Table 3 Up-regulated genes by both conventional and RGD-type Ad vectors in mouse peritoneal macrophages.

Affymetrix ID	Gene symbol	GenbankID	Gene name
1418930_at	Cxcl10	NM_021274	chemokine (C-X-C motif) ligand 10
1421008_at	Rsad2	BB741897	radical S-adenosyl methionine domain containing 2
1421009_at	Rsad2	BB741897	radical S-adenosyl methionine domain containing 2
1421578_at	Ccl4	AF128218	chemokine (C-C motif) ligand 4
1424339_at	Oasl1	AB067533	2'-5' oligoadenylate synthetase-like 1
1426208_x_at	Plagl1	AF147785	pleiomorphic adenoma gene-like 1
1427381_at	Irg1	L38281	immunoresponsive gene 1
1431591_s_at	G1p2	AK019325	interferon, alpha-inducible protein
1436058_at	Rsad2	BB132493	radical S-adenosyl methionine domain containing 2
1438511_a_at	1190002H23Rik	BB408123	RIKEN cDNA 1190002H23 gene
1449025_at	Ifit3	NM_010501	interferon-induced protein with tetratricopeptide repeats 3
1449317_at	Cflar	NM_009805	CASP8 and FADD-like apoptosis regulator
1449984_at	Cxcl2	NM_009140	chemokine (C-X-C motif) ligand 2
1450484_a_at	Tyki	AK004595	thymidylate kinase family LPS-inducible member

Table 4 The percentage of GFP-positive cells in various types of splenic cells in Ad-GFP1-injected mice.
Ad-GFP1 (5×10^{10} VP/mouse) was intravenously injected into mice. After 6 h post-injection, the splenic cells were collected and stained with PE-labeled monoclonal anti-mouse CD11c antibody and APC-labeled monoclonal anti-mouse CD86 antibody. The cells were then subjected to flowcytometric analysis. All data are represented as the means ± S.D. (n=5). *$p<0.05$ vs Mock

	% of GFP^+ cells	
Cell type	Mock	Ad-GFP1
cDCs ($CD11c^{high}$, $B220^-$)	0.17±0.29	13.02±1.67*
pDCs ($CD11c^{med}$, $B220^+$)	0.03±0.06	2.66±1.11*
B cell ($CD11c^-$, $B220^+$)	0.00±0.00	.12±0.13
Others	0.00±0.00	1.64±0.39

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., Tashiro K., Kawabata K., Yamaguchi T., Sakurai F., Nakagawa S., Mizuguchi H.	Adenoviral expression of suppressor of cytokine signaling-1 reduces adenovirus vector-induced innate immune response.	J. Immunol.	in press		
Sakurai H., Kawabata K., Sakurai F., Nakagawa S., Mizuguchi H.	Innate immune response induced by gene delivery vectors.	Int. J. Pharm.	in press		
水口裕之	遺伝子治療研究の動向	医薬ジャーナル新薬展望2008	44	235-242	2008
Yamaguchi T., Kawabata K., Koizumi N., Sakurai F., Nakashima K., Sasaki T., Okada N, Mizuguchi H.	Involvement of MyD88 and TLR9 in the innate immune response elicited by replication incompetent adenovirus vectors.	Hum. Gene Ther.	18	753-762	2007
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
水口裕之・櫻井文教・川端健二	カプシドタンパク質改変アデノウイルスベクター	遺伝子医学MOOK 別冊 絵で見てわかるナノDDS	7	235-242	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	22(2)	148-154	2007

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
Koizumi N., Kawabata K., Sakurai F., Watanebe Y., Hayakawa T., Mizuguchi H.	Modified adenovirus vectors with CAR-, αv integrin-, and heparan sulfate-binding ablation reduce in vivo tissue transduction and toxicity.	**Hum. Gene Ther.**	17	264-279	2006
Xu Z.L., Mizuguchi H., Koizumi N., Sakurai F., Hosono T., Kawabata K., Watanabe Y., Yamaguchi T., Hayakawa T.	Approaches to improve the kinetics of adenovirus delivered gene and gene product.	**Adv. Drug. Deli. Rev.**	57	781-802	2005

AQ: A

Adenoviral Expression of Suppressor of Cytokine Signaling-1 Reduces Adenovirus Vector-Induced Innate Immune Responses[1]

AQ: B

Haruna Sakurai,*† Katsuhisa Tashiro,*† Kenji Kawabata,* Tomoko Yamaguchi,*† Fuminori Sakurai,* Shinsaku Nakagawa,† and Hiroyuki Mizuguchi[2]*†

Adenovirus (Ad) vectors are among the most commonly used viral vectors in gene therapy clinical trials. However, the application of Ad vectors has been limited to local injection in many cases, because the systemic administration of Ad vectors triggers innate immune responses such as inflammatory cytokine production and tissue damage. To overcome this limitation, it will be necessary to develop safer Ad vectors less likely to induce the innate immune response. In the present study, we demonstrated that a suppressor of cytokine signaling-1 (SOCS1)-expressing Ad vector, Ad-SOCS1, reduces the innate immune response induced by Ad vectors. RAW264.7-SOCS1, a macrophage-like cell line that stably expresses SOCS1, was shown to produce lower levels of inflammatory cytokines after the transduction of Ad vectors. The systemic administration of Ad-SOCS1 into mice elicited the reduced production of inflammatory cytokines, as compared with that elicited by control Ad vectors, i.e., luciferase-expressing Ad vector, Ad-L2. Furthermore, the coadministration of Ad-L2 with Ad-SOCS1 attenuated inflammatory cytokine production and liver toxicity as compared with injection with Ad-L2 alone, and this was achieved without the suppression of luciferase production in various organs. The JAK/STAT pathway was involved in Ad vector-mediated cytokine production, which was impaired by the overexpression of SOCS1. These findings indicate that Ad-SOCS1 could be useful for reducing Ad vector-mediated innate immunity. ***The Journal of Immunology,* 2008, 180: 0000–0000.**

Adenovirus (Ad)[3] vectors are known to be a highly promising gene transfer system for in vitro and in vivo application, and thus are widely used not only in clinical trials, but also for basic research. However, the application of Ad vectors has been limited to the local injection delivery system (e.g., intratumoral injection) due to the concomitant induction of both innate and adaptive immune responses (1–3). The adaptive immune response against Ad vectors occurs a few weeks after the injection, and is characterized by elevated amounts of anti-Ad Abs and the induction of CTLs against Ad proteins and/or transgene products, which leads to multiorgan damage, in particular in the liver (4–6). Clarification of the molecular mechanism in adaptive immunity has been achieved with Ad vectors, and certain vectors have been developed to overcome these limitations, e.g., the helper-dependent (gutted) Ad vector, which deletes all of the viral protein-coding sequences. The humoral virus-neutralizing Ab responses against the Ad capsid itself are the other limitations, 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 (7). The Ad vectors belonging to types of the subgroup other than Ad type 5 or to species other than human (e.g., Ad types 11 and 35, and chimpanzee Ad) have been developed (8–12). As regards the innate immune response elicited by Ad vectors, it is characterized by an acute production of inflammatory cytokines/chemokines, such as IL-6 and IL-12; tissue damage occurs within several hours after the injection of Ad vectors. Splenic dendritic cells (DCs), especially conventional DCs (cDCs), are responsible for the Ad vector-mediated production of inflammatory cytokines/chemokines, leading to the activation of an innate immune response (2, 13–16). Unlike that of adaptive immunity, the molecular mechanism of innate immunity elicited by Ad vectors is not well-understood (2–4). Thus, it remains important to clarify the mechanism of the innate immune response by Ad vectors, as well to develop advanced Ad vectors that will produce less of an innate immune response.

The suppressor of cytokine signaling-1 (SOCS1) was initially identified as an intracellular negative-feedback molecule that inhibits the JAK-STAT activation initiated by various cytokines, including IFN-γ, IL-6, IL-4, and IL-12 (17–19). Although SOCS1-deficient (knockout (KO)) mice are normal at birth, they show stunted growth and die within 3 wk of age due to a syndrome characterized by severe lymphopenia, activation of peripheral T cells, fatty degeneration, and necrosis of the liver, as well as macrophage infiltration of the major organs (20, 21). These pathological alterations are reduced in IFN-γ-deficient SOCS1 KO mice (22, 23). SOCS1 in DCs negatively regulates antigenic peptide presentation and cytokine production in response to stimulation with cytokines and microbial products (24, 25). Moreover, the

---

*Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Ibaraki, Osaka, Japan; and †Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan

Received for publication December 4, 2007. Accepted for publication January 27, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan. T.Y. is the Research Fellow of the Japan Society for the Promotion of Science.

[2] Address correspondence and reprint requests to Dr. Hiroyuki Mizuguchi, Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. E-mail address: mizuguch@nibio.go.jp

[3] Abbreviations used in this paper: Ad, adenovirus; DC, dendritic cell; cDC, conventional DC; SOCS1, suppressor of cytokine signaling-1; GPT, glutamate pyruvate transaminase; VP, viral particle; DN, dominant negative; KO, knockout.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

suppression of LPS-induced innate immunity by SOCS1 has been reported by several groups (26–28), indicating that SOCS1 negatively regulates both adaptive immunity and innate immunity.

In the present study, the Ad vector-mediated innate immune response was successfully reduced not only by injection of Ad-SOCS1 alone, but also by coinjection of transgene-expressing Ad vectors with Ad-SOCS1. We also found that JAK/STAT signaling is involved in the inflammatory cytokine production induced by Ad vectors. Thus, Ad-SOCS1 might be a useful tool for attenuating innate immunity in the context of systemic administration of transgene-expressing Ad vectors.

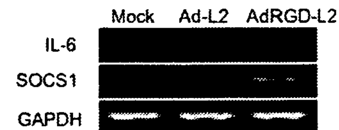

FIGURE 1. SOCS1 expression in Ad vector-transduced RAW264.7 cells. Total mRNA samples were isolated from RAW264.7 cells 6 h after the transduction of Ad-L2 or AdRGD-L2 (10,000 VP/cell). After the reverse transcriptase reaction, SOCS1 and IL-6 mRNA levels were measured by semiquantitative RT-PCR assay.

Materials and Methods

Animals and cell lines

C57BL/6 female mice aged 6–8 wk were obtained from Nippon SLC, and all animals were maintained under specific pathogen-free conditions. The mouse macrophage cell line, RAW264.7, were cultured in DMEM containing 10% FCS. RAW-STAT5DN cells (mutated RAW264.7 cells that stably express the dominant-negative (DN) mutant of STAT5) and RAW-neo cells were cultured in RPMI 1640 containing 10% FCS (both cell lines were donated by Dr. T. Naka, National Institute of Biomedical Innovation, Ibaraki, Osaka, Japan).

RAW264.7 transfectant cells

For the construction of the mouse SOCS1-expressing plasmid (pIRESneo-SOCS1), SOCS1 cDNA (provided by Dr. A. Yoshimura, Kyushu University, Fukuoka, Japan) was inserted into the multicloning site of pIRESneo (BD Clontech). RAW264.7 cells were transfected with pIRESneo-SOCS1 or pIRESneo (control plasmid) using Fugene 6 (Roche). Monoclonal RAW264.7-SOCS1 or RAW264.7-neo cells were obtained by geneticine (G418) selection. The expression of SOCS1 in the RAW264.7-SOCS1 cells was confirmed by Western blot analysis (see Fig. 2*A*).

Ad vectors

Two luciferase-expressing Ad vectors, Ad-L2 and AdRGD-L2, were constructed and generated as described previously (29). The CMV promoter-driven luciferase expression cassette, in which the luciferase gene was derived from the pGL3-Control (Promega), was inserted into the E1 deletion region of the Ad genome. Ad-L2 contains wild-type fibers, whereas AdRGD-L2 contains mutant fibers consisting of the RGD peptide, which has high affinity for integrins, in the HI loop of the fiber knob. AdRGD-L2 exhibits efficient transduction, even in cells that do not express coxsackievirus and adenovirus receptor, CAR (29). Ad-SOCS1, the SOCS1-expressing Ad vector, was constructed by an improved in vitro ligation method, as described previously (30). Briefly, mSOCS1 cDNA was cloned into a multicloning site of pHMCMV10, which contains the CMV promoter/enhancer and intron A sequences flanked by the I-*Ceu*I and PI-*Sce*I sites, thus yielding pHMCMV10-mSOCS1. pAdHM4-SOCS1 was constructed by ligation of I-*Ceu*I/PI-*Sce*I-digested pAdHM4 and I-*Ceu*I/PI-*Sce*I-digested pHMCMV10-mSOCS1. To generate the virus, pAdHM4-SOCS1 was digested with *Pac*I and purified by phenol-chloroform extraction and ethanol precipitation. Linearized DNAs were transfected into 293 cells with SuperFect (Qiagen) according to the manufacturer's instructions. The viruses were amplified in 293 cells. Before virus purification, cell lysates were centrifuged to remove cell debris and were digested 30 min at 37°C with 200 μg/ml DNase I and 200 μg/ml RNase A in the presence of 10 mM $MgCl_2$. Viruses were purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation. The purified viruses were dialyzed against a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol and were stored at −80°C. Viral particle (VP) and biological titers were determined by a spectrophotometrical method (31) and by using an Adeno-X Rapid Titer kit (BD Clontech), respectively. The ratios of the biological-to-particle titer were 1:8, 1:8, 1:12, 1:7, and 1:11 for Ad-L2, AdRGD-L2, Ad-SOCS1, Ad-GFP1, and Ad-null, respectively.

AQ: C

Ad vector-mediated transduction in vivo

Ad vectors (5×10^{10} VP/mouse) were injected into C57BL/6 mice via the tail vain. The heart, lung, liver, kidney, spleen, and peripheral blood were subsequently isolated at the appropriate times. The organs were then homogenized with a hand-held homogenizer in lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris-HCl (pH 7.8)). After the samples were frozen and thawed, the homogenates were centrifuged at 15,000 × *g* at 4°C for 10 min, and the supernatants were collected. Luciferase production in the supernatants was determined by using a luciferase assay system (PicaGene 5500; Toyo Ink). The protein content was measured with a BCA protein assay (Pierce) using BSA as a standard.

Cytokine measurement

RAW264.7 cells (1×10^5 cells) seeded on 24-well plates were pretreated with IFN-γ (200 ng/ml) overnight and the cells were then washed with medium. After treatment with JAK2 inhibitor II (50 μM; Calbiochem) for 1 h, the cells were stimulated with 10,000 VP/cell of Ad vector for 24 h, and the cultured medium was then collected. RAW264.7-SOCS1, RAW264.7-neo, RAW-STAT5DN, and RAW-neo cells ($5-10 \times 10^4$ cells) were seeded on 24-well plates and pretreated with IFN-γ (200 ng/ml) overnight and the cells were then washed with medium. The cells were then stimulated with 10,000 VP/cell of Ad vector for 24 h, and the cultured medium was collected. Blood samples were collected via the inferior vena cava or the fundus oculi at the indicated times after the i.v. injection of Ad vectors, followed by placement of the samples on ice for 2–3 h for serum collection. The levels of IL-6, IL-12p40, and TNF-α in the culture medium and serum were measured using an ELISA kit (R&D Research Systems) according to the manufacturer's instructions. The concentrations of other cytokines and chemokines were measured with Bio-plex (Bio-Rad). Glutamate pyruvate transaminase (GPT) activity in the serum was measured using the Transaminase CII test (Wako).

Preparation of paraffin sections of liver

At 24 h following injection, mice were sacrificed and their livers were collected. Each liver was washed, fixed in 10% formalin, and embedded in paraffin. After sectioning, the tissue was dewaxed in ethanol, rehydrated,

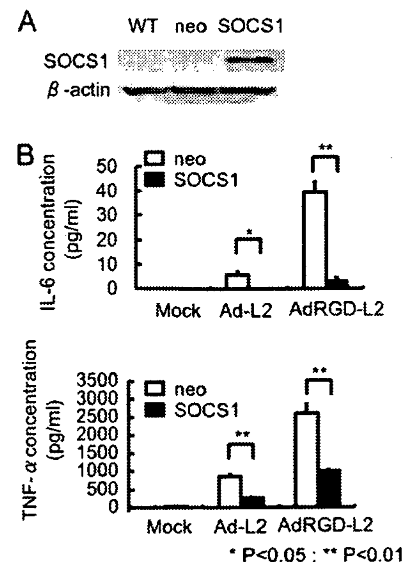

FIGURE 2. Ad vector-mediated induction of cytokine production in RAW264.7-SOCS1 cells. The expression of SOCS1 in RAW264.7-SOCS1 cells was detected by Western blot analysis (*A*). IFN-γ-activated RAW264.7-neo cells and RAW264.7-SOCS1 cells were stimulated by Ad-L2 or AdRGD-L2 (10,000 VP/cell) for 24 h. The concentration of IL-6 and TNF-α in the cultured supernatants was measured by ELISA (*B*). All data are represented as the means ± SD (*n* = 3).

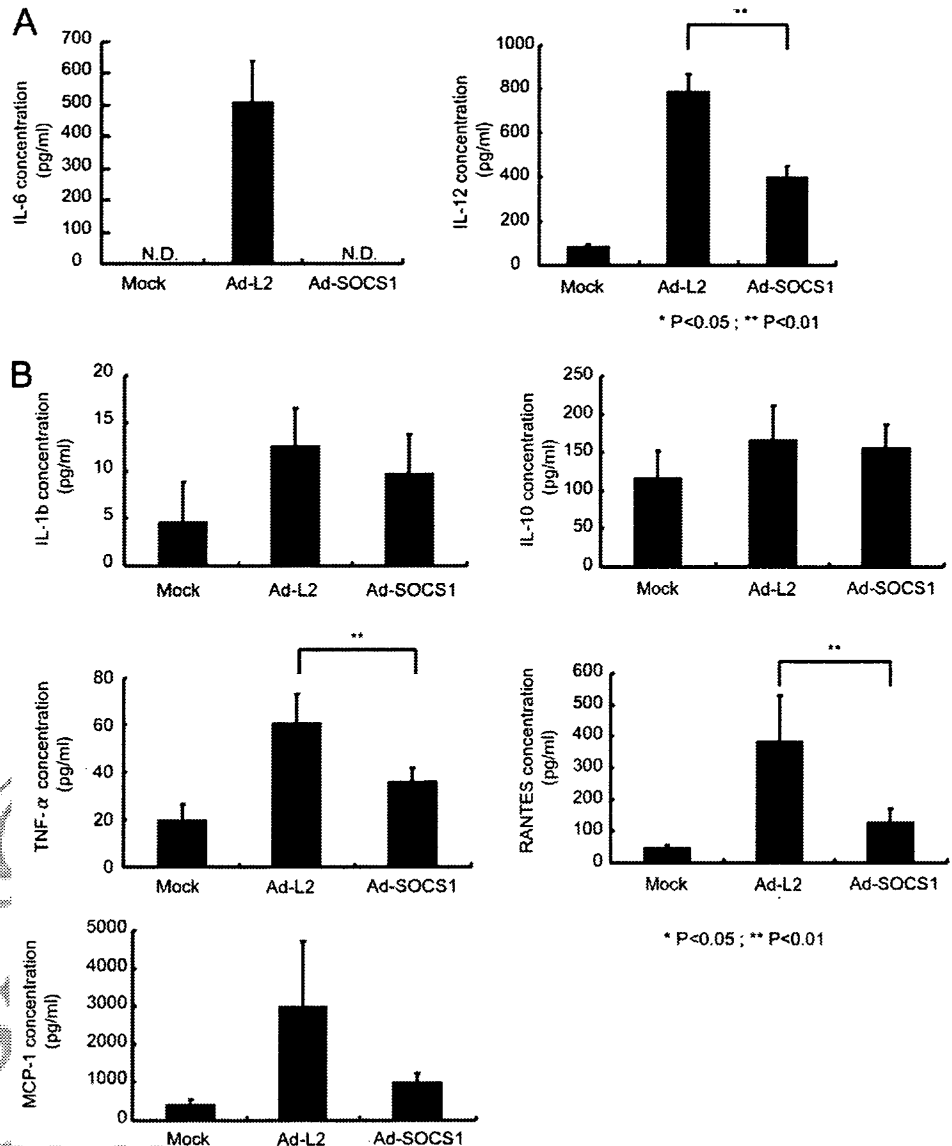

FIGURE 3. Inflammatory cytokine production after the systemic administration of Ad-L2 or Ad-SOCS1 into mice. Ad-L2 or Ad-SOCS1 (5×10^{10} VP/mouse) was i.v. injected into mice. Six hours later, blood samples were collected from the inferior vena cava, and the concentrations of IL-6 and IL-12 in the serum was measured by ELISA (*A*). The concentrations of IL-1β, IL-10, TNF-α, RANTES, and MCP-1 in serum of Ad vector-injected mice were measured by Bio-plex (*B*). All data are represented as the means ± SD (n = 3–4).

and stained with H&E. This process was commissioned to Applied Medical Research Laboratory (Osaka, Japan).

Western blot analysis

The organs or cells were homogenized with lysis buffer (organ: PBS containing 1% Triton X-100 and 2 mM EDTA; cells: 1% Nonidet P-40, 1 mM EDTA, 25 mM Tris-HCl, 5 mM NaF, and 150 mM NaCl) containing protease inhibitor mixture (Sigma-Aldrich). After being frozen and thawed, the homogenates were centrifuged at 15,000 × g at 4°C for 10 min, and the supernatants were collected. The lysates were subjected to SDS-PAGE on 15% polyacrylamide gel and were then transferred onto polyvinylidene fluoride membranes (Millipore). After the reaction was blocked with 5% skim milk in TBS containing 0.1% Tween 20 at room temperature for 1 h, the membranes were incubated with rabbit anti-SOCS1 Ab (diluted 1/20; Immuno-Biological Laboratories), mouse anti-α-tubulin Ab (diluted 1/200; Santa Cruz Biotechnology), or mouse anti-β actin Ab (diluted 1/5000; Sigma-Aldrich) at 4°C overnight, followed by reaction with HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Cell Signaling Technology) at room temperature for 1 h. The band was visualized by ECL Plus Western blotting detection reagents (GE Healthcare) and the signals were read using a LAS-3000 imaging system (FUJI Film).

In vitro luciferase assay

RAW264.7 cells (1×10^5 cells) seeded on 24-well plates were pretreated with IFN-γ (200 ng/ml) for overnight and the cells were then washed with medium. After treatment with JAK2 inhibitor II (50 μM; Calbiochem) for 1 h, the cells were stimulated with 10,000 VP/cell of the Ad vectors for 24 h and were then washed with PBS. Cells were incubated with 100 μl of cell lysis buffer (LC-β; Toyo Ink) for 15 min and were then scraped off the plates using a cell scraper. After being frozen and thawed, the cell lysates were centrifuged at 15,000 × g at 4°C for 10 min, and the supernatants were collected. Luciferase production in each supernatant was determined using a luciferase assay system (PicaGene 5500; Toyo Ink).

Flow cytometric analysis of transgene expression in Ad vector-transduced cDCs

Six hours following the i.v. injection of Ad-GFP1 (5×10^{10} VP/mouse), the mice were sacrificed and the spleens were collected. After the hemolysis, splenic cells were stained with PE-labeled monoclonal anti-mouse CD11c Ab (BD Biosciences) and allophycocyanin-labeled monoclonal anti-mouse B220 Ab (BD Biosciences) in the presence of anti-CD16/32 to block any nonspecific binding. The stained cells were washed and analyzed by FACSCanto (BD Biosciences), followed by analysis using CellQuest software (BD Biosciences). AQ: D

Results

Ad vector-mediated inflammatory cytokine production in macrophages was suppressed by the overexpression of SOCS1

Transduction of Ad vectors into immune cells such as macrophages and DCs induces the production of inflammatory cytokines (14), although the molecular mechanism is not well-understood. Recently, we performed a DNA microarray analysis of the liver

and spleen of Ad vector-injected mice and found that SOCS1 was up-regulated in the liver of Ad vector-injected mice (our unpublished data). Because SOCS1 has been shown to negatively regulate the immune response of macrophages in pathological stimulation (28, 32), we hypothesized that SOCS1 might suppress Ad vector-mediated inflammatory cytokine production in immune cells. Initially, we examined the induction of SOCS1 mRNA expression after the transduction of Ad vectors into immune cells. We selected a mouse macrophage-like cell line, RAW264.7, as a model of immune cells, because this cell line is commonly used in studies of innate immune responses to pathogens (28, 33). We used the luciferase-expressing Ad vector containing the RGD peptide in the HI loop of the fiber knob (AdRGD-L2) (29) as well as the luciferase-expressing conventional Ad vector (Ad-L2) for in vitro analysis. AdRGD-L2 transduces well even into macrophages that do not express coxsackievirus and adenovirus receptor, CAR, via interaction between the RGD peptide and α_v integrin on the cells. AdRGD-L2 induces a stronger immune response than does the conventional Ad vector when immune cells lacking CAR expression are treated with the same dose of Ad vectors. Six hours after the transduction of AdRGD-L2, the up-regulation of IL-6 mRNA was detected in RAW264.7 cells, indicating that the transduction of AdRGD-L2 elicited the innate immune response in RAW264.7 cells (Fig. 1). The up-regulation of SOCS1 mRNA was also detected. However, no up-regulation of either gene was detected in RAW264.7 cells transduced with Ad-L2 most likely because Ad-L2 inefficiently transduces RAW264.7 cells lacking CAR expression. These results suggest that the immune signal activated by Ad vectors might induce SOCS1 to negatively regulate the immune response.

To examine the suppressive effect of SOCS1 on Ad vector-mediated inflammatory cytokine production in RAW264.7 cells, we next established RAW264.7 cells that constitutively express SOCS1 (RAW264.7-SOCS1) (Fig. 2A) and observed Ad vector-mediated cytokine production in these cells (Fig. 2B). The RAW264.7 cells were pretreated with IFN-γ (IFN-γ), because no cytokine production was detected by ELISA in the absence of IFN-γ. After the 24 h transduction of Ad vectors in IFN-γ-pretreated RAW264.7 cells, the levels of IL-6 and TNF-α were significantly reduced by the overexpression of SOCS1 (Fig. 2B). It has been reported that although SOCS1 suppresses IL-6 production in RAW264.7 cells treated with LPS, it does not suppress the production of other cytokines, including TNF-α (28). These results suggest that SOCS1 inhibits Ad vector-mediated inflammatory cytokine production in RAW264.7 cells and that the immune signal activated by Ad vector transduction in RAW264.7 cells might differ from that activated by LPS.

SOCS1-expressing Ad vector suppresses Ad vector-mediated innate immune response

In animals, the systemic administration of Ad vectors results in the initiation of a strong innate immune response and inflammation (2), and this toxicity limits the utility of Ad vectors for gene therapy. Because SOCS1 suppresses the inflammatory cytokine production by Ad vectors in vitro, we expected that SOCS1 might suppress the in vivo innate immune response triggered by Ad vectors. To test this hypothesis, we injected the SOCS1-expressing Ad vector, Ad-SOCS1, i.v. into mice, and the serum levels of IL-6 and IL-12 at 6 h postinjection were examined. We could not detect any type I IFNs at early time points (1–6 h) after the injection (data not shown). The complete elimination of IL-6 was observed in the serum of Ad-SOCS1-injected mice 6 h postinjection, while only a partial elimination of IL-12 was observed in the serum of Ad-SOCS1-injected mice (Fig. 3A). The Ad vector-mediated induction

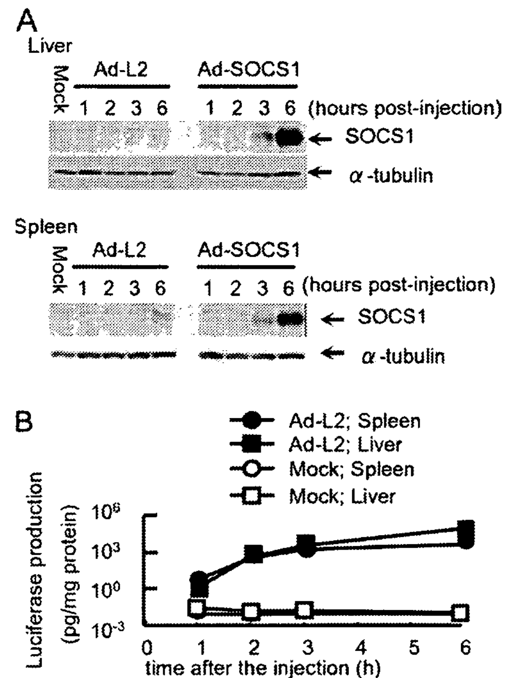

FIGURE 4. Transgene expression in the liver and spleen by the systemic administration of Ad vectors. Ad-SOCS1-mediated SOCS1 expression in the liver and the spleen was examined at 1, 2, 3, and 6 h after i.v. injection of Ad vectors, as determined by Western blotting (*A*). Luciferase production in the liver and the spleen was examined at 1, 2, 3, and 6 h after i.v. injection of Ad-L2, as determined by luciferase assay (*B*).

of MCP-1, RANTES, and TNF-α was also suppressed by the expression of SOCS1; however, the production of IL-1β was not inhibited (Fig. 3B). We did not detect any induction of IL-10 in the serum of Ad vector-injected mice at 6 h postinjection.

We previously demonstrated that cytokines are mainly produced in the spleen after the systemic injection of Ad vectors (13). Because the suppressive effect of SOCS1 in Ad vector-mediated cytokine production in vivo was observed 6 h after the injection, Ad vector-mediated SOCS1 expression must occur before the 6 h postinjection time point. Thus, we performed a Western blot analysis to examine the expression of SOCS1 in the liver and the spleen of Ad vector-injected mice at early time points (Fig. 4A). The liver and the spleen were selected because Ad vector-mediated transgene expression mainly occurs in the liver (34), while Ad vector-induced cytokine production mainly occurs in the spleen (13). We also examined the luciferase production of Ad-L2-injected mice at time points earlier than 6 h postinjection, because luciferase assay is much more sensitive than is Western blot analysis. SOCS1 expression was almost negligible until 3 h postinjection by Western blot analysis (Fig. 4A); however, luciferase production by Ad-L2 was detected even 1 h after the injection (Fig. 4B), indicating that Ad vector-mediated SOCS1 expression could potentially occur at least 1 h after the injection although expression levels of SOCS1 were below the Western blot detection limit. A faint band showing SOCS1 expression was also detected in the spleen at 6 h following Ad-L2 injection (Fig. 4A). We previously revealed that splenic cDCs mainly produce inflammatory cytokines in response to the systemic injection of Ad vectors (13). It is possible that Ad-SOCS1 mediates SOCS1 expression in splenic cDCs and reduces cytokine production via the systemic injection of Ad vectors. To confirm this possibility, we examined the cell type of splenic cells transduced with Ad vectors in the spleens of Ad vector-injected mice. GFP-expressing Ad vectors, Ad-GFP1,

Table I. *The percentage of GFP-positive cells in various types of splenic cells in Ad-GFP1-injected mice*[a]

Cell Type	% of GFP⁺ Cells	
	Mock	Ad-GFP1
cDCs (CD11c^{high}, B220$^-$)	0.17 ± 0.29	13.02 ± 1.67*
pDCs (CD11c^{med}, B220$^+$)	0.03 ± 0.06	2.66 ± 1.11*
B cell (CD11c$^-$, B220$^+$)	0.00 ± 0.00	0.12 ± 0.13
Others	0.00 ± 0.00	1.64 ± 0.39

[a] Ad-GFP1 (5×10^{10} VP/mouse) was i.v. injected into mice. After 6 h postinjection, the splenic cells were collected and stained with PE-labeled monoclonal anti-mouse CD11c Ab and allophycocyanin-labeled monoclonal anti-mouse CD86 Ab. The cells were then subjected to flow cytometric analysis. All data are represented as the means ± SD ($n = 5$). *, $p < 0.01$ vs Mock.

were i.v. injected into the mice, and FACS analysis was performed. At 6 h postinjection of Ad-GFP1, 13.2% of the splenic cDCs expressed GFP (Table I), suggesting that Ad vector-mediated SOCS1 expression in splenic cDCs does suppress the Ad vector-mediated innate immune response in vivo.

We next hypothesized that the coinjection of Ad-L2 with Ad-SOCS1 might inhibit the innate immune response while retaining luciferase production. To examine this possibility, we injected mixed Ad vectors which were created by mixing Ad-L2 and Ad-SOCS1 at a VP ratio of 1:9, and the production of inflammatory cytokines was measured. We used Ad-null, a transgene-deficient Ad vector, as a control vector. At 6 h after the injection of the mixed Ad vectors, IL-6 levels were completely diminished when Ad-L2 was coinjected with Ad-SOCS1, whereas the levels of IL-12 were only partially reduced (Fig. 5*A*). These data are similar to those obtained with Ad-SOCS1-only-injected mice (Fig. 3*A*). Importantly, luciferase production in the organs was maintained at high levels, even with the coinjection of Ad-SOCS1; these results were similar to those observed in the control group, in which mice were injected with Ad-null and Ad-L2, although in the kidney and spleen, luciferase production was somewhat reduced (Fig. 5*B*). We also examined the suppressive effects of SOCS1 in Ad vector-induced liver damage, because liver damage is to some extent caused by the Ad vector-induced innate immune response (13). Therefore, GPT activity in the serum was measured. Coinjection of Ad-L2 with Ad-null led to high levels of serum GPT activity at 24 h postinjection, while that of Ad-L2 with Ad-SOCS1 did not induce significant GPT activity (Fig. 5*C*). Histological analysis showed severe damages, including cell death, in the livers from Ad-L2 with Ad-null-coinjected mice, whereas less liver damage was detected in the livers from Ad-L2 with Ad-SOCS1-coinjected mice (Fig. 5*D*). These results indicate that Ad-SOCS1 suppresses

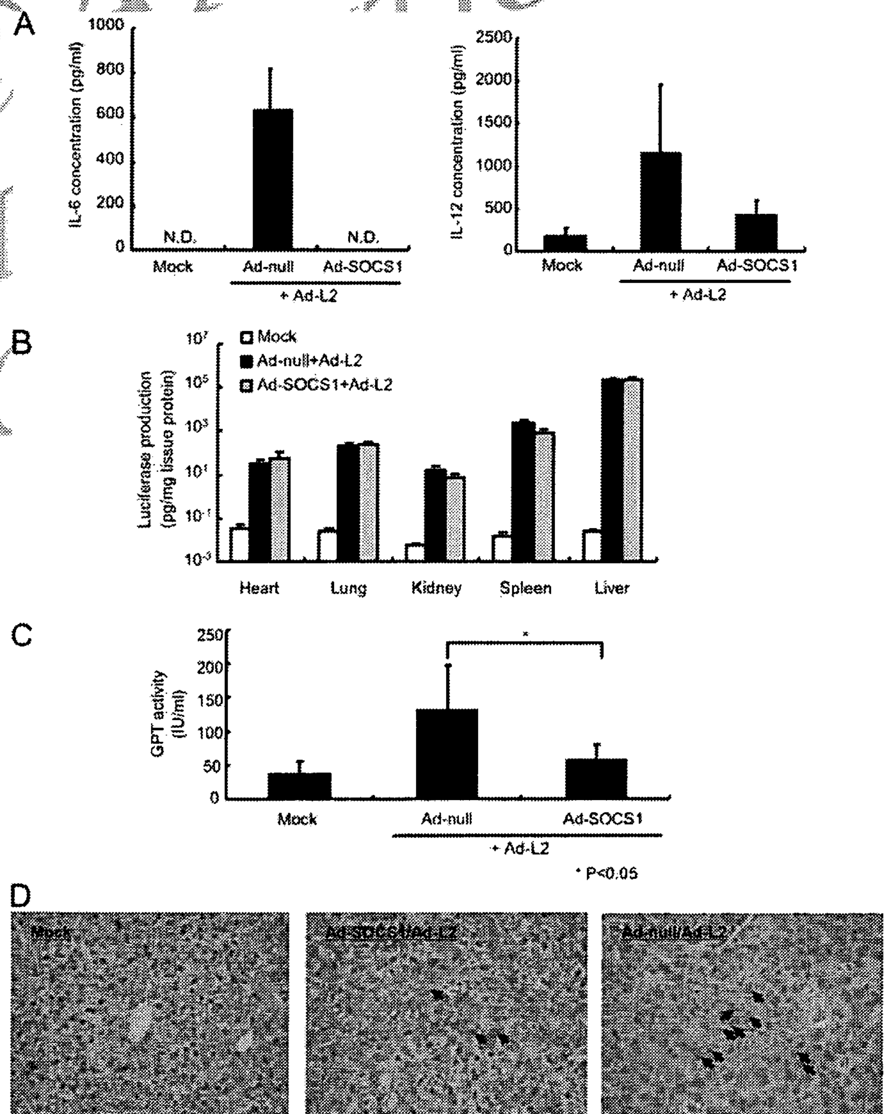

FIGURE 5. Inflammatory cytokine production, luciferase production, and liver toxicity after the systemic coinjection of Ad-L2 and Ad-SOCS1 into mice. Ad-L2/Ad-null or Ad-L2/Ad-SOCS1 were i.v. coinjected into mice (Ad-L2: Ad-SOCS1 = 1:9; total 5×10^{10} VP/mouse). Blood samples were collected from the fundus oculi at 6 h (*A*) or 24 h (*C*) after injection. The heart, lungs, kidneys, liver, and spleen were harvested at 24 h (*B* and *D*) following the injection. *A*, The concentrations of IL-6 and IL-12 in the serum were measured by ELISA. *B*, Luciferase production in each organ was measured using a luciferase assay system. *C*, GPT levels in the serum were measured using a Transaminase CII kit. *D*, Paraffin sections of the livers were prepared, and each section was stained with H&E. The arrowheads indicate dead cells. All data are represented as the means ± SD ($n = 3$).

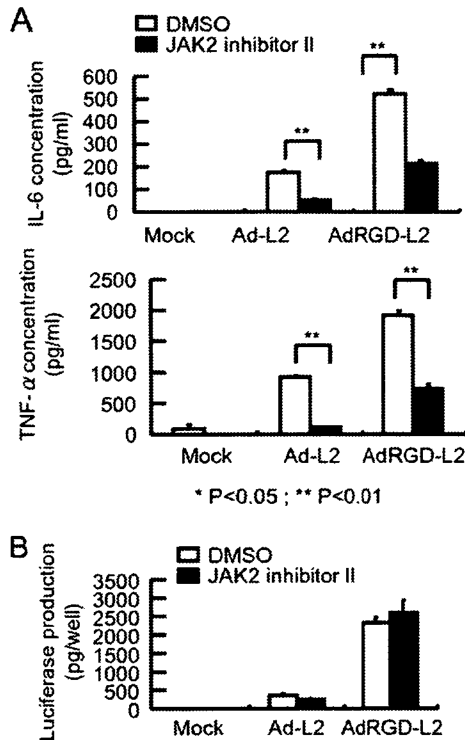

FIGURE 6. JAK2 inhibitor II suppresses the Ad vector-mediated cytokine production in RAW264.7 cells. RAW264.7 cells pretreated with IFN-γ were incubated with JAK2 inhibitor II (50 μM) for 1 h and the cells were stimulated by Ad vectors for 24 h. *A*, The concentrations of IL-6 and TNF-α in the cultured supernatants were measured by ELISA. *B*, Luciferase production in the cells was examined using a luciferase assay system. All data are represented as the means ± SD (*n* = 3).

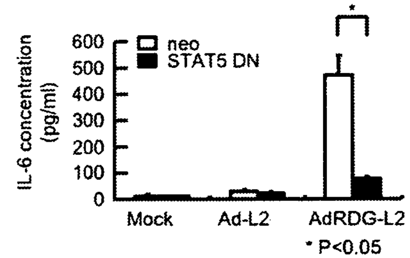

FIGURE 7. Ad vector-mediated induction of cytokine production and luciferase production in RAW264.7-STAT5DN cells. IFN-γ-activated RAW-neo cells and RAW-STAT5DN cells were stimulated by Ad-L2 or AdRGD-L2 (10,000 VP/cell) for 24 h. The concentration of IL-6 in the cultured supernatants was measured by ELISA (*A*). All data are represented as the means ± SD (*n* = 3).

not only the innate immune response itself induces, but also that induced by the coinjected Ad vector.

Involvement of JAK-STAT signaling in macrophage activation

To date, the molecular mechanism of the Ad vector-mediated innate immune response has remained unclear. JAK-STAT signaling is expected to participate in the Ad vector-induced innate immune response, because JAK proteins are the target of SOCS1 (18, 19). We examined cytokine production in Ad vector-transduced RAW264.7 cells under the condition that JAK2 autophosphorylation was inhibited by JAK2 inhibitor II, which is known to be the tyrosine kinase inhibitor specific for JAK2 (35) (Fig. 6*A*). IFN-γ-stimulated RAW264.7 cells were transduced by Ad vectors with or without preincubation with JAK2 inhibitor II, and then inflammatory cytokine levels in the cultured medium were measured. The levels of IL-6 and TNF-α were significantly reduced with the inhibition of JAK2 autophosphorylation by JAK2 inhibitor II (Fig. 6*A*). Reduced cytokine production in Ad vector-transduced RAW264.7 cells with the inhibition of JAK2 autophosphorylation was also observed at the mRNA level (data not shown). Luciferase production in the cells was unchanged with the inhibition of JAK2 autophosphorylation (Fig. 6*C*), indicating that Ad vector-mediated immune cell activation does not affect the transduction efficiency of Ad vectors. These results suggest that JAK2 is indeed involved in the innate immune response by Ad vectors.

Because STAT proteins are activated by JAK in various types of cytokine signaling (36), we investigated the involvement of STAT in Ad vector-activated signaling. We speculated that STAT5 might be a molecule downstream of Ad vector-activated JAK2, because JAK2/STAT5 signaling is reported to be involved in IL-6 production triggered by LPS (28). To examine the involvement of STAT5 in cytokine production induced by Ad vectors, RAW264.7 cells expressing a STAT5 DN mutant (RAW-STAT5DN) were transduced by Ad vectors. When STAT5DN was overexpressed, IL-6 production induced by Ad vectors was significantly eliminated (Fig. 7). These findings indicate that the JAK2/STAT5 pathway is involved in the cytokine production by Ad vectors.

F6 AQ: G F7

Discussion

The major limitation of the use of Ad vectors as gene therapy vectors is the innate immune response thereby elicited, which causes inflammatory cytokine production and tissue damage; however, the precise mechanism of this induction remains to be clarified. In this study, we found that SOCS1 suppresses the in vitro and in vivo production of inflammatory cytokines by Ad vectors. Furthermore, coinjection with Ad-SOCS1 enabled a successful reduction in the innate immune response in vivo while retaining the transduction efficiency of the transgene. Thus, Ad-SOCS1 is expected to be useful for attenuating the innate immune response induced by the systemic administration of Ad vectors.

Because SOCS1 is known to be a negative regulator of LPS-induced innate immunity as well as being a cytokine signal (17–19, 28, 33), we speculated that SOCS1 might suppress Ad vector-induced inflammatory cytokine production. However, we expected that the Ad-SOCS1-mediated expression of SOCS1 might occur too late for the successful inhibition of cytokine production, because it appeared that Ad vector-induced acute inflammatory cytokine production would take place earlier than Ad-SOCS1-mediated SOCS1 expression. However, a substantial reduction in cytokine production was observed when Ad-SOCS1 was used for the injection (Fig. 3). We and other groups have previously reported that the spleen is responsible for the Ad vector-induced production of inflammatory cytokines (13, 16). To determine whether Ad-SOCS1-mediated SOCS1 expression occurs before the induction of cytokine production, we examined the time course of luciferase (and SOCS1) expression in the spleens of Ad-L2-injected mice. We found that Ad vector-mediated luciferase expression in the spleen starts within 1 h postinjection (Fig. 4*B*). Interestingly, at 1 h after the injection of Ad-L2, luciferase production in the spleen was greater than that in the liver, whereas luciferase production in the liver was 1 order of magnitude higher than in the spleen 6 h after the injection (Fig. 4*B*). The transduction of Ad vectors in immune cells might occur earlier than that in other cells, resulting in the prevention of acute inflammatory cytokine production. A previous report also indicated that splenic cDCs produce inflammatory cytokines via the transduction of Ad vectors in vivo (13, 14). Our results showed that ~13% of the splenic

AAI DISTRIBUTION PROHIBITED

cDCs collected from Ad-GFP1-injected mice expressed GFP (Table I). Although we were unable to detect GFP expression in the remaining 85% of the splenic cDCs by FACS analysis, a small amount of SOCS1 was expressed in the splenic cDCs of Ad-SOCS1-injected mice, which could suffice for the suppression of Ad vector-induced inflammatory cytokine production. Taken together, these results indicate that Ad-SOCS1-mediated SOCS1 expression in splenic cDCs inhibits inflammatory cytokine production. Even though the Ad vector-mediated induction of IL-6 was completely eliminated by Ad-SOCS1 (Figs. 3*A* and 5*A*), only partial suppression of the induction of other cytokines (IL-1β, IL-12, and TNF-α) and chemokines (RANTES and MCP-1) by Ad-SOCS1 was detected (Fig. 3). These findings suggest that there are several signal pathways for the production of cytokines/chemokines by Ad vectors, and that differences in these pathways are due to their respective cytokine/chemokine dependence. Although SOCS1 was reported to negatively regulate the adaptive immunity by suppressing Ag presentation in DCs, no suppression of anti-Ad Ab production by Ad-SOCS1 was observed. It might be due to the partial suppression of inflammatory cytokine production other than IL-6.

The molecular mechanism of immune cell activation following the transduction of Ad vectors remains to be elucidated (2–4). Because we observed a reduction in cytokine production in Ad vector-transduced RAW264.7 cells by the overexpression of SOCS1, we predicted that the JAK/STAT signal might be involved in Ad vector-activated immune signaling. The activation of JAK2 leads to the production of IL-6 in LPS stimulation which is impaired by the overexpression of SOCS1 (28, 33). In this study, we found that the inhibition of JAK2 autophosphorylation by JAK2 inhibitor II results in a reduction in the Ad vector-mediated production of IL-6 and TNF-α (Fig. 6*A*). Moreover, the overexpression of the DN mutant of STAT5 resulted in the elimination of Ad vector-mediated IL-6 production in RAW264.7 cells (Fig. 7). These results suggest that the JAK2/STAT5 pathway is involved in the Ad vector-mediated activation of immune cells, which is inhibited by SOCS1.

We previously demonstrated that Ad vector-induced IL-6 plays an important role in the liver toxicity induced by the systemic administration of Ad vectors (13). Ad vector-mediated liver damage was avoided by coinjection of Ad-L2 with Ad-SOCS1 (Fig. 5, *C* and *D*). The liver damage caused by the effects of Ad vectors has been reported as occurring via two major pathways: 1) the activation of infiltrating lymphocytes by cytokines such as IL-6 (13), and 2) the activation and the cell death of Kupffer cells by Ad vectors (37, 38). In the first scenario, Ad-induced cytokine/chemokine production (especially that of IL-6) recruits immune effector cells (neutrophils, monocytes/macrophages, and NK cells) into Ad-transduced cells (mainly in the liver), resulting in acute hepatic toxicity. Because coinjection with Ad-SOCS1 eliminated IL-6 production, the activation of lymphocytes infiltrating into the liver might have been inhibited, resulting in the absence of liver damage. Moreover, our results indicate that Ad vector-induced IL-6 and TNF-α production in RAW264.7 cells was impaired by the overexpression of SOCS1 (Fig. 2*B*). These results suggest that SOCS1 also prevents Kupffer cells from undergoing Ad vector-induced activation and cell death.

Another important finding is that transgene (luciferase) expression by coinjected Ad vector is not disturbed by Ad-SOCS1 (Fig. 5*B*). We addressed the suppression of cytokine production by coinjecting Ad-SOCS1 with only a ninth the amount of Ad-L2 (Fig. 5*A*). The optimum ratio of Ad-L2 to Ad-SOCS1 should be identified in the future. Our findings suggest that Ad-SOCS1 should be a useful tool for attenuating innate immunity in the context of systemic administration of transgene-expressing Ad vectors.

Our present study offers a new strategy for reducing the Ad vector-mediated innate immune response. The results of the present study also provide new insights into the cellular biological mechanism of the cytokine production induced by Ad vectors. SOCS1-expressing Ad vectors thus possess great potential for the development of safe gene therapies.

Acknowledgments

We thank Dr. E. Mukai (National Institute of Biomedical Innovation, Ibaraki, Osaka, Japan) and Dr. N. Koizumi (Showa Pharmaceutical University, Tokyo, Japan) for helpful discussion. We thank Dr. S. Nakamura (Research Center for Animal Life Science, Shiga University of Medical Science, Shiga, Japan) for technical supports with pathological analysis. AQ: H

Disclosures

The authors have no financial conflict of interest.

References

1. Nazir, S. A., and J. P. Metcalf. 2005. Innate immune response to adenovirus. *J. Investig. Med.* 53: 292–304.
2. Sakurai, H., K. Kawabata, F. Sakurai, S. Nakagawa, and H. Mizuguchi. Innate immune response induced by gene delivery vectors. *Int. J. Pharm.* In press. AQ: I
3. Muruve, D. A. 2004. The innate immune response to adenovirus vectors. *Hum. Gene Ther.* 15: 1157–1166.
4. Liu, Q., and D. A. Muruve. 2003. Molecular basis of the inflammatory response to adenovirus vectors. *Gene Ther.* 10: 935–940.
5. Jooss, K., and N. Chirmule. 2003. Immunity to adenovirus and adeno-associated viral vectors: implications for gene therapy. *Gene Ther.* 10: 955–963.
6. Chen, D., B. Murphy, R. Sung, and J. S. Bromberg. 2003. Adaptive and innate immune responses to gene transfer vectors: role of cytokines and chemokines in vector function. *Gene Ther.* 10: 991–998.
7. Roberts, D. M., A. Nanda, M. J. Havenga, P. Abbink, D. M. Lynch, B. A. Ewald, J. Liu, A. R. Thorner, P. E. Swanson, D. A. Gorgone, et al. 2006. Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature* 441: 239–243.
8. Palmer, D. J., and P. Ng. 2005. Helper-dependent adenoviral vectors for gene therapy. *Hum. Gene Ther.* 16: 1–16.
9. Sakurai, F., H. Mizuguchi, and T. Hayakawa. 2003. Efficient gene transfer into human $CD34^+$ cells by an adenovirus type 35 vector. *Gene Ther.* 10: 1041–1048.
10. Sakurai, F., K. Kawabata, and H. Mizuguchi. 2007. Adenovirus vectors composed of subgroup B adenoviruses. *Curr. Gene Ther.* 7: 229–238.
11. Vogels, R., D. Zuijdgeest, R. van Rijnsoever, E. Hartkoorn, I. Damen, M. P. de Bethune, S. Kostense, G. Penders, N. Helmus, W. Koudstaal, et al. 2003. Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. *J. Virol.* 77: 8263–8271.
12. Farina, S. F., G. P. Gao, Z. Q. Xiang, J. J. Rux, R. M. Burnett, M. R. Alvira, J. Marsh, H. C. Ertl, and J. M. Wilson. 2001. Replication-defective vector based on a chimpanzee adenovirus. *J. Virol.* 75: 11603–11613.
13. Koizumi, N., T. Yamaguchi, K. Kawabata, F. Sakurai, S. T., Y. Watanabe, T. Hayakawa, and H. Mizuguchi. 2007. Fiber-modified adenovirus vectors decrease liver toxicity through reduced interleukin 6 production. *J. Immunol.* 178: 1767–1773.
14. Yamaguchi, T., K. Kawabata, N. Koizumi, F. Sakurai, K. Nakashima, H. Sakurai, T. Sasaki, N. Okada, K. Yamanishi, and H. Mizuguchi. 2007. Involvement of MyD88 and TLR9 in the innate immune response elicited by replication-incompetent adenovirus vectors. *J. Immunol.* In press. AQ: J
15. Basner-Tschakarjan, E., E. Gaffal, M. O'Keeffe, D. Tormo, A. Limmer, H. Wagner, H. Hochrein, and T. Tuting. 2006. Adenovirus efficiently transduces plasmacytoid dendritic cells resulting in TLR9-dependent maturation and IFN-α production. *J. Gene Med.* 8: 1300–1306.
16. De Geest, B., J. Snoeys, S. Van Linthout, J. Lievens, and D. Collen. 2005. Elimination of innate immune responses and liver inflammation by PEGylation of adenoviral vectors and methylprednisolone. *Hum. Gene Ther.* 16: 1439–1451.
17. Starr, R., T. A. Willson, E. M. Viney, L. J. Murray, J. R. Rayner, B. J. Jenkins, T. J. Gonda, W. S. Alexander, D. Metcalf, N. A. Nicola, and D. J. Hilton. 1997. A family of cytokine-inducible inhibitors of signalling. *Nature* 387: 917–921.
18. Endo, T. A., M. Masuhara, M. Yokouchi, R. Suzuki, H. Sakamoto, K. Mitsui, A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misawa, et al. 1997. A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* 387: 921–924.
19. Naka, T., M. Narazaki, M. Hirata, T. Matsumoto, S. Minamoto, A. Aono, N. Nishimoto, T. Kajita, T. Taga, K. Yoshizaki, et al. 1997. Structure and function of a new STAT-induced STAT inhibitor. *Nature.* 387: 924–929.
20. Starr, R., D. Metcalf, A. G. Elefanty, M. Brysha, T. A. Willson, N. A. Nicola, D. J. Hilton, and W. S. Alexander. 1998. Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. *Proc. Natl. Acad. Sci. USA* 95: 14395–14399.

balt6/zim-jimm/zim-jimm/zim00708/zim6868-08z | xppws | S=1 | 2/20/08 | 7:13 | 4/Color Figure(s): F5 | Art: 6868 | Input-gaj

21. Naka, T., T. Matsumoto, M. Narazaki, M. Fujimoto, Y. Morita, Y. Ohsawa, H. Saito, T. Nagasawa, Y. Uchiyama, and T. Kishimoto. 1998. Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice. *Proc. Natl. Acad. Sci. USA* 95: 15577–15582.
22. Marine, J. C., D. J. Topham, C. McKay, D. Wang, E. Parganas, D. Stravopodis, A. Yoshimura, and J. N. Ihle. 1999. SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. *Cell* 98: 609–616.
23. Alexander, W. S., R. Starr, J. E. Fenner, C. L. Scott, E. Handman, N. S. Sprigg, J. E. Corbin, A. L. Cornish, R. Darwiche, C. M. Owczarek, et al. 1999. SOCS1 is a critical inhibitor of interferon γ signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* 98: 597–608.
24. Shen, L., K. Evel-Kabler, R. Strube, and S. Y. Chen. 2004. Silencing of SOCS1 enhances antigen presentation by dendritic cells and antigen-specific anti-tumor immunity. *Nat. Biotechnol.* 22: 1546–1553.
25. Hanada, T., H. Yoshida, S. Kato, K. Tanaka, K. Masutani, J. Tsukada, Y. Nomura, H. Mimata, M. Kubo, and A. Yoshimura. 2003. Suppressor of cytokine signaling-1 is essential for suppressing dendritic cell activation and systemic autoimmunity. *Immunity* 19: 437–450.
26. Kinjyo, I., T. Hanada, K. Inagaki-Ohara, H. Mori, D. Aki, M. Ohishi, H. Yoshida, M. Kubo, and A. Yoshimura. 2002. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity* 17: 583–591.
27. Nakagawa, R., T. Naka, H. Tsutsui, M. Fujimoto, A. Kimura, T. Abe, E. Seki, S. Sato, O. Takeuchi, K. Takeda, et al. 2002. SOCS-1 participates in negative regulation of LPS responses. *Immunity* 17: 677–687.
28. Kimura, A., T. Naka, T. Muta, O. Takeuchi, S. Akira, I. Kawase, and T. Kishimoto. 2005. Suppressor of cytokine signaling-1 selectively inhibits LPS-induced IL-6 production by regulating JAK-STAT. *Proc. Natl. Acad. Sci. USA* 102: 17089–17094.
29. Mizuguchi, H., N. Koizumi, T. Hosono, N. Utoguchi, Y. Watanabe, M. A. Kay, and T. Hayakawa. 2001. A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. *Gene Ther.* 8: 730–735.
30. Mizuguchi, H., and M. A. Kay. 1999. A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum. Gene Ther.* 10: 2013–2017.
31. Maizel, J. V., Jr., D. O. White, and M. D. Scharff. 1968. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 36: 115–125.
32. Yoshimura, A., H. M. Ohishi, D. Aki, and T. Hanada. 2004. Regulation of TLR signaling and inflammation by SOCS family proteins. *J. Leukocyte Biol.* 75: 422–427.
33. Okugawa, S., Y. Ota, T. Kitazawa, K. Nakayama, S. Yanagimoto, K. Tsukada, M. Kawada, and S. Kimura. 2003. Janus kinase 2 is involved in lipopolysaccharide-induced activation of macrophages. *Am. J. Physiol.* 285: C399–C408.
34. Sakurai, H., F. Sakurai, K. Kawabata, T. Sasaki, N. Koizumi, H. Huang, K. Tashiro, S. Kurachi, S. Nakagawa, and H. Mizuguchi. 2007. Comparison of gene expression efficiency and innate immune response induced by Ad vector and lipoplex. *J Control Release* 117: 430–437.
35. Parker, A. L., S. N. Waddington, C. G. Nicol, D. M. Shayakhmetov, S. M. Buckley, L. Denby, G. Kemball-Cook, S. Ni, A. Lieber, J. H. McVey, et al. 2006. Multiple vitamin K-dependent coagulation zymogens promote adenovirus-mediated gene delivery to hepatocytes. *Blood* 108: 2554–2561.
36. Schindler, C., D. E. Levy, and T. Decker. 2007. JAK-STAT signaling: from interferons to cytokines. *J. Biol. Chem.* 282: 20059–20063.
37. Manickan, E., J. S. Smith, J. Tian, T. L. Eggerman, J. N. Lozier, J. Muller, and A. P. Byrnes. 2006. Rapid Kupffer cell death after intravenous injection of adenovirus vectors. *Mol. Ther.* 13: 108–117.
38. Lieber, A., C. Y. He, L. Meuse, D. Schowalter, I. Kirillova, B. Winther, and M. A. Kay. 1997. The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. *J. Virol.* 71: 8798–8807.

©AAI DISTRIBUTION PROHIBITED

ELSEVIER

Available online at www.sciencedirect.com

International Journal of Pharmaceutics xxx (2007) xxx–xxx

INTERNATIONAL JOURNAL OF PHARMACEUTICS

www.elsevier.com/locate/ijpharm

Mini review

Innate immune response induced by gene delivery vectors

Haruna Sakurai [a,b], Kenji Kawabata [a], Fuminori Sakurai [a], Shinsaku Nakagawa [b], Hiroyuki Mizuguchi [a,b,*]

[a] *Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka, Japan*
[b] *Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan*

Received 6 June 2007; received in revised form 6 June 2007; accepted 11 June 2007

Abstract

Gene therapy is a clinical strategy that has the potential to treat an array of genetic and nongenetic diseases. Vectors for gene transfer are the essential tools of gene therapy. For gene therapy to be successful, an appropriate amount of the therapeutic gene must be delivered into the target cells without substantial toxicity. A major limitation of the use of gene therapy vectors is the innate immune responses triggered by systemic administration of such vectors. It is essential to overcome vector-mediated innate immune responses, such as production of inflammatory cytokines, the maturation of antigen-presenting cells and tissue damage, because the induction of these responses not only shortens the period of gene expression but also leads to serious side effects. Viral vectors (for example, adenovirus (Ad) vectors) have been assumed to be more potent in inducing innate immune responses in spite of their high transduction efficiency since they contain pathogenic proteins. However, recent studies have demonstrated that not only viral vectors but also nonviral vectors, such as lipoplex (liposome/plasmid DNA complex), can induce innate immune responses. Indeed, nonviral vectors including lipoplex induce comparable or larger levels of innate immune response than viral vectors. In this review, we present an overview of the innate immune responses induced by Ad vector and lipoplex, which are used primarily for *in vivo* gene transfer.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Adenovirus vector; Lipoplex; Innate immunity

1. Introduction

Gene therapy provides new hope as a therapeutic method for both genetic and nongenetic diseases. Various types of gene delivery vectors have been developed to improve the efficiency of *in vivo* gene expression, and have been employed in many clinical trials. The vectors for gene delivery are usually classified as viral or nonviral vectors. Viral vectors, at present, dominate in clinical trials because they are highly efficient in transducing cells; however, viral vectors are immunogenic and potentially mutagenic. In 1999, there was a fatal accident in Pennsylvania caused by the systemic administration of adenovirus (Ad) vector (Marshall, 1999; Raper et al., 2003). This accident was due to the over-activation of innate immunity triggered by the injection of heavy doses of Ad vectors. Thus, the immune response induced by gene therapy vectors is a significant problem, which must be overcome (Marshall, 1999).

The systemic administration of Ad vectors induces both innate and adaptive immune responses with its humoral and cell-mediated components. In the case of adaptive immune response, capsid antigens are largely responsible for specific immunity toward Ad vectors. In the first generation Ad vector lacking the E1 gene, leaky expression of viral genes from the vector stimulates an immune response against Ad vector-transduced cells (Yang et al., 1994; Yang et al., 1995). The cytotoxic T lymphocyte (CTL) response can be elicited against viral gene product and/or transgene products expressed in the transduced cells. To reduce cell-mediated immune response against viral gene products expressed in the transduced cells, "helper-dependent (HD)" or "gutted" Ad vectors, in which all viral genes are deleted except the inverted terminal repeat (ITR) sequences at both ends and the packaging signal, have been developed. The deletion of all viral protein-coding regions from the Ad genome improves the prospects of Ad vectors for long-term gene expression,

* Corresponding author at: Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. Tel.: +81 72 641 9815; fax: +81 72 641 9816.
E-mail address: mizuguch@nibio.go.jp (H. Mizuguchi).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.06.012

suggesting that immunogenic toxicities induced by HD Ad vectors are greatly reduced (Palmer and Ng, 2005). Humoral virus-neutralizing antibody responses against the Ad capsid itself are another limitation, preventing transduction upon the subsequent administration of vectors of the same serotype. Because hexons are primarily targeted by neutralizing antibodies, hexon modification has been reported to allow for escape from neutralizing antibodies (Roberts et al., 2006). As other strategies, Ad vectors belonging to subgroups other than Ad type 5, such as Ad type 11 or 35, or to species other than human, have also been developed (Farina et al., 2001; Sakurai et al., 2003a; Seshidhar Reddy et al., 2003; Vogels et al., 2003; Holterman et al., 2004). Compared with adaptive immunity to Ad vectors, the mechanism of innate immune response is less understood. It is essential to elucidate the complete mechanism of Ad vector-mediated innate immune response in order to develop safe Ad vectors.

Nonviral vectors have recently gained increasing attention since they do not contain any pathogenic proteins and are therefore more likely to be safe (Niidome and Huang, 2002). However, Li et al. demonstrated that the systemic administration of lipoplex induces high levels of inflammatory cytokines (Li et al., 1999). The production of inflammatory cytokines mediates the suppression of gene expression and tissue damage (Qin et al., 1997; Loisel et al., 2001; Sellins et al., 2005). Thus, innate immune response is the most important problem to be overcome for both viral and nonviral vectors. In this paper, we review innate immune response induced by Ad vector and lipoplex, which are used for *in vivo* gene transfer in contrast with other vector systems.

2. Innate immune response to Ad vectors

Ad is a nonenveloped virus containing an icosahedral protein capsid with a diameter of approximately 80 nm (Shenk, 2001). At least 51 serotypes of human Ad have been identified, and Ad serotype 5 (Ad5) and Ad2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. Ad vectors are the most efficient class of vector in terms of delivering genes into both dividing and non-dividing cells. They have a large packaging ability for the incorporation of foreign genes and can easily be grown to high titers (Wilson, 1996). Additionally, they can transduce foreign genes efficiently into both cultured cells *in vitro* and many target organs *in vivo*. These advantageous features lead to increasing numbers of clinical applications for Ad vectors. By July of 2006, Ad vectors had been used in 26% of all gene therapy protocols (out of a total of 305 protocols) in gene therapy (Journal of Gene Medicine, Website http://www.wiley.co.uk/genmed/clinical/) worldwide. Systemic Ad vector application, however, is limited due to its activation of cellular, humoral and innate immune responses (Schnell et al., 2001; Zhang et al., 2001; Liu et al., 2003; Muruve, 2004; Xu et al., 2005). Among these, innate immune response against Ad vectors is the most poorly understood. It is essential to clarify the mechanism of innate immune response triggered by the systemic administration of Ad vectors in order to achieve a safe method of gene therapy using Ad vectors.

2.1. Origin of cytokine/chemokine production induced by Ad vectors

The intravenous injection of Ad vectors results in the immediate production (1–6 h post-injection) of cytokines/chemokines (De Geest et al., 2005; Shayakhmetov et al., 2005; Hartman et al., 2007b; Kiang et al., 2006; Koizumi et al., 2006, 2007; Manickan et al., 2006; Sakurai et al., 2007; Yamaguchi et al., 2007). As shown in Table 1, various kinds of cytokines/chemokines are released by the systemic administration of Ad vector.

Intravenously injected Ad vectors are delivered primarily to the liver and spleen. In the liver, Ad vector is likely to be equally distributed to the parenchymal (hepatocytes) and non-parenchymal (Kupffer and endothelial) cells, depending on the dose injected (Koizumi et al., 2003, 2006). Since these tissues contain many immune cells including liver Kupffer cells, splenic dendritic cells (DCs) and macrophages, these cells are assumed to be responsible for the production of inflammatory cytokines.

The depletion of Kupffer cells in mice by intravenous injection of gadolinium chloride ($GdCl_3$) eliminates the Ad vector-induced release of tumor necrosis factor (TNF)-α, but does not suppress the production of interleukin (IL)-6, suggesting that there might be other sites of inflammatory cytokine production (Lieber et al., 1997). The depletion of DCs and tissue macrophages in mice by intravenous injection of liposomes encapsulating dichloromethylene-bisphosphonate (Cl_2MDP) results in a marked inhibition of IL-6 and IL-12 production (Zhang et al., 2001). Splenic DCs and macrophages isolated from Ad vector-injected mice secrete high levels of inflammatory cytokines (Zhang et al., 2001). When the mice are splenectomized, IL-6 production is decreased upon Ad vector injection (De Geest et al., 2005). Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis of the liver and spleen after systemic Ad injection suggests that the spleen, but not the liver, is a major site of inflammatory cytokine production (Koizumi et al., 2007). These results indicate that immune cells in the spleen should be a major source of inflammatory cytokine production.

Excessive complement activation has also been reported to be involved in Ad vector-mediated innate immune responses (Kiang et al., 2006). Ad vectors bind to blood factors, such as factor IX, factor X and lactoferrin, leading to liver transduction and hepatotoxicity (Shayakhmetov et al., 2005; Johansson et al., 2007; Parker et al., 2006). The interaction of Ad vector with blood factors might also play a role in the induction of innate immune responses.

2.2. Intracellular mechanism of cytokine/chemokine production

Toll-like receptors (TLRs), which sense specific molecular patterns present in microbial components, are major receptors involved in the activation of innate immune response. Following the recognition of microbial components by TLRs, they, except for TLR3, transduce intracellular signaling through the adaptor protein, myeloid differentiation primary response gene 88 (MyD88), leading to the production of inflammatory cytokines

Please cite this article in press as: Sakurai, H. et al., Innate immune response induced by gene delivery vectors, Int. J. Pharmaceut. (2007), doi:10.1016/j.ijpharm.2007.06.012

Table 1
Level of cytokine production *in vivo* by systemic administration of Ad vector or lipoplex

Vector	Strain of mice used	Injected dose of vectors (/mouse)	Peak level of cytokine productions (pg/ml)			Other cytokines/chemokines determined	Reference
			IL-6	IL-12	TNF-α		
Ad-hAAT	C3H/HeJ	10^{10} VP	1300	–	1250	–	Lieber et al. (1997)
Ad-LacZ	DBA/2	10^{8-9} PFU	–	–	–	MIP-2, IL-10, RANTES, MCP-1	Muruve et al. (1999)
Ad-LacZ	C57BL/6	3×10^{11} VP	1200	1000	–	–	Zhang et al. (2001)
Ad-LacZ	DBA/2	10^{11} VP	–	–	–	IP-10, MIP-2	Tibbles et al. (2002)
Ad-AT_4	C57BL/6	10^{11} VP	2500	–	–	–	De Geest et al. (2005)
Ad-GFP	C57BL/6	10^{11} VP	1700	–	–	IFN-γ	Shayakhmetov et al. (2005)
Ad-LacZ	C57/BL6J	1.5×10^{11} VP	1000	1200	–	CXCL1, MIP-1α, MCP-1, RANTES, IL-5	Hartman et al. (2007b)
Ad'Luc	C57BL/6	10^{11} VP	600	–	–	–	Koizumi et al. (2006)
Ad-LacZ	C57BL/6	1.5×10^{11} VP	1200	1000	–	CXCL1, IFN-γ, RANTES, IL-1β	Kiang et al. (2006)
Ad-GFP	C57BL/6Ncr	10^{11} VP	5000	1000	–	–	Manickan et al. (2006)
Ad'Luc	C57BL/6	10^{11} VP	800	800	ND	MIP-2, IFN-α, IFN-β, IFN-γ	Koizumi et al. (2007)
Ad'Luc	C57BL/6	5×10^{10} VP	500	1000	ND	–	Sakurai et al. (2007)
Ad-Luc	C57BL/6	3×10^{10} VP	200	–	–	–	Yamaguchi et al. (2007)
Liposome–protamine–DNA complex	CD-1	50 μg DNA	–	–	20000	IFN-γ	Li et al. (1999)
Liposome–protamine–DNA complex	C57BL/6	25 μg DNA	–	1200	5000	IFN-γ	Whitmore et al. (1999)
Lipoplex	Swiss mouse	50 μg DNA	4500	–	350	IFN-γ	Loisel et al. (2001)
Lipoplex	CDF1	25 μg DNA	–	3000	7000	IFN-γ	Sakurai et al. (2002)
Lipoplex	CDF1	25 μg DNA	–	–	900	IFN-γ	Sakurai et al. (2003a,b)
Lipoplex	CD-1	25 μg DNA	–	2000	1500	IFN-γ	Liu et al. (2004)
Lipoplex	C57BL/6	33 μg DNA	10000	250	300	MIP-1α, RANTES, IL-10, IFN-γ	Zhao et al. (2004)
Lipoplex	BALB/c	25 μg DNA	–	–	500	–	Kuramoto et al. (2006)
Lipoplex	C57BL/6	25 μg DNA	800	3000	600	–	Sakurai et al. (2007)

VP: viral particle; PFU: plaque forming unit; ND: not detectable.

and interferons (IFNs) by activating nuclear factor kappa B (NF-κB) and interferon regulatory factors (IRFs), respectively (Hemmi et al., 2000; Zhao et al., 2004; Kawai and Akira, 2006) (Fig. 1).

Recently, it has become clear that DCs are divided into two major subsets, conventional DCs (cDCs) and plasmacytoid DCs (pDCs); the former play a role as professional antigen-presenting cells, while the latter act as major type I IFN producers in viral infection (Colonna et al., 2004; Yoneyama et al., 2004, 2005). The stimulation of bone marrow precursors *in vitro* with Flt3-ligand leads to differentiation into both cDCs (Flt3L-cDCs) and pDCs (Flt3L-pDCs). IL-6 production in Flt3L-cDCs is TLR9/MyD88-dependent, while type I IFN production is TLR9-indepedent (Basner-Tschakarjan et al., 2006; Yamaguchi et al., 2007). On the other hand, IL-6 production in Flt3L-pDCs and peripheral macrophages by Ad vectors occurs in a TLR9/MyD88-independent manner (Yamaguchi et al., 2007). These results suggest that the recognition of Ad vector by immune cells occurs not only in a TLR-dependent manner, but also in a TLR-independent manner (Fig. 1). The specific sensor receptor and/or signaling pathway used for the activation of innate immune responses to Ad vector might depend on the type of cell. In contrast to the MyD88-dependent IL-6 production in cDCs, TLR9- or MyD88-deficient mice show no decrease in serum IL-6 levels after Ad vector administration (Yamaguchi et al., 2007), suggesting that not only DCs, but also other kinds of cells such as macrophages and endothelial cells might also produce cytokines *in vivo*.

The activation of intracellular signaling for cytokine production by Ad vector has also been studied in nonimmune cells such as HeLa cells and A549 cells (Bruder and Kovesdi, 1997; Bowen et al., 2002; Hartman et al., 2007a). The activation of the Raf/mitogen-activated protein kinase (MAPK) pathway by Ad vectors results in the production of IL-8 or IP-10 in nonimmune cells (Bruder and Kovesdi, 1997; Tibbles et al., 2002). The activation of Akt/protein kinase B, protein kinase A (PKA) and the p38/MAPK pathway are also involved in the production of inflammatory cytokines (Suomalainen et al., 2001; Liu et al., 2005). The Ad vector-mediated production of inflammatory cytokines/chemokines in nonimmune cells is associated with NF-κB activation, as in the case of immune cells (Borgland et al., 2000; Morelli et al., 2000; Bowen et al., 2002; Liu et al., 2005; Hartman et al., 2007a). Taken together, these findings suggest that nonimmune cells might also be involved in the innate immune response induced by the systemic administration of Ad vector.

Please cite this article in press as: Sakurai, H. et al., Innate immune response induced by gene delivery vectors, Int. J. Pharmaceut. (2007), doi:10.1016/j.ijpharm.2007.06.012

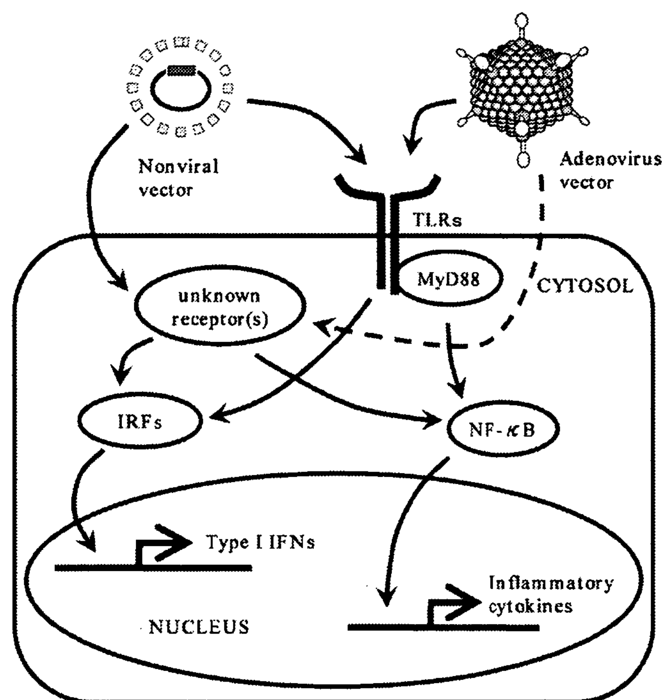

Fig. 1. General scheme of Ad vector- or nonviral vector-induced inflammatory gene expression in immune cells. TLR: toll-like receptor; MyD88: myeloid differentiation primary response gene 88; IRF: interferon regulatory factor; NF-κB: nuclear factor kappa B; IFN: interferon.

2.3. Development of an improved Ad vector which induces less innate immune response

An understanding of the biology of host response to Ad vectors will impact the design of future generations of these agents by allowing researchers to focus on reducing their immunogenicity and improving their safety. To reduce the production of inflammatory cytokines by the systemic administration of Ad vectors, we and others have developed new types of Ad vectors (De Geest et al., 2005; Koizumi et al., 2006, 2007). The non-specific distribution of Ad vectors in tissue after *in vivo* gene transfer is due to the relatively broad expression of coxsackievirus and adenovirus receptor (CAR) (the primary receptor), α_v integrin (the secondary receptor) and heparan sulfate (the tertiary receptor). The modified Ad vector ablated for CAR, α_v integrin and heparan sulfate binding, which we have named "triple mutant Ad vector", reduces cytokine production *in vivo*, suggesting that the binding of Ad vector with these receptors might be involved in the induction of innate immune response, although the mechanism behind this remains unknown (Koizumi et al., 2006). The fiber-modified Ad vector containing a stretch of lysine residues (K7 (KKKKKKK) peptide), AdK7, decreases the production of inflammatory cytokines (IL-6, macrophage inflammatory protein (MIP)-2 and IFN-γ, but not TNF-α, regulated on activation, normal T cell expressed and secreted (RANTES), IFN-α and IFN-β) due to the reduced spleen distribution of AdK7 compared with that with conventional Ad (Koizumi et al., 2007). Since the interaction of fiber with CAR is reported to be responsible for cytokine production in nonimmune cells (Liu et al., 2003; Tamanini et al., 2006), the substitution of Ad5 fiber for the fiber of other types of Ad vectors which do not bind to CAR, such as Ad7, Ad35 and Ad41, is another strategy for reducing innate immune responses (Shayakhmetov et al., 2004; Schoggins et al., 2005; Ni et al., in press). The intravenous injection of modified Ad vector containing Ad type 35 fiber shows lower IL-6 and TNF-α levels compared to the injection of conventional Ad vector (Shayakhmetov et al., 2004; Ni et al., in press), suggesting that CAR-binding activity might participate in the activation of innate immune responses *in vivo*. Another approach is to modify Ad vector with monomethoxypolyethylene glycol (MPEG). PEGylation reduces vector uptake in the spleen, resulting in the suppression of cytokine production (De Geest et al., 2005). The development of improved Ad vectors targeting a specific tissue or cell type with reduced distribution to immune cells is an important approach to avoid the induction of innate immune responses (Mizuguchi and Hayakawa, 2004).

3. Innate immune response to lipoplex

Although viral vectors have high transduction efficiency, safety concerns regarding their use in humans make nonviral vectors an attractive alternative. Cationic liposome has proven to be a useful tool for the delivery of genes into cells in nonviral forms (Felgner et al., 1987, 1995; Zhu et al., 1993; Liu et al., 1995). Nonviral vectors have advantages with respect to simplicity of use, ease of large-scale production, and lack of specific immune response (Liu and Huang, 2002). Genetic immunization with plasmid DNA vaccines has proven to be a promising tool in conferring protective immunity in various experimental animal models of infectious diseases or tumors, however this indicates that plasmid DNA has the ability to induce immune responses (Sakurai et al., 2003b; Prud'homme, 2005; Bolesta et al., 2006). As in the case of viral vectors, innate immune responses and tissue damages are induced by the systemic injection of lipoplex even though it contains no viral components (Li et al., 1999; Whitmore et al., 1999; Loisel et al., 2001; Sakurai et al., 2002, 2003b, 2007; Liu et al., 2004; Zhao et al., 2004; Kuramoto et al., 2006). The systemic administration of cationic polymer/plasmid DNA complex (polyplex) is also known induce innate immune response (Gautam et al., 2001).

The deletion of macrophages in tissue by intravenous injection of $GdCl_3$ decreases the production of TNF-α and IL-12 by lipoplex, suggesting that tissue macrophages containing liver Kupffer cells and spleen macrophages are closely involved in inflammatory cytokine production following the systemic administration of lipoplex (Sakurai et al., 2002). The trigger of the innate immune response is likely to be the bacterial origin of the plasmid DNA, which is incorporated in the lipoplex. Hemmi et al. report that bacterial DNA, such as plasmid DNA, is recognized by TLR 9 (Hemmi et al., 2000). Plasmid DNA and bacterial DNA contains a much higher frequency of unmethylated CpG motifs (also known as immunostimulatory CpG motifs) than does mammalian DNA (Scheule, 2000; Zhao et al., 2004; Yasuda et al., 2005). The production of inflammatory cytokines induced by the systemic administration of lipoplex is greatly, but not completely, suppressed in $TLR9^{-/-}$ mice (Zhao

Please cite this article in press as: Sakurai, H. et al., Innate immune response induced by gene delivery vectors, Int. J. Pharmaceut. (2007), doi:10.1016/j.ijpharm.2007.06.012

et al., 2004), indicating that the recognition of CpG motifs in plasmid DNA by TLR9 is crucial for the induction of innate immune responses induced by lipoplex. In contrast, the absence of CpG motif in plasmid DNA greatly reduces cytokine production, although it also does not completely eliminate it (Sakurai et al., 2007). Another study has shown that the methylation of CpG motifs in plasmid DNA partly suppresses the production of inflammatory cytokines (Whitmore et al., 1999). These findings indicate that the interaction of the CpG motifs in the plasmid DNA with TLR9 plays a role in the innate immune responses, but that there is another as yet unknown mechanism underlying the induction of innate immune responses, independently of the CpG motifs. Recently, Ishii et al. reported that double-stranded B-form DNA triggers the production of type I IFNs and chemokines through a TLR-independent mechanism (Ishii et al., 2006). They suggest that there is (are) unknown receptor(s) in cytoplasm for the recognition of DNA, leading to the activation of innate immune responses (Fig. 1). The identification of these unknown receptors which sense foreign DNAs would provide a strategy for reducing the innate immune response induced by lipoplex.

As a strategy to reduce the innate immune response induced by lipoplex, Liu et al. developed a new type of lipoplex, safeplex, which efficiently delivers genes with less induction of innate immune response by co-delivering DNA and the inflammatory suppressor dexamethasone (Liu et al., 2004). Recent reports demonstrate that lipopolysaccharide-induced TNF-α production is suppressed with the pre-injection of NF-κB decoy, whose double-stranded oligonucleotides contain an NF-κB binding sequence (Higuchi et al., 2005, 2006), suggesting that NF-κB decoy might be another suppressor of lipoplex-induced cytokine production.

As described above, both Ad vector and lipoplex have the ability to activate innate immune responses by systemic administration of the vectors. Table 1 shows the levels of cytokine production induced by Ad vector or lipoplex. These results clearly indicate that the induction of inflammatory cytokines, such as IL-6, IL-12 and TNF-α, is greater when lipoplex is injected, than with Ad vector, even though it is commonly believed that nonviral vectors are safer to use in gene therapy than viral vectors. Thus, it is essential to pay close attention to the innate immune responses induced by nonviral vectors as well as to those induced by viral vectors.

4. Conclusion

To achieve the desired therapeutic effects, gene therapy vectors must be able to safely deliver genes of interest to the designated target and to ensure their safe expression for an appropriate amount of time. Recently, improved Ad vectors and lipoplex have been developed to decrease inflammatory toxicity. However, those new vectors do not completely suppress the induction of the innate immune responses that may occur with the systemic administration of the vectors. A greater understanding of the mechanism of induction of innate immune responses by gene therapy vectors is essential for the development of next-generation safe gene therapy vectors.

Acknowledgement

This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan.

References

Basner-Tschakarjan, E., Gaffal, E., O'Keeffe, M., Tormo, D., Limmer, A., Wagner, H., Hochrein, H., Tuting, T., 2006. Adenovirus efficiently transduces plasmacytoid dendritic cells resulting in TLR9-dependent maturation and IFN-alpha production. J. Gene. Med. 8, 1300–1306.

Bolesta, E., Kowalczyk, A., Wierzbicki, A., Eppolito, C., Kaneko, Y., Takiguchi, M., Stamatatos, L., Shrikant, P.A., Kozbor, D., 2006. Increased level and longevity of protective immune responses induced by DNA vaccine expressing the HIV-1 Env glycoprotein when combined with IL-21 and IL-15 gene delivery. J. Immunol. 177, 177–191.

Borgland, S.L., Bowen, G.P., Wong, N.C., Libermann, T.A., Muruve, D.A., 2000. Adenovirus vector-induced expression of the C–X–C chemokine IP-10 is mediated through capsid-dependent activation of NF-kappaB. J. Virol. 74, 3941–3947.

Bowen, G.P., Borgland, S.L., Lam, M., Libermann, T.A., Wong, N.C., Muruve, D.A., 2002. Adenovirus vector-induced inflammation: capsid-dependent induction of the C–C chemokine RANTES requires NF-kappaB. Hum. Gene. Ther. 13, 367–379.

Bruder, J.T., Kovesdi, I., 1997. Adenovirus infection stimulates the Raf/MAPK signaling pathway and induces interleukin-8 expression. J. Virol. 71, 398–404.

Colonna, M., Trinchieri, G., Liu, Y.J., 2004. Plasmacytoid dendritic cells in immunity. Nat. Immunol. 5, 1219–1226.

De Geest, B., Snoeys, J., Van Linthout, S., Lievens, J., Collen, D., 2005. Elimination of innate immune responses and liver inflammation by PEGylation of adenoviral vectors and methylprednisolone. Hum. Gene. Ther. 16, 1439–1451.

Farina, S.F., Gao, G.P., Xiang, Z.Q., Rux, J.J., Burnett, R.M., Alvira, M.R., Marsh, J., Ertl, H.C., Wilson, J.M., 2001. Replication-defective vector based on a chimpanzee adenovirus. J. Virol. 75, 11603–11613.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U.S.A. 84, 7413–7417.

Felgner, P.L., Tsai, Y.J., Sukhu, L., Wheeler, C.J., Manthorpe, M., Marshall, J., Cheng, S.H., 1995. Improved cationic lipid formulations for in vivo gene therapy. Ann. NY Acad. Sci. 772, 126–139.

Gautam, A., Densmore, C.L., Waldrep, J.C., 2001. Pulmonary cytokine responses associated with PEI-DNA aerosol gene therapy. Gene. Ther. 8, 254–257.

Hartman, Z.C., Black, E.P., Amalfitano, A., 2007a. Adenoviral infection induces a multi-faceted innate cellular immune response that is mediated by the toll-like receptor pathway in A549 cells. Virology 358, 357–372.

Hartman, Z.C., Kiang, A., Everett, R.S., Serra, D., Yang, X.Y., Clay, T.M., Amalfitano, A., 2007b. Adenovirus infection triggers a rapid, MyD88 regulated, transcriptome response critical to acute phase and adaptive immune responses in vivo. J. Virol. 81, 1796–1812.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S., 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408, 740–745.

Higuchi, Y., Kawakami, S., Nishikawa, M., Yamashita, F., Hashida, M., 2005. Intracellular distribution of NFkappaB decoy and its inhibitory effect on TNFalpha production by LPS stimulated RAW 264.7 cells. J. Control. Release 107, 373–382.

Higuchi, Y., Kawakami, S., Oka, M., Yamashita, F., Hashida, M., 2006. Suppression of TNFalpha production in LPS induced liver failure in mice after intravenous injection of cationic liposomes/NFkappaB decoy complex. Pharmazie 61, 144–147.

Holterman, L., Vogels, R., van der Vlugt, R., Sieuwerts, M., Grimbergen, J., Kaspers, J., Geelen, E., van der Helm, E., Lemckert, A., Gillissen, G., Verhaagh, S., Custers, J., Zuijdgeest, D., Berkhout, B., Bakker, M., Quax, P.,

Goudsmit, J., Havenga, M., 2004. Novel replication-incompetent vector derived from adenovirus type 11 (Ad11) for vaccination and gene therapy: low seroprevalence and non-cross-reactivity with Ad5. J. Virol. 78, 13207–13215.
Ishii, K.J., Coban, C., Kato, H., Takahashi, K., Torii, Y., Takeshita, F., Ludwig, H., Sutter, G., Suzuki, K., Hemmi, H., Sato, S., Yamamoto, M., Uematsu, S., Kawai, T., Takeuchi, O., Akira, S., 2006. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nat. Immunol. 7, 40–48.
Johansson, C., Jonsson, M., Marttila, M., Persson, D., Fan, X., Skog, J., Frangsmyr, L., Wadell, G., Arnberg, N., 2007. Adenoviruses use lactoferrin as a bridge for CAR-independent binding to and infection of epithelial cells. J. Virol 81, 954–963.
Kawai, T., Akira, S., 2006. Innate immune recognition of viral infection. Nat. Immunol. 7, 131–137.
Kiang, A., Hartman, Z.C., Everett, R.S., Serra, D., Jiang, H., Frank, M.M., Amalfitano, A., 2006. Multiple innate inflammatory responses induced after systemic adenovirus vector delivery depend on a functional complement system. Mol. Ther. 14, 588–598.
Koizumi, N., Mizuguchi, H., Sakurai, F., Yamaguchi, T., Watanabe, Y., Hayakawa, T., 2003. Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and alphav integrin-binding ablation. J. Virol. 77, 13062–13072.
Koizumi, N., Kawabata, K., Sakurai, F., Watanabe, Y., Hayakawa, T., Mizuguchi, H., 2006. Modified adenoviral vectors ablated for coxsackievirus-adenovirus receptor, alphav integrin, and heparan sulfate binding reduce in vivo tissue transduction and toxicity. Hum. Gene. Ther. 17, 264–279.
Koizumi, N., Yamaguchi, T., Kawabata, K., Sakurai, F.T.S., Watanabe, Y., Hayakawa, T., Mizuguchi, H., 2007. Fiber-modified adenovirus vectors decrease liver toxicity through reduced interleukin 6 production. J. Immunol. 178, 1767–1773.
Kuramoto, T., Nishikawa, M., Thanaketpaisarn, O., Okabe, T., Yamashita, F., Hashida, M., 2006. Use of lipoplex-induced nuclear factor-kappaB activation to enhance transgene expression by lipoplex in mouse lung. J. Gene. Med. 8, 53–62.
Li, S., Wu, S.P., Whitmore, M., Loeffert, E.J., Wang, L., Watkins, S.C., Pitt, B.R., Huang, L., 1999. Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors. Am. J. Physiol. 276, L796–L804.
Lieber, A., He, C.Y., Meuse, L., Schowalter, D., Kirillova, I., Winther, B., Kay, M.A., 1997. The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. J. Virol. 71, 8798–8807.
Liu, F., Huang, L., 2002. Development of non-viral vectors for systemic gene delivery. J. Control. Release. 78, 259–266.
Liu, F., Shollenberger, L.M., Huang, L., 2004. Non-immunostimulatory nonviral vectors. FASEB J. 18, 1779–1781.
Liu, Q., Zaiss, A.K., Colarusso, P., Patel, K., Haljan, G., Wickham, T.J., Muruve, D.A., 2003. The role of capsid–endothelial interactions in the innate immune response to adenovirus vectors. Hum. Gene. Ther. 14, 627–643.
Liu, Q., White, L.R., Clark, S.A., Heffner, D.J., Winston, B.W., Tibbles, L.A., Muruve, D.A., 2005. Akt/protein kinase B activation by adenovirus vectors contributes to NFkappaB-dependent CXCL10 expression. J. Virol. 79, 14507–14515.
Liu, Y., Liggitt, D., Zhong, W., Tu, G., Gaensler, K., Debs, R., 1995. Cationic liposome-mediated intravenous gene delivery. J. Biol. Chem. 270, 24864–24870.
Loisel, S., Le Gall, C., Doucet, L., Ferec, C., Floch, V., 2001. Contribution of plasmid DNA to hepatotoxicity after systemic administration of lipoplexes. Hum. Gene. Ther. 12, 685–696.
Manickan, E., Smith, J.S., Tian, J., Eggerman, T.L., Lozier, J.N., Muller, J., Byrnes, A.P., 2006. Rapid Kupffer cell death after intravenous injection of adenovirus vectors. Mol. Ther. 13, 108–117.
Marshall, E., 1999. Gene therapy death prompts review of adenovirus vector. Science 286, 2244–2245.
Mizuguchi, H., Hayakawa, T., 2004. Targeted adenovirus vectors. Hum. Gene. Ther. 15, 1034–1044.
Morelli, A.E., Larregina, A.T., Ganster, R.W., Zahorchak, A.F., Plowey, J.M., Takayama, T., Logar, A.J., Robbins, P.D., Falo, L.D., Thomson, A.W., 2000. Recombinant adenovirus induces maturation of dendritic cells via an NF-kappaB-dependent pathway. J. Virol. 74, 9617–9628.
Muruve, D.A., 2004. The innate immune response to adenovirus vectors. Hum. Gene. Ther. 15, 1157–1166.
Muruve, D.A., Barnes, M.J., Stillman, I.E., Liberman, T.A., 1999. Adenoviral gene delivery leads to rapid induction of multiple chemokines and acute neutrophil dependent hepatic injury in vivo. Hum. Gen. Ther. 10, 965–976.
Ni, S., Gaggar, A., Paolo, N.D., Li, Z., Liu, Y., Strauss, R., Sova, P., Morihara, J., Feng, Q., Kiviat, N., Toure, P., Sow, P., Lieber, A., in press. Evaluation of adenovirus vectors containing serotype 35 fibers for tumor targeting. Cancer Gene. Ther.
Niidome, T., Huang, L., 2002. Gene therapy progress and prospects: nonviral vectors. Gene. Ther. 9, 1647–1652.
Palmer, D.J., Ng, P., 2005. Helper-dependent adenoviral vectors for gene therapy. Hum. Gene. Ther. 16, 1–16.
Parker, A.L., Waddington, S.N., Nicol, C.G., Shayakhmetov, D.M., Buckley, S.M., Denby, L., Kemball-Cook, G., Ni, S., Lieber, A., McVey, J.H., Nicklin, S.A., Baker, A.H., 2006. Multiple vitamin K-dependent coagulation zymogens promote adenovirus-mediated gene delivery to hepatocytes. Blood 108, 2554–2561.
Prud'homme, G.J., 2005. DNA vaccination against tumors. J. Gene. Med. 7, 3–17.
Qin, L., Ding, Y., Pahud, D.R., Chang, E., Imperiale, M.J., Bromberg, J.S., 1997. Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. Hum. Gene. Ther. 8, 2019–2029.
Raper, S.E., Chirmule, N., Lee, F.S., Wivel, N.A., Bagg, A., Gao, G.P., Wilson, J.M., Batshaw, M.L., 2003. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol. Genet. Metab. 80, 148–158.
Roberts, D.M., Nanda, A., Havenga, M.J., Abbink, P., Lynch, D.M., Ewald, B.A., Liu, J., Thorner, A.R., Swanson, P.E., Gorgone, D.A., Lifton, M.A., Lemckert, A.A., Holterman, L., Chen, B., Dilraj, A., Carville, A., Mansfield, K.G., Goudsmit, J., Barouch, D.H., 2006. Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. Nature 441, 239–243.
Sakurai, F., Terada, T., Yasuda, K., Yamashita, F., Takakura, Y., Hashida, M., 2002. The role of tissue macrophages in the induction of proinflammatory cytokine production following intravenous injection of lipoplexes. Gene. Ther. 9, 1120–1126.
Sakurai, F., Mizuguchi, H., Hayakawa, T., 2003a. Efficient gene transfer into human CD34+ cells by an adenovirus type 35 vector. Gene. Ther. 10, 1041–1048.
Sakurai, F., Terada, T., Maruyama, M., Watanabe, Y., Yamashita, F., Takakura, Y., Hashida, M., 2003b. Therapeutic effect of intravenous delivery of lipoplexes containing the interferon-beta gene and poly I: poly C in a murine lung metastasis model. Cancer Gene. Ther. 10, 661–668.
Sakurai, H., Sakurai, F., Kawabata, K., Sasaki, T., Koizumi, N., Huang, H., Tashiro, K., Kurachi, S., Nakagawa, S., Mizuguchi, H., 2007. Comparison of gene expression efficiency and innate immune response induced by Ad vector and lipoplex. J. Control. Release. 117, 430–437.
Scheule, R.K., 2000. The role of CpG motifs in immunostimulation and gene therapy. Adv. Drug Deliv. Rev. 44, 119–134.
Schnell, M.A., Zhang, Y., Tazelaar, J., Gao, G.P., Yu, Q.C., Qian, R., Chen, S.J., Varnavski, A.N., LeClair, C., Raper, S.E., Wilson, J.M., 2001. Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. Mol. Ther. 3, 708–722.
Schoggins, J.W., Nociari, M., Philpott, N., Falck-Pedersen, E., 2005. Influence of fiber detargeting on adenovirus-mediated innate and adaptive immune activation. J. Virol. 79, 11627–11637.
Sellins, K., Fradkin, L., Liggitt, D., Dow, S., 2005. Type I interferons potently suppress gene expression following gene delivery using liposome(−)DNA complexes. Mol. Ther. 12, 451–459.
Seshidhar Reddy, P., Ganesh, S., Limbach, M.P., Brann, T., Pinkstaff, A., Kaloss, M., Kaleko, M., Connelly, S., 2003. Development of adenovirus serotype 35 as a gene transfer vector. Virology 311, 384–393.

Shayakhmetov, D.M., Li, Z.Y., Ni, S., Lieber, A., 2004. Analysis of adenovirus sequestration in the liver, transduction of hepatic cells, and innate toxicity after injection of fiber-modified vectors. J. Virol. 78, 5368–5381.

Shayakhmetov, D.M., Gaggar, A., Ni, S., Li, Z.Y., Lieber, A., 2005. Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. J. Virol. 79, 7478–7491.

Shenk, T.E., 2001. In: Howley, D.M.Ka.P.M. (Ed.), Adenoviridae: The Viruses and their Replication, vol. 2. Lippincott Williams & Wilkins, Philadelphia, pp. 2265–2300.

Suomalainen, M., Nakano, M.Y., Boucke, K., Keller, S., Greber, U.F., 2001. Adenovirus-activated PKA and p38/MAPK pathways boost microtubule-mediated nuclear targeting of virus. EMBO J. 20, 1310–1319.

Tamanini, A., Nicolis, E., Bonizzato, A., Bezzerri, V., Melotti, P., Assael, B.M., Cabrini, G., 2006. Interaction of adenovirus type 5 fiber with the coxsackievirus and adenovirus receptor activates inflammatory response in human respiratory cells. J. Virol. 80, 11241–11254.

Tibbles, L.A., Spurrell, J.C., Bowen, G.P., Liu, Q., Lam, M., Zaiss, A.K., Robbins, S.M., Hollenberg, M.D., Wickham, T.J., Muruve, D.A., 2002. Activation of p38 and ERK signaling during adenovirus vector cell entry lead to expression of the C–X–C chemokine IP-10. J. Virol. 76, 1559–1568.

Vogels, R., Zuijdgeest, D., van Rijnsoever, R., Hartkoorn, E., Damen, I., de Bethune, M.P., Kostense, S., Penders, G., Helmus, N., Koudstaal, W., Cecchini, M., Wetterwald, A., Sprangers, M., Lemckert, A., Ophorst, O., Koel, B., van Meerendonk, M., Quax, P., Panitti, L., Grimbergen, J., Bout, A., Goudsmit, J., Havenga, M., 2003. Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. J. Virol. 77, 8263–8271.

Whitmore, M., Li, S., Huang, L., 1999. LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth. Gene. Ther. 6, 1867–1875.

Wilson, J.M., 1996. Adenoviruses as gene-delivery vehicles. N. Engl. J. Med. 334, 1185–1187.

Xu, Z.L., Mizuguchi, H., Sakurai, F., Koizumi, N., Hosono, T., Kawabata, K., Watanabe, Y., Yamaguchi, T., Hayakawa, T., 2005. Approaches to improving the kinetics of adenovirus-delivered genes and gene products. Adv. Drug Deliv. Rev. 57, 781–802.

Yamaguchi, T., Kawabata, K., Koizumi, N., Sakurai, F., Nakashima, K., Sasaki, T., Okada, N., Mizuguchi, H., in press. Involvement of MyD88 and TLR9 in the innate immune response elicited by replication-incompetent adenovirus vectors. Hum. Gene. Ther.

Yang, Y., Ertl, H.C., Wilson, J.M., 1994. MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. Immunity 1, 433–442.

Yang, Y., Li, Q., Ertl, H.C., Wilson, J.M., 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J. Virol. 69, 2004–2015.

Yasuda, K., Ogawa, Y., Yamane, I., Nishikawa, M., Takakura, Y., 2005. Macrophage activation by a DNA/cationic liposome complex requires endosomal acidification and TLR9-dependent and -independent pathways. J. Leukoc. Biol. 77, 71–79.

Yoneyama, H., Matsuno, K., Zhang, Y., Nishiwaki, T., Kitabatake, M., Ueha, S., Narumi, S., Morikawa, S., Ezaki, T., Lu, B., Gerard, C., Ishikawa, S., Matsushima, K., 2004. Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. Int. Immunol. 16, 915–928.

Yoneyama, H., Matsuno, K., Toda, E., Nishiwaki, T., Matsuo, N., Nakano, A., Narumi, S., Lu, B., Gerard, C., Ishikawa, S., Matsushima, K., 2005. Plasmacytoid DCs help lymph node DCs to induce anti-HSV CTLs. J. Exp. Med. 202, 425–435.

Zhang, Y., Chirmule, N., Gao, G.P., Qian, R., Croyle, M., Joshi, B., Tazelaar, J., Wilson, J.M., 2001. Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. Mol. Ther. 3, 697–707.

Zhao, H., Hemmi, H., Akira, S., Cheng, S.H., Scheule, R.K., Yew, N.S., 2004. Contribution of Toll-like receptor 9 signaling to the acute inflammatory response to nonviral vectors. Mol. Ther. 9, 241–248.

Zhu, N., Liggitt, D., Liu, Y., Debs, R., 1993. Systemic gene expression after intravenous DNA delivery into adult mice. Science 261, 209–211.

Please cite this article in press as: Sakurai, H. et al., Innate immune response induced by gene delivery vectors, Int. J. Pharmaceut. (2007), doi:10.1016/j.ijpharm.2007.06.012

HUMAN GENE THERAPY 18:753–762 (August 2007)
© Mary Ann Liebert, Inc.
DOI: 10.1089/hum.2007.016

Role of MyD88 and TLR9 in the Innate Immune Response Elicited by Serotype 5 Adenoviral Vectors

TOMOKO YAMAGUCHI,[1,2,*] KENJI KAWABATA,[1,*] NAOYA KOIZUMI,[1,3] FUMINORI SAKURAI,[1] KAZUKO NAKASHIMA,[1,4] HARUNA SAKURAI,[1,2] TOMOMI SASAKI,[1] NAOKI OKADA,[2] KOICHI YAMANISHI,[2,5] and HIROYUKI MIZUGUCHI[1,2]

ABSTRACT

A replication-incompetent adenoviral (Ad) vector is generating interest for both gene therapy and immunotherapy. A major limitation of the use of Ad vectors is the innate immune response, which causes inflammatory cytokine production and tissue damage; however, the precise mechanism of the innate immune response remains to be clarified. Here, we show that serotype 5 human Ad vectors elicit innate immune responses through a myeloid differentiating factor 88 (MyD88)/Toll-like receptor (TLR)-9-dependent and/or -independent manner according to cell type. After stimulation with Ad vectors, the production of interleukin (IL)-6 and IL-12 was significantly decreased in MyD88- or TLR9-deficient dendritic cells (DCs), compared with wild-type DCs. In addition, the surface expression of maturation marker proteins, such as CD40, CD80, CD86, and MHC class II, in MyD88- or TLR9-deficient granulocyte-macrophage colony-stimulating factor (GM-CSF)-DCs was similar to that in wild-type DCs. On the other hand, MyD88- or TLR9-deficient peritoneal macrophages produced the same level of IL-6 as wild-type macrophages after infection with Ad vectors. We did not find any differences in the mRNA expression levels of the molecules involved in innate immunity, such as MyD88, TLR3, TLR7, and TLR9, between DCs and macrophages. The intravenous injection of luciferase-expressing Ad vectors into MyD88- or TLR9-deficient mice resulted in almost comparable levels of IL-6 and IL-12 production and luciferase expression with wild-type mice. These results suggest that Ad vectors can activate innate immunity via MyD88/TLR9-dependent and -independent mechanisms.

OVERVIEW SUMMARY

***In vivo* application of adenoviral (Ad) vectors elicits innate immune responses, typified by elevated inflammatory cytokines; however, the precise mechanism remains to be clarified. Here, we show that Ad vectors elicit innate immune responses through myeloid differentiating factor 88 (MyD88)/Toll-like receptor (TLR)-9-dependent and/or -independent pathway according to cell type. Absence of MyD88 or TLR9 significantly decreased the secretion of interleukin (IL)-6 and IL-12 in conventional dendritic cells after Ad vector infection. However, neither MyD88 nor TLR9 was required for Ad vector-induced IL-6 production in peritoneal macrophages. IL-6 and IL-12 secretion *in vivo* by MyD88- or TLR9-deficient mice infected with Ad vector was not significantly different from that by wild-type mice. These results provide important clues for elucidation of Ad vector-mediated innate immune responses.**

INTRODUCTION

ADENOVIRAL (Ad) VECTORS have proved to be a highly promising gene transfer system for *in vitro* and *in vivo* transduction. However, an *in vivo* application of Ad vectors is usually associated with systemic toxicity caused by two types

[1]Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka 567-0085, Japan.
[2]Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 567-0871, Japan.
[3]Present address: Showa Pharmaceutical University, Machida, Tokyo, 194-8543, Japan.
[4]Japan Health Sciences Foundation, Tokyo 103-0001, Japan
[5]National Institute of Biomedical Innovation, Osaka 567-0085, Japan.
[6]T.Y. and K.K. contributed equally in this work.

of immune responses: adaptive and innate immune responses. The adaptive immune response to Ad vectors is well characterized (Bessis *et al.*, 2004), whereas the mechanism of the innate immune response to Ad vectors remains unclarified. The innate immune response to Ad vectors is characterized by the acute production of several inflammatory cytokines, including interleukin (IL)-6 and IL-12, independently of viral gene expression within several hours after Ad vector injection, and the maturation of antigen-presenting cells, such as dendritic cells (DCs) and macrophages (Muruve, 2004; Nazir and Metcalf, 2005). The production of inflammatory cytokines leads to systemic toxicity, such as multiorgan damage. It is highly important for successful gene therapy using Ad vectors to prevent the innate immune response. However, the precise mechanisms of the Ad vector-mediated innate immune response remain to be elucidated.

Innate immune responses triggered by Ad vectors, such as inflammatory cytokine production, are largely due to antigen-presenting cells, including DCs and liver Kupffer cells. Systemically administered Ad vectors are captured by antigen-presenting cells and removed from the blood circulation (Alemany *et al.*, 2000; Koizumi *et al.*, 2003), leading to the production of inflammatory cytokines, although it remains unclear which types of cells are mainly responsible for Ad clearance and cytokine production. Previous studies demonstrated that antigen-presenting cells express various types of pattern-recognizing receptors (PRRs), such as Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I), and the nucleotide-binding oligomerization domain (NOD)-1, and that innate immune responses in antigen-presenting cells occur by recognition of pathogens by PRRs (Yoneyama *et al.*, 2004; Kato *et al.*, 2005; Akira *et al.*, 2006; Meylan *et al.*, 2006). After the recognition of pathogens, PRRs subsequently activate NF-κB, leading to the transcription of inflammatory cytokine genes. Among the PRRs, TLRs are the most extensively characterized. TLRs recognize microbial components and trigger the signal cascade that activates innate immune responses. After the recognition of microbial components by TLRs, they, except for TLR3, transduce intracellular signaling through the adaptor protein myeloid differentiating factor-88 (MyD88), which initiates a signaling cascade leading to NF-κB activation (Yamamoto *et al.*, 2002, 2003; Oshiumi *et al.*, 2003; Takeda and Akira, 2004; Kawai and Akira, 2006). Among 13 members of TLRs, TLR2, TLR3, TLR4, TLR7, TLR8, and TLR9 have been demonstrated to be involved in the recognition of viral components. Viral double-stranded RNA (dsRNA), the fusion protein of respiratory syncytial virus (RSV), and single-stranded RNA (ssRNA) are recognized by TLR3, TLR4, and TLR7, respectively (Kurt-Jones *et al.*, 2000; Alexopoulou *et al.*, 2001; Diebold *et al.*, 2004; Heil *et al.*, 2004). One study has reported that herpes simplex virus (HSV) is recognized by TLR2 (Sato *et al.*, 2006). TLR9 has been demonstrated to be involved in the production of inflammatory cytokines by DNA viruses, including HSV-1, HSV-2, and murine cytomegalovirus (MCMV) (Lund *et al.*, 2003; Hochrein *et al.*, 2004; Krug *et al.*, 2004a,b; Tabeta *et al.*, 2004). TLR9 has been identified as a PRR for double-stranded DNA containing unmethylated CpG motifs (Hemmi *et al.*, 2000), which is also possessed by the Ad genome. Therefore, it has been hypothesized that TLRs, especially TLR9, might be involved in Ad vector-induced innate immune responses.

In the present study, we examined the role of MyD88 and TLR9 in the *in vitro* and *in vivo* innate immune responses caused by Ad vectors, and showed that Ad vector induced innate immune responses via both MyD88/TLR9-dependent and -independent pathways according to cell type.

MATERIALS AND METHODS

Mice

C57BL/6 wild-type mice were purchased from Nippon SLC (Shizuoka, Japan). MyD88- or TLR9-deficient mice were originally generated by S. Akira (Osaka University, Osaka, Japan) (Adachi *et al.*, 1998; Hemmi *et al.*, 2000) and purchased from Oriental Yeast (Tokyo, Japan). All mice used in this study were 5–10 weeks of age.

Adenoviral vectors

Luciferase-expressing serotype 5 human Ad vector, Ad-L2, was constructed as described previously (Mizuguchi *et al.*, 2001). Briefly, the CMV promoter-driven luciferase gene derived from pGL3-Control (Promega, Madison, WI) was inserted into the E1 deletion region of the Ad genome. Ad-L2 was purified by $CsCl_2$ step gradient ultracentrifugation, dialyzed with a solution containing 10 m*M* Tris (pH 7.5), 1 m*M* $MgCl_2$, and 10% glycerol, and sorted into aliquots at −80°C. Determination of virus particle titer (VP) and infectious titer was accomplished spectrophotometrically by the method of Maizel *et al.* (1968) and with an Adeno-X rapid titer kit (Clontech, Palo Alto, CA), respectively. The ratio of infectious titer to particle titer was 1:18. The viral stocks were free of endotoxin (<0.03 endotoxin unit/ml), as determined by use of a *Limulus* Color KY test (Wako, Osaka, Japan).

Reagents

Synthesized oligodeoxynucleotide, CpG-DNA, was purchased from Hokkaido System Science (Hokkaido, Japan). The sequence of the CpG-DNA was as follows: 5′-GGTGCATCGATGCAGGGGGG-3′ (Honda *et al.*, 2005). Anti-mouse CD16/32 antibody was purchased from BioLegend (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated antibody against CD11c (clone N418), phycoerythrin (PE)-conjugated antibody against CD86 (clone GL1), and antibody against CD45R/B220 (clone RA3-6B2) were purchased from eBioscience (San Diego, CA).

Cells

Peritoneal macrophages were collected from wild-type, MyD88-deficient, and TLR9-deficient mice 3 days after intraperitoneal injection of thioglycollate medium (Nissui, Tokyo, Japan). Isolated macrophages were activated with interferon (IFN)-γ (R&D Systems, Abingdon, UK) (200 ng/ml) for 24 hr before Ad vector infection. Bone marrow cells were prepared from the femurs and tibias of wild-type, MyD88-deficient, and TLR9-deficient mice. These cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, and human Flt3 ligand (Flt3L, 100 ng/ml; PeproTech, Rocky Hill, NJ) or murine granulocyte-macrophage colony-