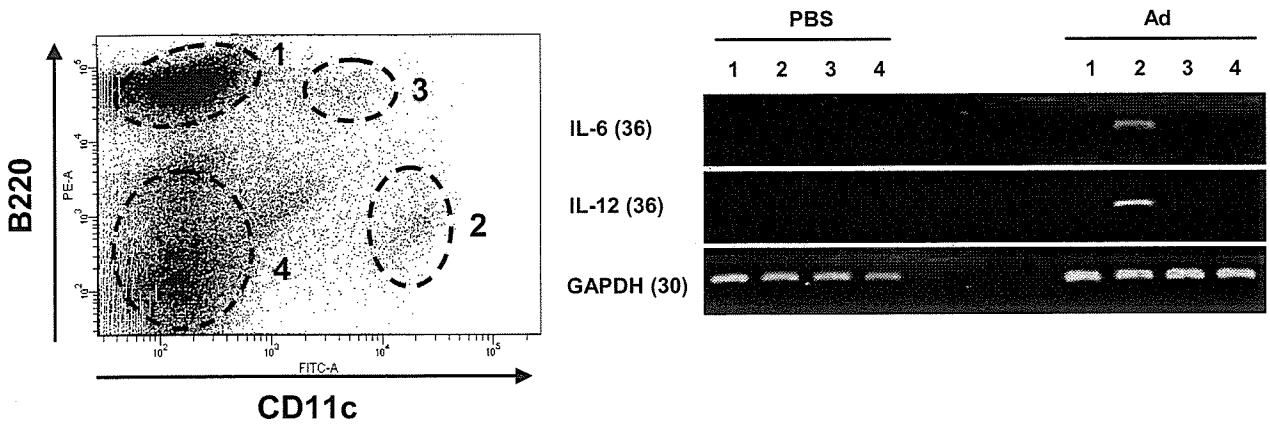

Fig.8 IL-6 and IL-12 mRNA levels in splenic CD11c positive cells after the systemic administration of Ad-L2 into mice. Total mRNA samples were isolated from sorted splenic cells at 3 hours after intravenous administration of Ad-L2 ($1.0x10^{11}$ VP). The expression levels of IL-6 and IL-12 mRNA were 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 cell ($B220^-$, $CD11c^-$). ( ); cycle number

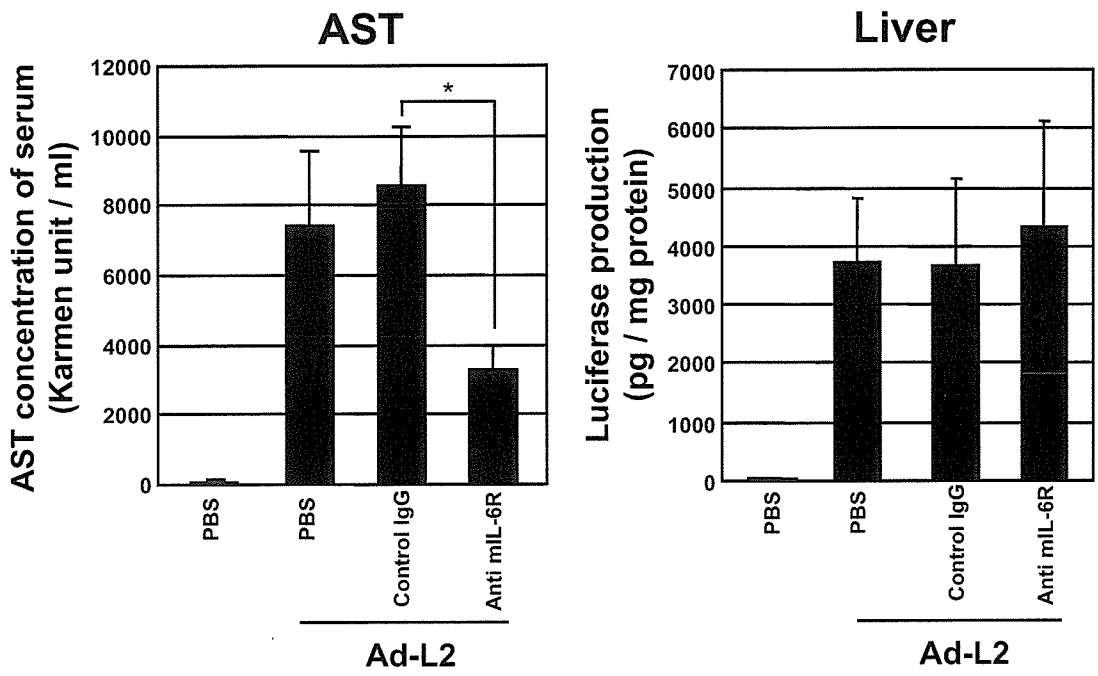

Fig.9 Effects of serum IL-6 on serum AST levels and liver luciferase production after the systemic administration of Ad-L2 into mice. C57Bl6 mice were intraperitoneally administered with 100 μg/mouse of anti-IL-6 receptor antibody (clone; D7715A7), which was specific for blocking IL-6 signaling, or rabbit IgG as a control (clone; R3-34). Ad-L2 or AdK7-L2 ($3.0x10^{10}$ VP) was intravenously injected into the mice 1.5 hours later. Blood samples and liver tissue were collected 48 hours 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 ± S.D. of 3-4 mice. *, $p<0.01$.

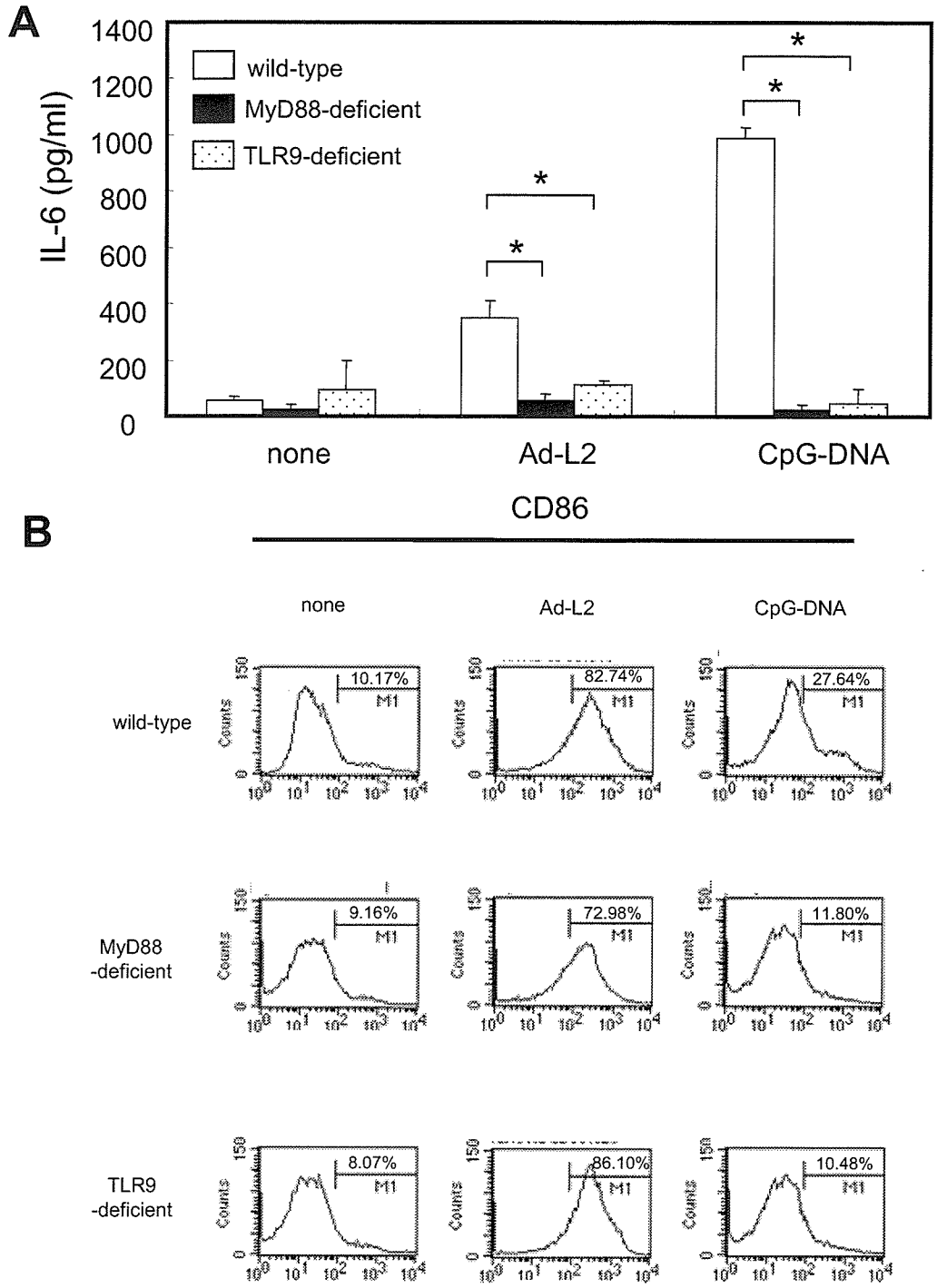

Fig10 MyD88- and TLR9-dependent activation of GMCSF-DCs after Ad infection. (A) Ad-induced IL-6 production. GMCSF-DCs from wild-type, MyD88-deficient and TLR9-deficient mice were stimulated with Ad-L2 (10,000 VP/cell) or CpG-DNA (2.5mM) for 48 hours, and the production of IL-6 in the culture supernatants was measured by ELISA. All data represent the means ± S.D. (n=3). (B) Ad-induced maturation of GMCSF-DCs. GMCSF-DCs from wild-type, MyD88-deficient and TLR9-deficient mice were stimulated with Ad-L2 (10,000 VP/cell) or CpG-DNA (2.5mM). After 48 hours 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 flowcytometric analysis. The results were representative of two independent experiments.
* $p<0.01$.

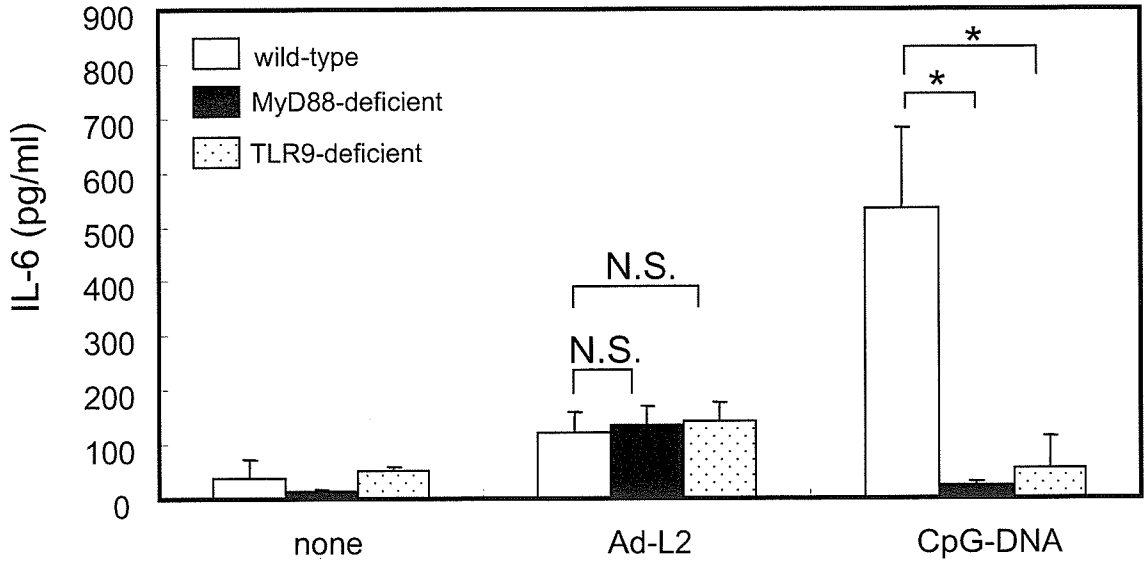

Fig.11 MyD88- and TLR9-independent IL-6 production in peritoneal macrophages stimulated with Ad vectors. IFN-γ-activated peritoneal macrophages from wild-type, MyD88-deficient and TLR9-deficient mice were incubated with Ad-L2 (10,000 VP/cell) or CpG-DNA (2.5mM) for 48 hours, and the amounts of IL-6 in the culture supernatants were measured by ELISA. All data represent the means ± S.D. (n=3). N.S., not significantly different. * $p<0.01$.

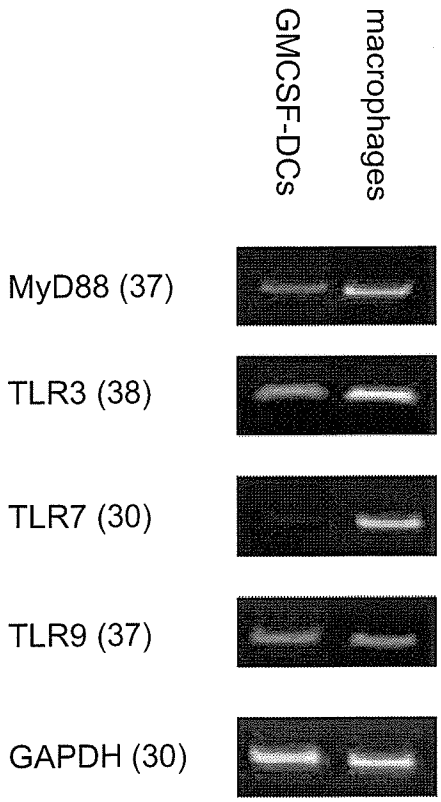

Fig.12 MyD88, TLR3, TLR7, and TLR9 mRNA expression in immune cells. Total RNA samples were isolated from GMCSF-DCs and peritoneal macrophages. The expression of MyD88, TLR3, TLR7, and TLR9 mRNA were tested by RT-PCR. (lane 1) GMCSF-DCs; (lane 2) peritoneal macrophages. ( ); cycle number.

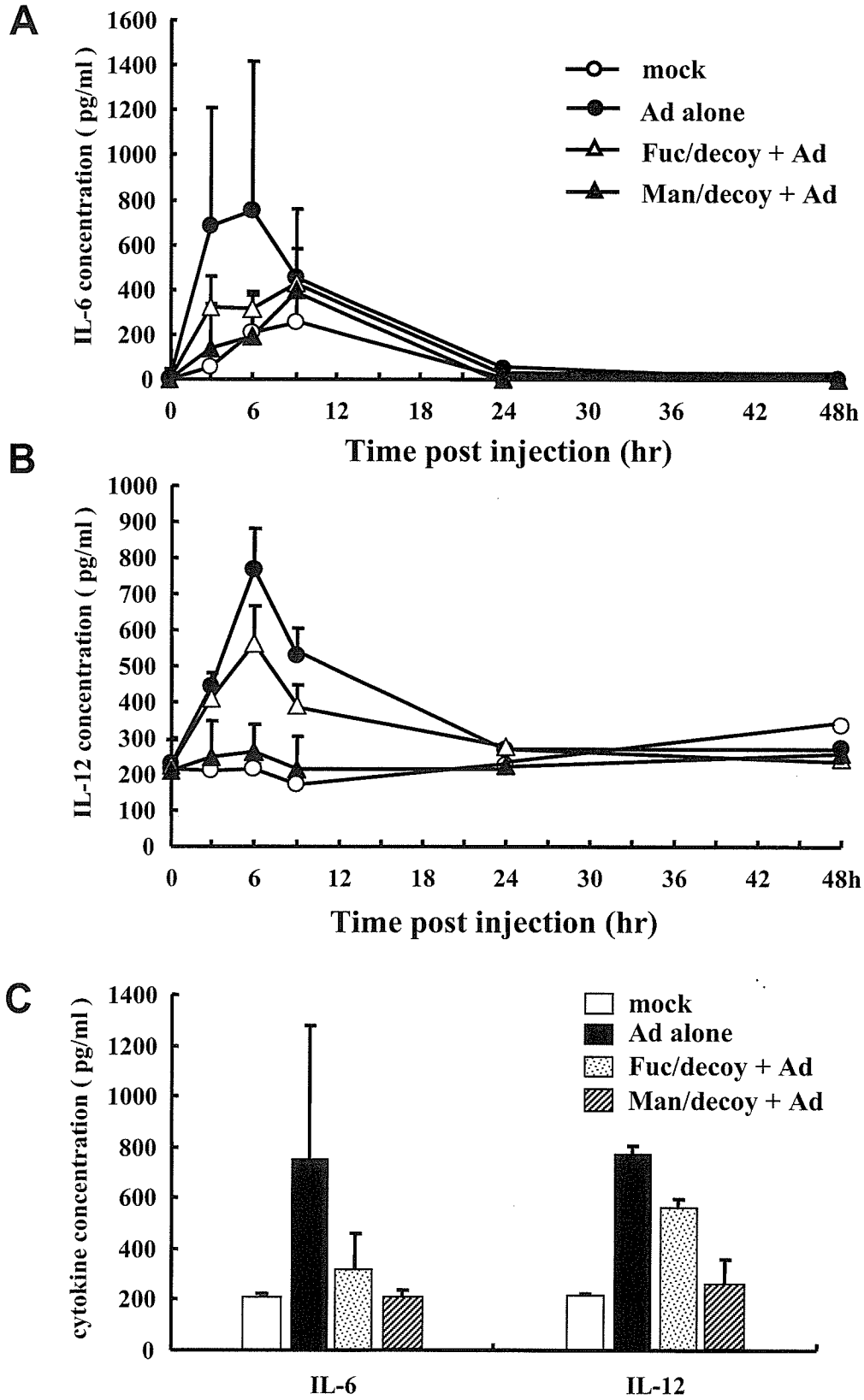

Fig.13 Induction of various inflammatory cytokines by intravenous injection of Ad vectors. A final volume of 200 μl of Ad vectors (5×10^{10} VP/mouse) was injected intravenously into each mouse 10 min after complexes pre-injection. After the indicated time had passed following the Ad injection, peripheral blood serum was collected, and the concentration of IL-6 (A) and IL-12 (B) were measured by ELISA. Data of 6 h after the injection was shown in C. The white bar indicates mock treatment. All data are expressed as mean ±S.D. of 6 mice per group.

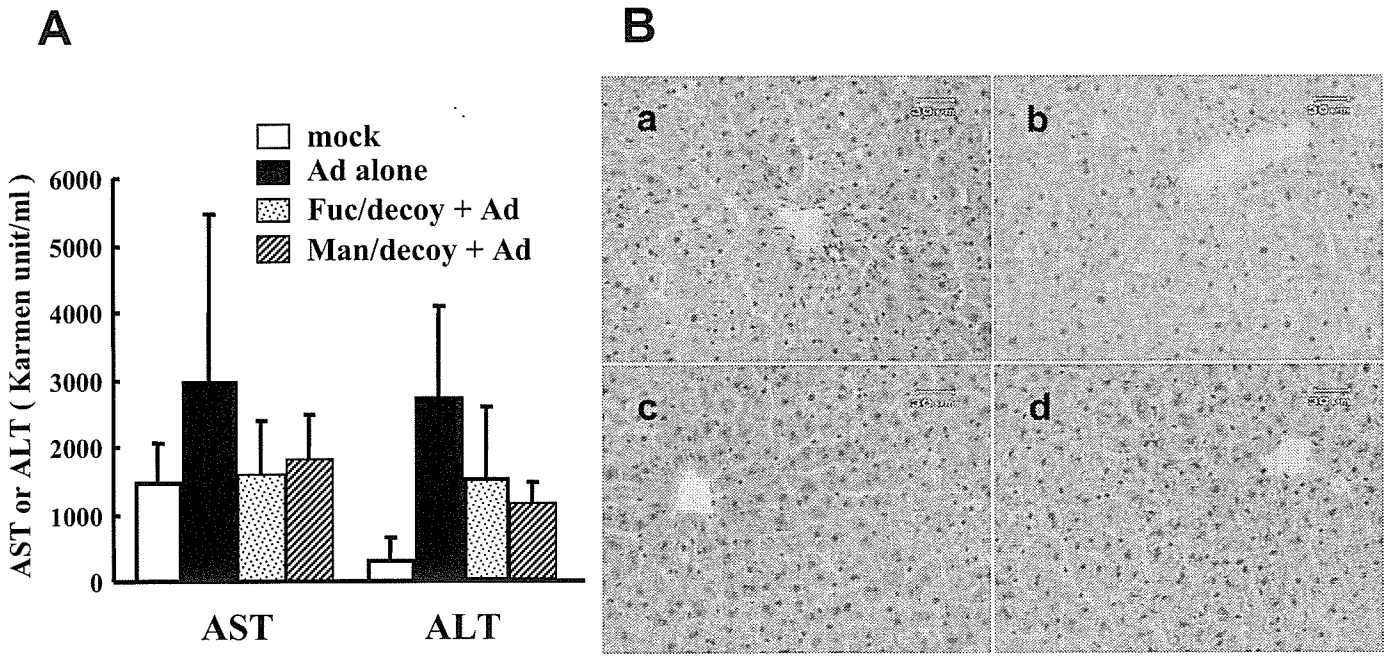

Fig.14 Hepatotoxicity by intravenous injection of Ad vectors. (A) A final volume of 200 μl of Ad vectors (5×10^{10} VP/mouse) was injected intravenously into each mouse at 10 min after complexes pre-injection. Fourty-eight h after the Ad injection, peripheral blood serum was collected, and the ALT and AST activity were measured. The white bar indicates mock treatment. Data are expressed as mean ±S.D. of 6 mice per group. (B) The frozen sections of livers of 48 h after the Ad were prepared, and stained with Hematoxylin and Eosin. (a) mock, (b) Ad vector alone, (c) Fuc/decoy complex + Ad vector, (d) Man/decoy complex + Ad vector.

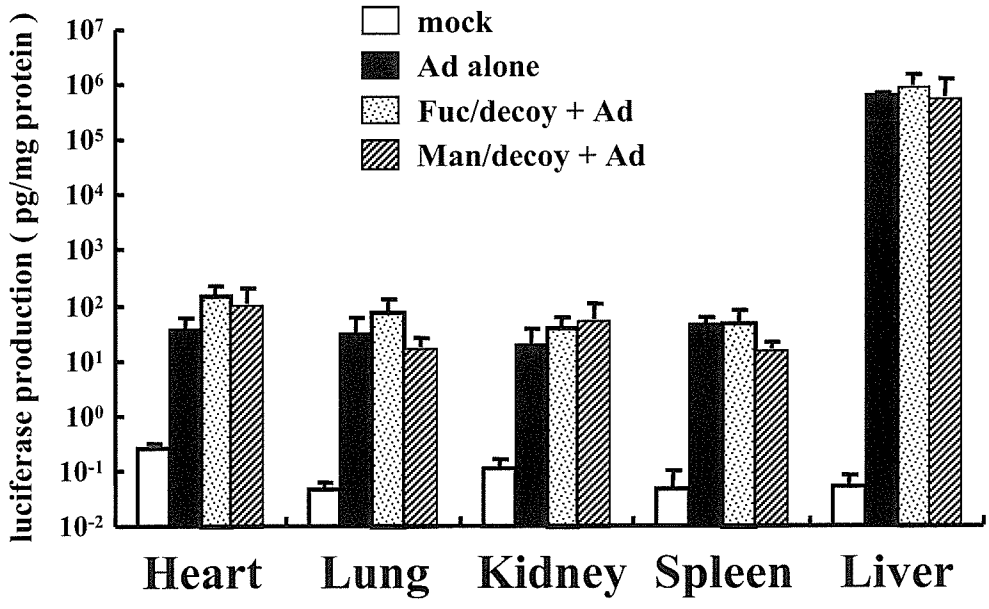

Fig.15 Luciferase production in various organs after intravenous injection of Ad vectors. A final volume of 200 μl of Ad vectors (5×10^{10} VP/mouse) was injected intravenously into each mouse at 10 min after complexes pre-injection. Organs were collected 48 h after the Ad injection, and luciferase activity and protein concentration were measured. The white bar indicates mock treatment. All data are expressed as mean ±S.D. of 6 mice per group.

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sakurai H., Kawabata K., Sakurai F., Nakagawa S., Mizuguchi H.	Innate immune response induced by gene delivery vectors.	Int. J. Pharm.	in press		
Koizumi N., Yamaguchi T., Kawabata K., Sakurai F., Sasaki T., Watanabe Y., Hayakawa T., Mizuguchi H.	Fiber-modified adenovirus vectors decrease liver toxicity through reduced interleukin 6 production.	J. Immunol.	178	1767-1773	2007
Sakurai H., Sakurai F., Kawabata K., Sasaki T., Koizumi N., Huang H., Tashiro K., Kurachi S., Nakagawa S., Mizuguchi H.	Comparison of gene expression efficiency and innate immune response induced by Ad vector and lipoplex.	J. Control. Release.	117	430-437	2007
川端健二・櫻井文教・水口裕之	改良型アデノウイルスベクターを用いた遺伝子デリバリー	Drug Delivery System	印刷中		
水口裕之	アデノウイルスベクター開発の最前線	バイオテクノロジージャーナル	7(2)	168-173	2007

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

Naoya Koizumi,*† Tomoko Yamaguchi,* Kenji Kawabata,* Fuminori Sakurai,* Tomomi Sasaki,* Yoshiteru Watanabe,† Takao Hayakawa,‡ and Hiroyuki Mizuguchi[2]*§

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.

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.

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).

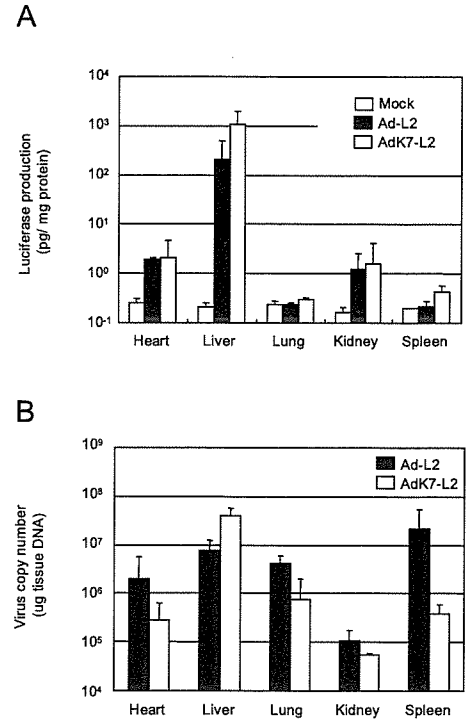

FIGURE 1. Luciferase production and biodistribution of viral DNA after the i.v. administration of Ad-L2 or AdK7-L2 into mice. Ad-L2 or AdK7-L2 (1.0×10^{10} VP) was i.v. injected into the mice. Forty-eight hours later, the heart, lung, liver, kidney, and spleen were harvested, and luciferase production (*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.

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.

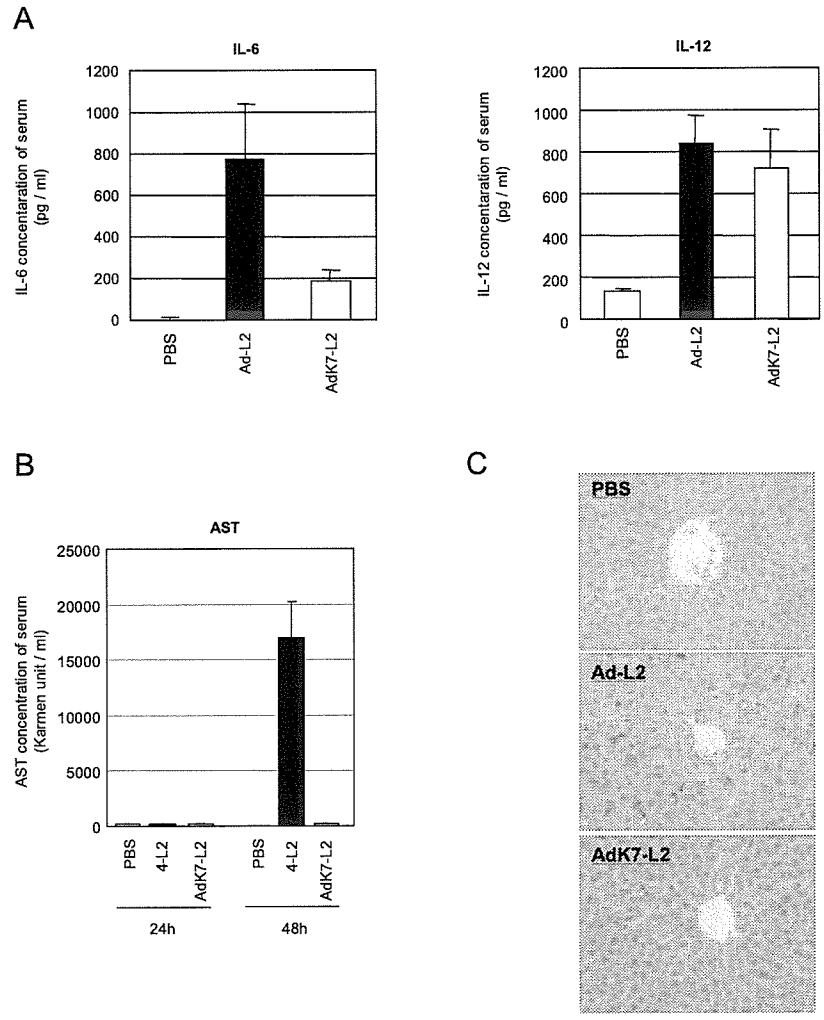

FIGURE 2. Cytokines and liver enzyme levels in serum after the systemic administration of Ad-L2 or AdK7-L2 into mice. Blood samples were collected by inferior vena cave at 3 h (*A*) or 24 and 48 h (*B*) after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP for *A* or 3.0×10^{10} VP for *B*). 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

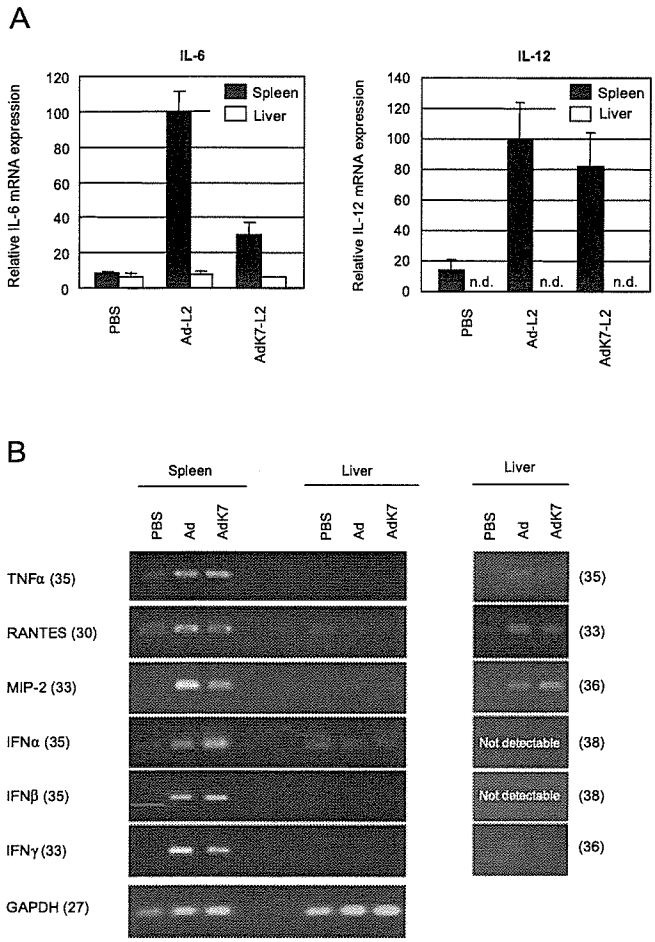

FIGURE 3. Cytokine, chemokine, and IFN mRNA levels in liver and spleen after the systemic administration of Ad-L2 or AdK7-L2 into mice. Total mRNA samples were isolated from liver and spleen at 3 h after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP). After the reverse transcriptase reaction, 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.

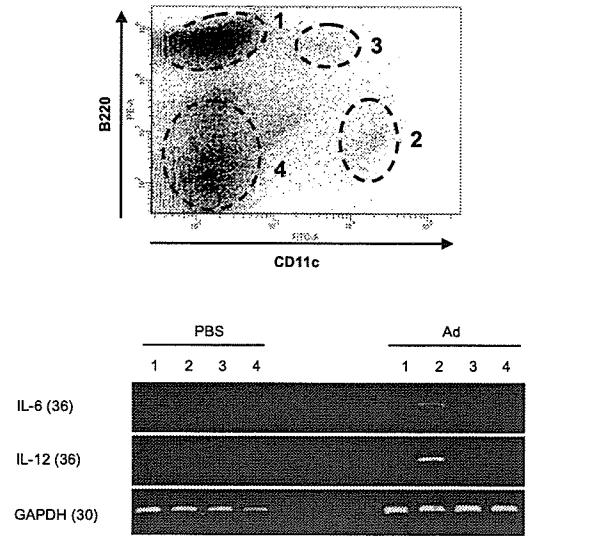

FIGURE 4. IL-6 and IL-12 mRNA levels in splenic CD11c-positive cells after the systemic administration of Ad-L2 into mice. Total mRNA samples were isolated from sorted splenic cells 3 h after i.v. administration of Ad-L2 (1.0×10^{11} VP). The expression levels of IL-6 and IL-12 mRNA were measured by 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

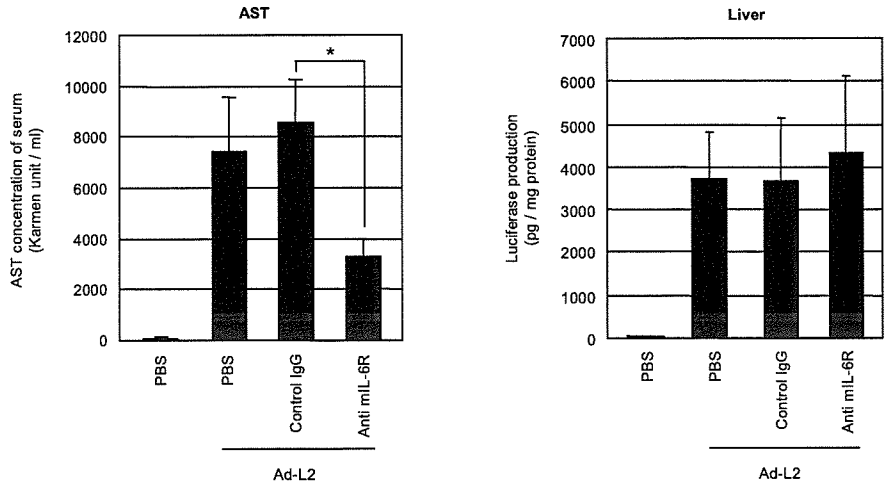

FIGURE 5. Effects of serum IL-6 on serum AST levels and liver luciferase production after the systemic administration of Ad-L2 into mice. C57BL/6 mice were i.p. administered 100 μg per mouse of anti-IL-6R Ab (clone D7715A7), which was specific for blocking IL-6 signaling, or rabbit IgG as a control (clone; 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 $\pm$ 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

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