E. 結論

1) Ad ベクターによる自然免疫応答のメカニズムを解明するため、昨年度行った DNA マイクロアレイ解析により抽出された自然免疫応答関連候補遺伝子を詳細に検討したところ、Ad ベクターにより誘導される炎症性サイトカイン産生には JAK2/STAT5 経路が関与しており、このシグナルは JAK2 の細胞内活性化阻害分子である SOCS により抑制されることが明らかとなった。また、SOCS1 を搭載した Ad ベクターである Ad-SOCS1 を全身投与すると、ルシフェラーゼを搭載した Ad ベクターである Ad-L2 を投与した場合と比較して、炎症性サイトカイン産生が抑制されることが明らかとなった。さらに、Ad-L2 と Ad-SOCS1 を共投与することにより、Ad-L2 による遺伝子発現を妨げることなく、炎症性サイトカイン産生および肝傷害を抑制することに成功した。

2) DNA マイクロアレイ解析により、Ad ベクターを in vivo で作用させたときに引き起こされる自然免疫応答に関与する候補遺伝子を選び出した。そして遺伝子 X および Y について in vitro での解析を進めるため、まず、マウスマクロファージ様細胞である RAW264.7 細胞を LPS で刺激し発現動向を調べた。遺伝子 X および Y どちらも（特に X）LPS 刺激後、早期に発現が誘導されることがわかり、LPS 応答におけるサイトカイン産生の一連のシグナル経路の初期段階に関与していることが示唆された。X を高発現する RAW264.7 細胞の stable clone の作製を試みたところ高い X の発現を示すクローンを樹立することができたため今後の解析に利用していく。

3) 炎症性サイトカイン産生に重要な転写因子の NF-κB に特異的に結合する RNA アプタマー（eA-p50）を搭載した Ad ベクターは、in vivo での Ad ベクター投与による炎症性サイトカイン IL-12 産生を抑制した。このことから、eA-p50 搭載 Ad ベクターを用いることで、炎症性サイトカイン産生の抑制できる可能性が示唆された。

F. 健康危険情報

該当事項なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得

該当事項なし

2. 実用新案登録

該当事項なし

3. その他

該当事項なし

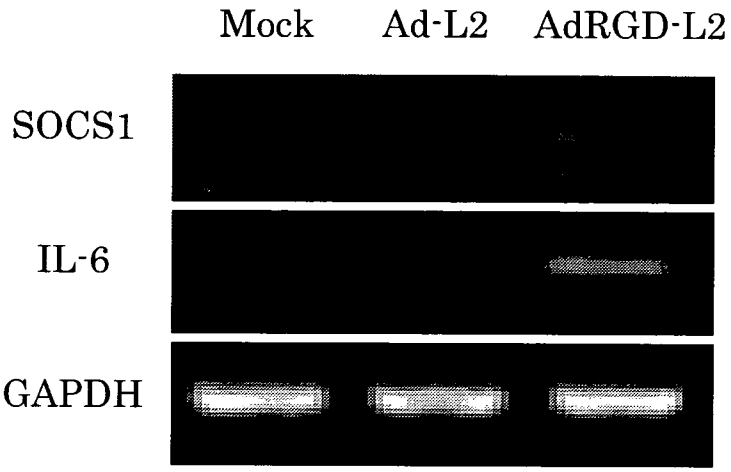

Fig. 1 SOCS1 expression in Ad vector-transduced RAW264.7 cells.
Total mRNA samples were isolated from RAW264.7 cells 6 h after the transduction of Ad-L2 or AdRGD-L2 (10,000 VP/cell). After the reverse transcriptase reaction, SOCS1 and IL-6 mRNA levels were measured by semiquantitative RT-PCR assay.

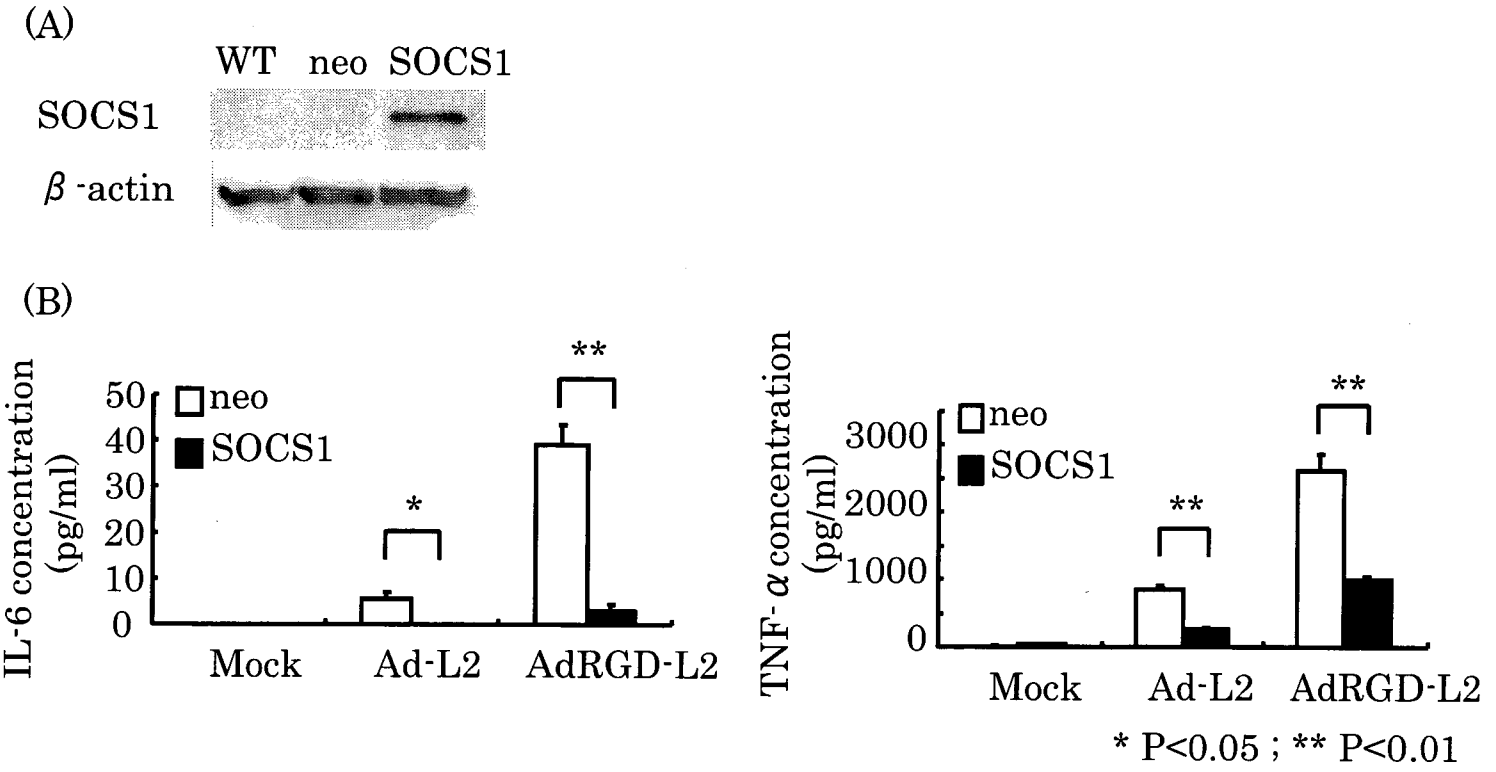

Fig. 2 Ad vector-mediated induction of cytokine production in RAW264.7-SOCS1 cells.
The expression of SOCS1 in RAW264.7-SOCS1 cells was detected by Western blot analysis (A). IFN-γ-activated RAW264.7-neo cells and RAW264.7-SOCS1 cells were stimulated by Ad-L2 or AdRGD-L2 (10,000 VP/cell) for 24 h. The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

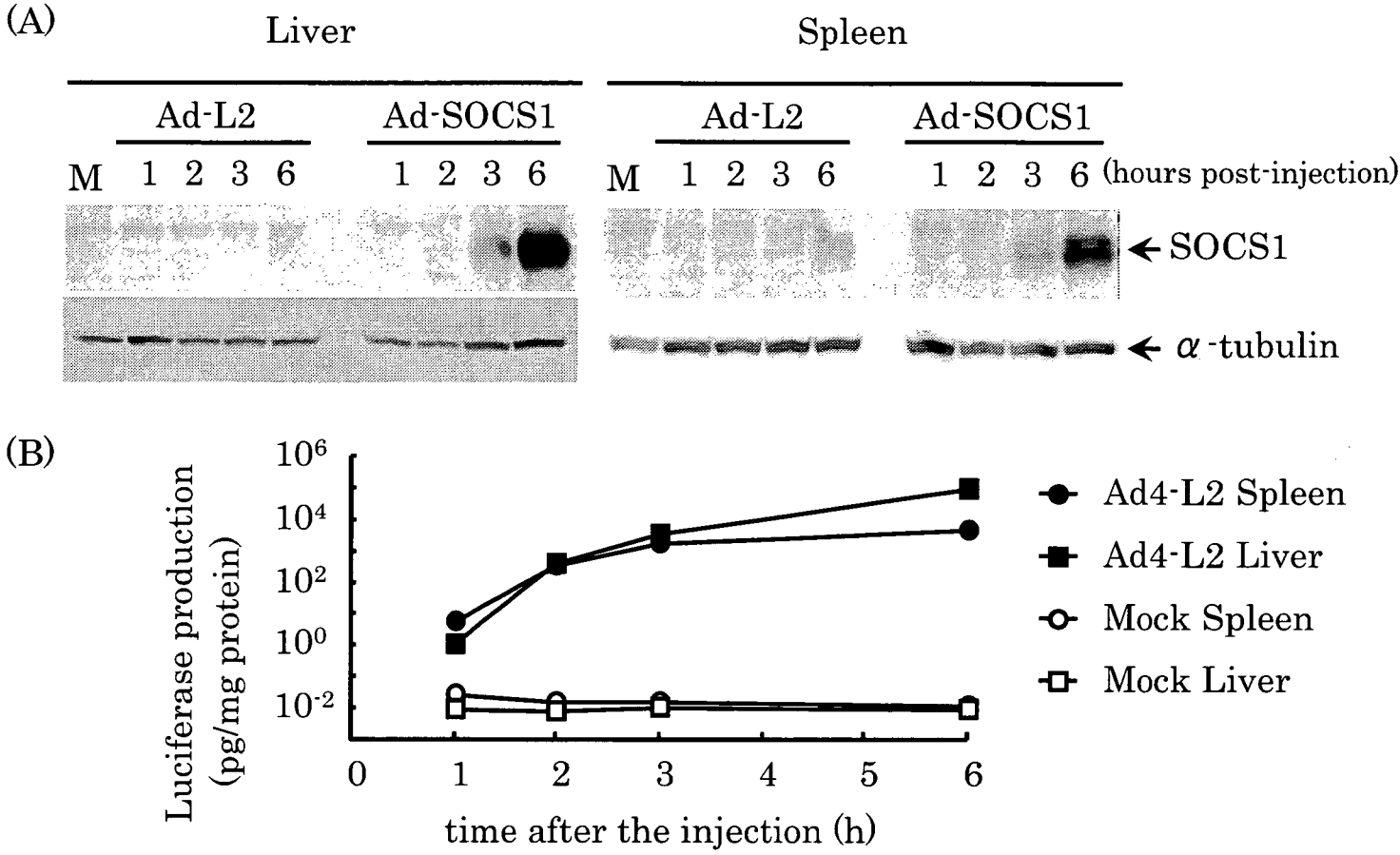

Fig. 3 Transgene expression in the liver and spleen by the systemic administration of Ad vectors.

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). M; Mock

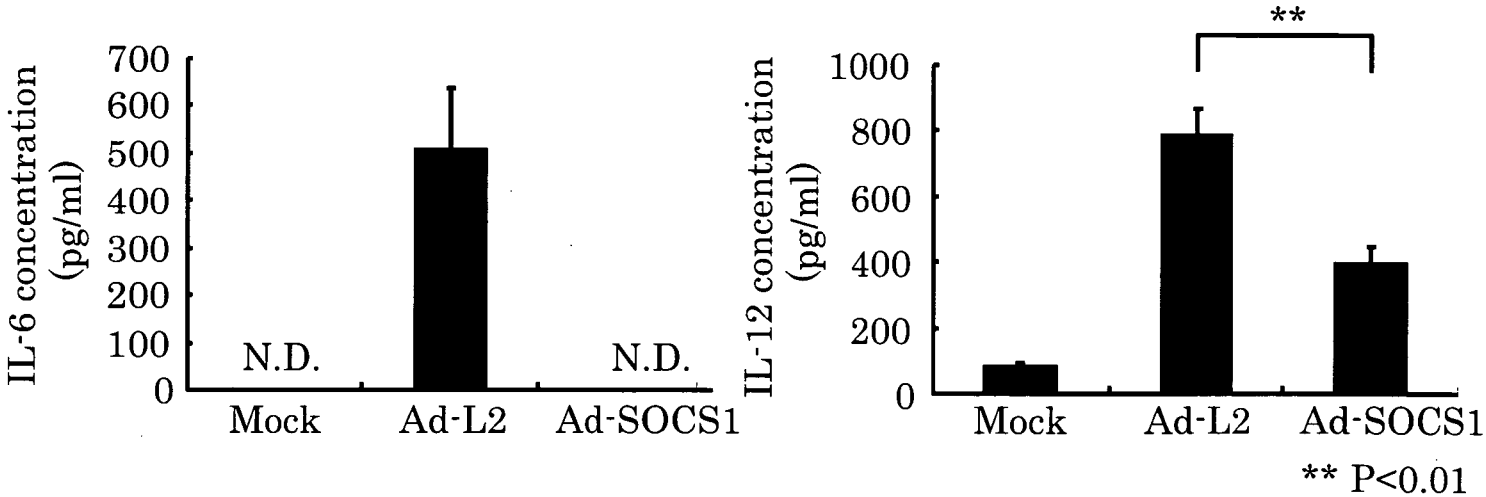

Fig. 4 Inflammatory cytokine production after the systemic administration of Ad-L2 or Ad-SOCS1 into mice.

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. All data are represented as the means ± S.D. (n=3~4).

N.D.; not detectable

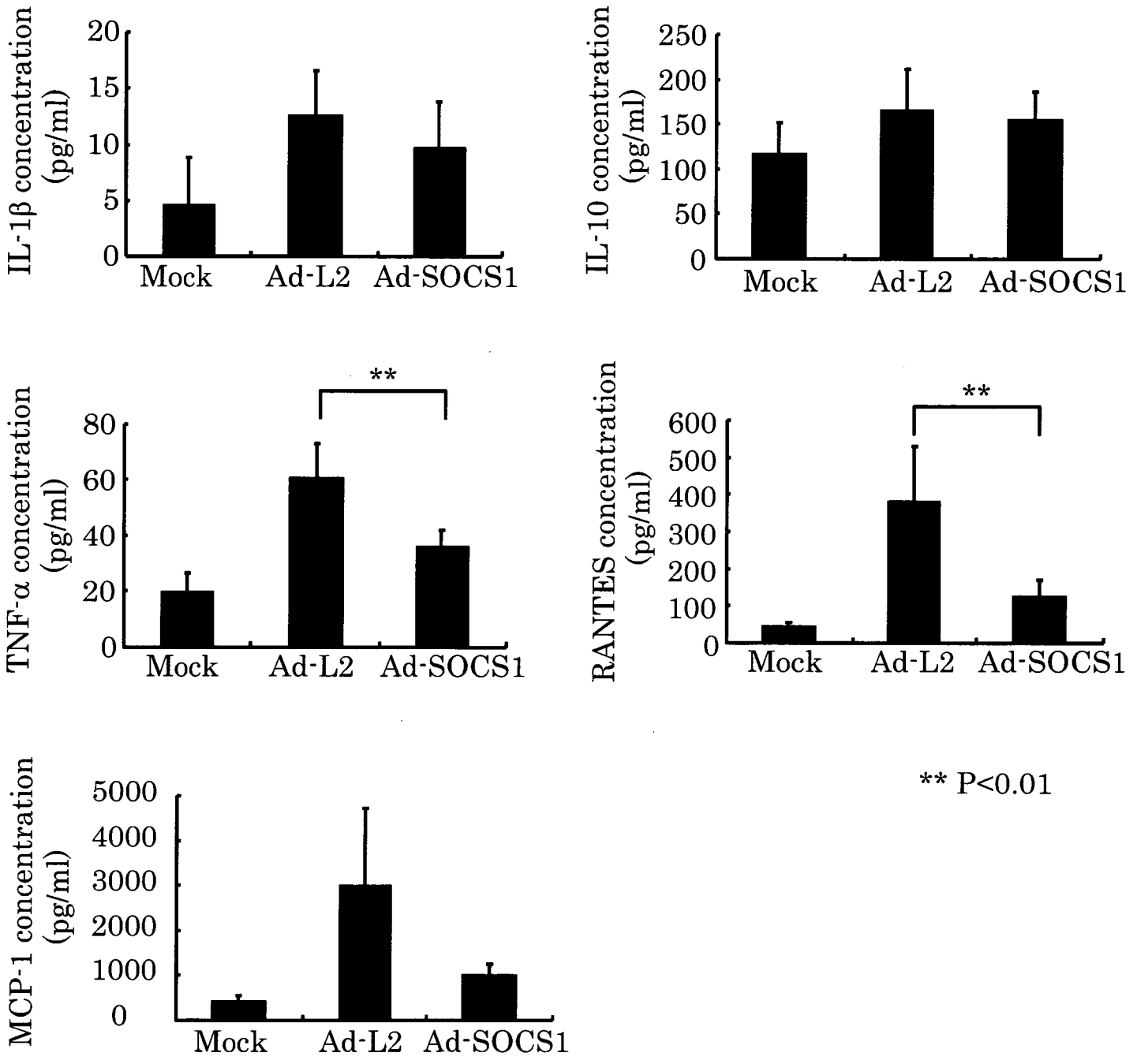

Fig. 5 Inflammatory cytokine/chemokine production after the systemic administration of Ad-L2 or Ad-SOCS1 into mice.

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-1b, IL-10, TNF-α, RANTES, and MCP-1 in serum of Ad vector-injected mice were measured by Bio-plex. All data are represented as the means ± S.D. (n=3~4).

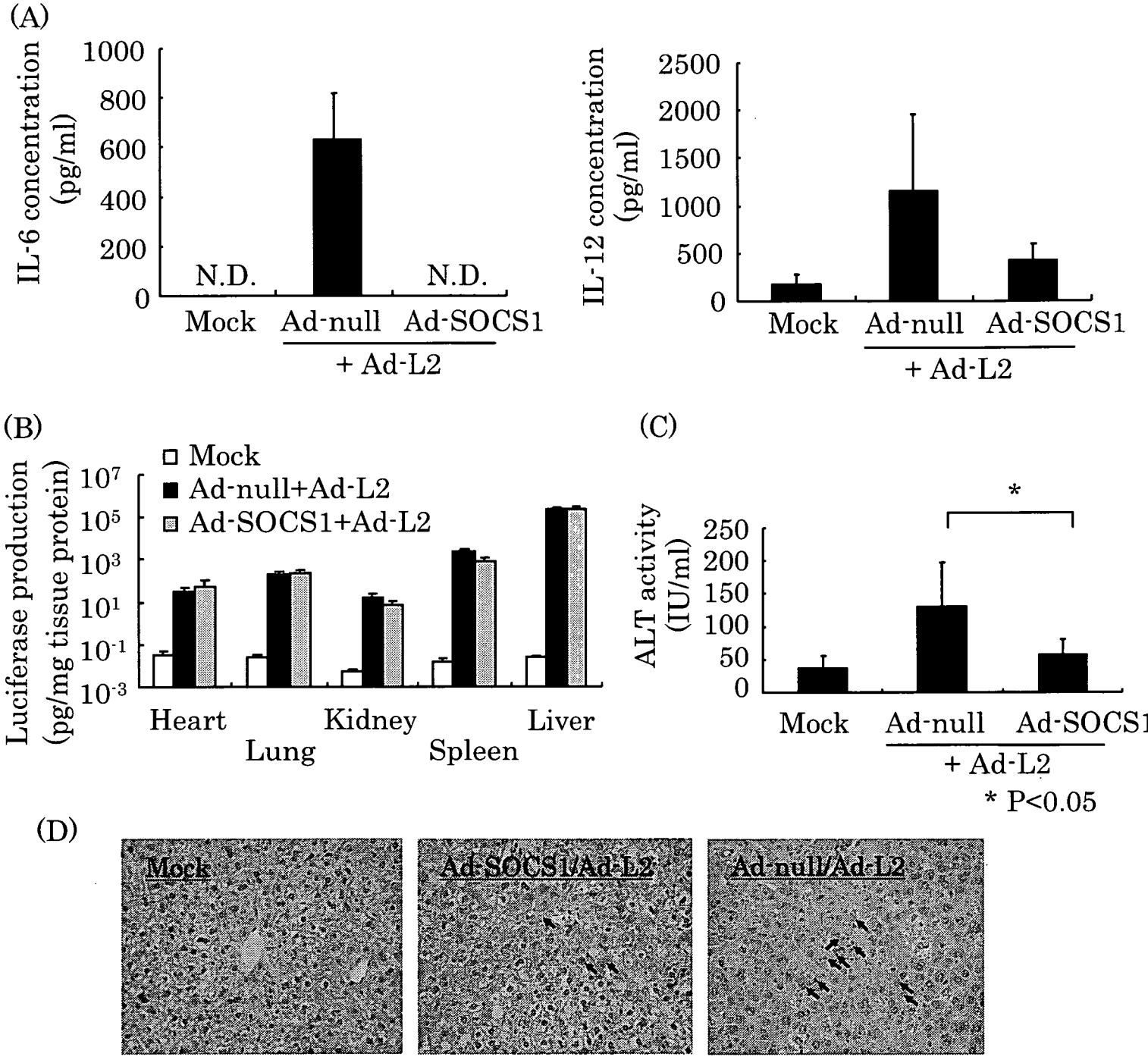

Fig. 6 Inflammatory cytokine production, luciferase production, and liver toxicity after the systemic co-injection of Ad-L2 and Ad-SOCS1 into mice.

Ad-L2/Ad-null or Ad-L2/Ad-SOCS1 were intravenously co-injected 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, ALT 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 ± S.D. (n=3).

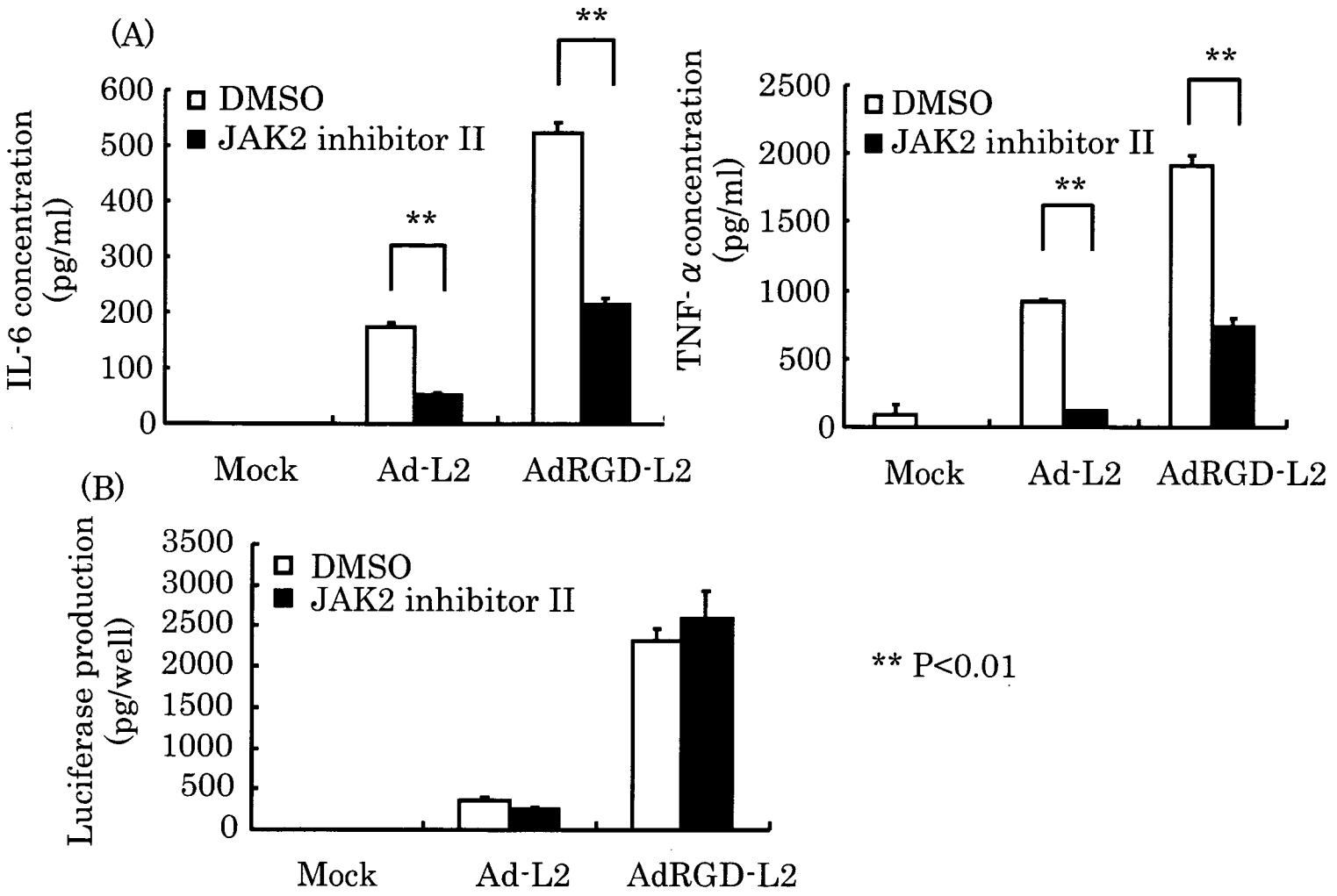

Fig. 7 JAK2 inhibitor II suppresses the Ad vector-mediated cytokine production and luciferase production in RAW264.7 cells.

RAW264.7 cells pre-treated with IFN-g 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 ± S.D. (n=3).

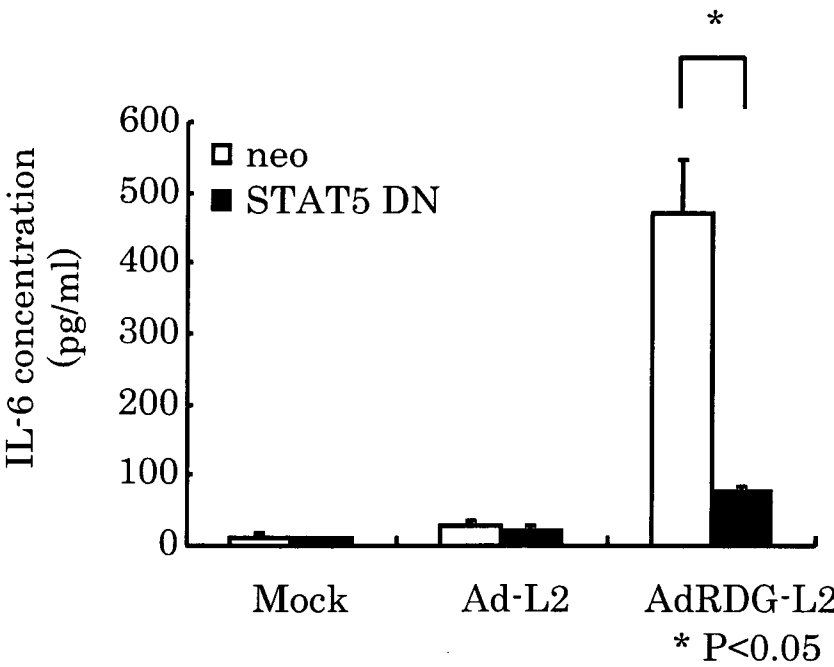

Fig. 8 Ad vector-mediated induction of cytokine production in RAW-STAT5DN cells.

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 ± S.D. (n=3).

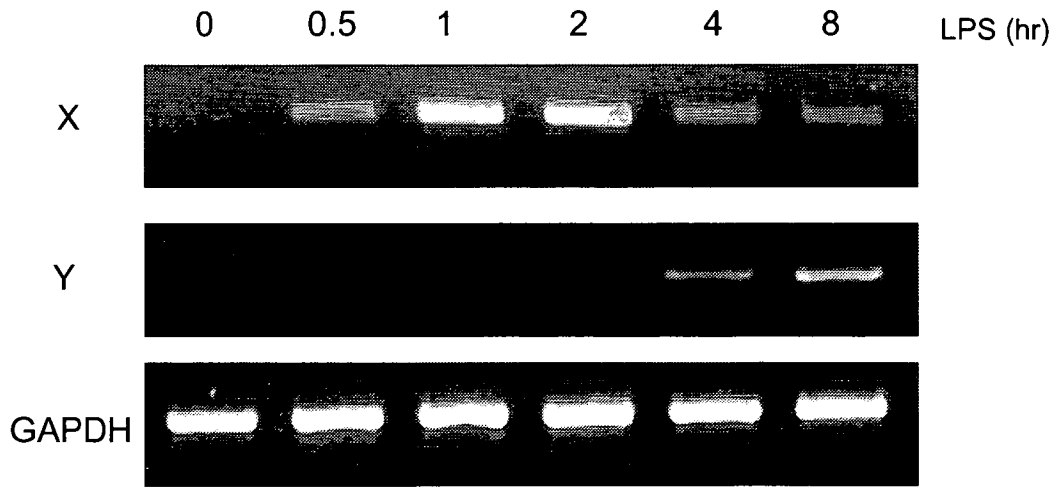

Fig. 9 X and Y mRNA expression was induced in RAW264.7 cells upon LPS stimulation.
RAW264.7 cells were cultured with LPS (1 mg/ml) for the indicated length of time (hours). Total RNA from these cells was converted into cDNA. Equal amounts of cDNA from each sample was used as a template for PCR to amplify X (top), Y (middle), and GAPDH as a dose control (bottom).

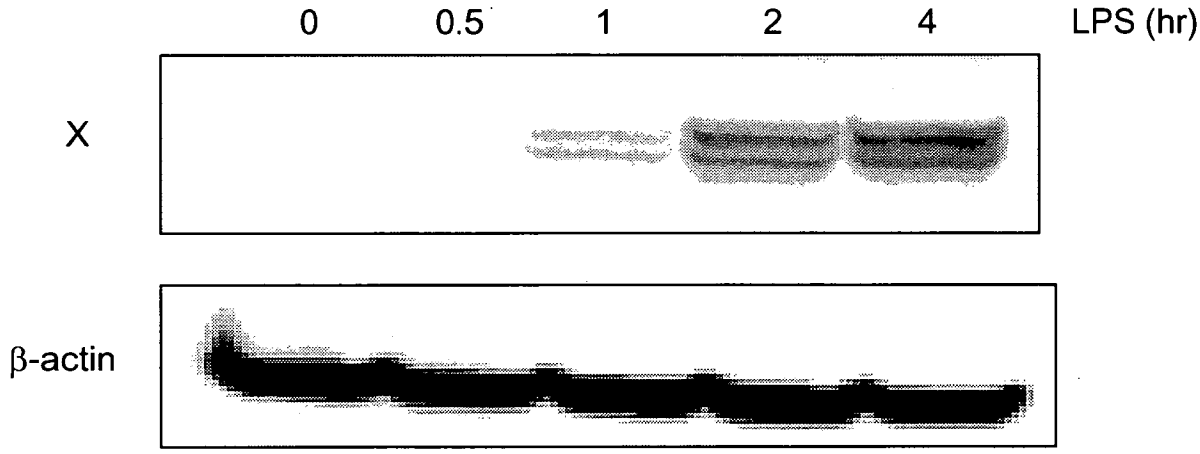

Fig. 10 Protein expression of X was induced in RAW264.7 cells upon LPS stimulation.
RAW264.7 cells were cultured with LPS (1 mg/ml) for the indicated length of time (hours). Cell extracts were prepared from these cells and subjected to immunoblot analysis with anti-X antibody (top). This blot was reprobed with anti-b-actin antibody to control for the amount of the extracts used (bottom).

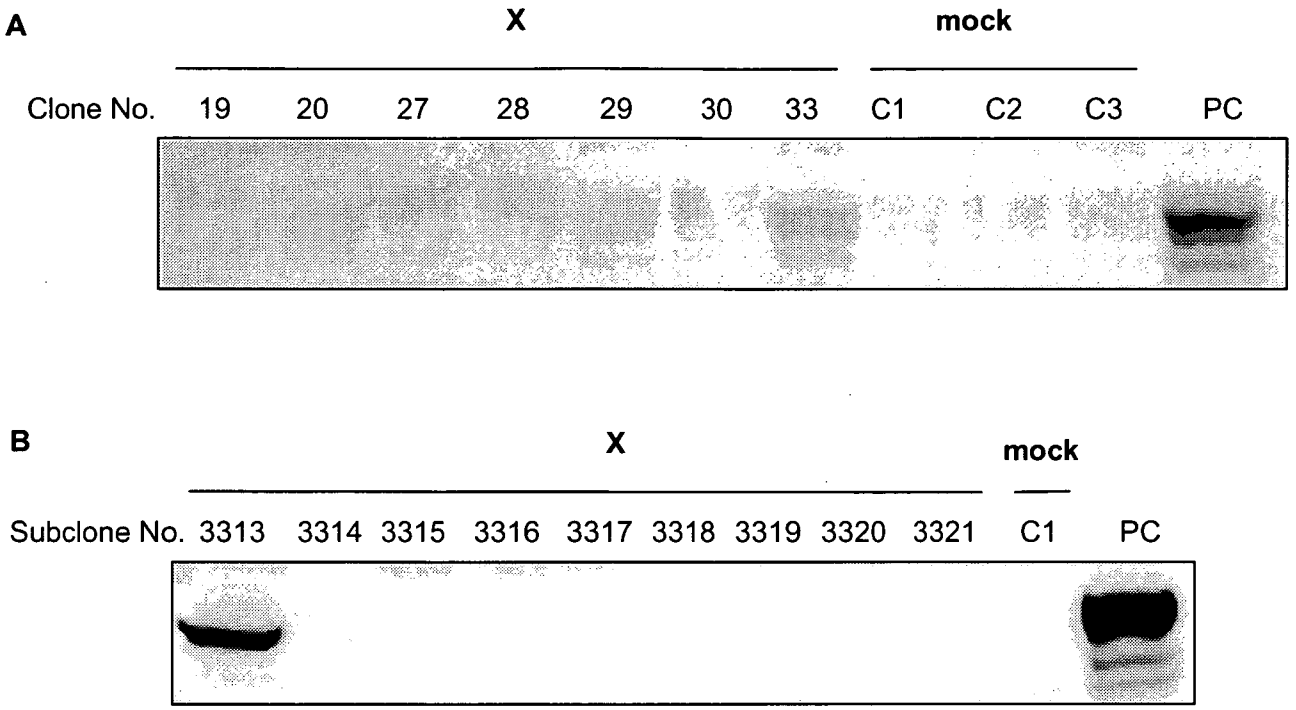

Fig. 11 Establishment of RAW264.7- stable transfectant overexpressing protein X.

RAW264.7 cells were stably transfected with cDNA encoding X as described in materials and methods. (A) Cell extracts from G418 resistant cells were subjected to western blot analysis with anti-X antibody. (B) Subcloning of X/RAW clone No.33. Cell extract from RAW264.7 cells stimulated with LPS for 4 hr was used as a positive control (PC), and stably transfected with mock vector (pcDNA3) was used as a negative control (NC).

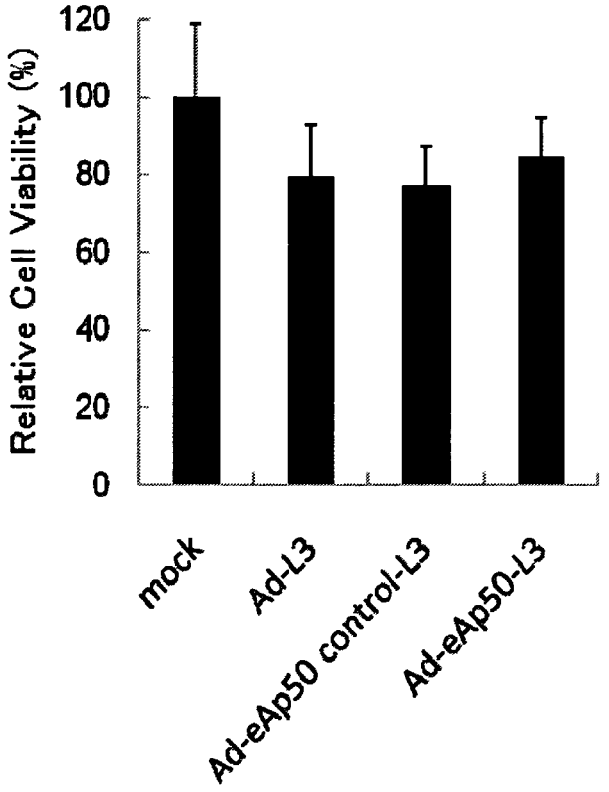

Fig. 12 Cell viability of A549 cells transduced with Ad-eAp50-L3.
A549 cells were transduced with 3000 vp/cell od Ad-L3, Ad-eAp50-L3, Ad-eAp50control-L3 for 1.5h. After 24 h-cultivation, the cell viability was measured using the Alamar blue assay. Data are expressed as means ± SD of triplicate culture.

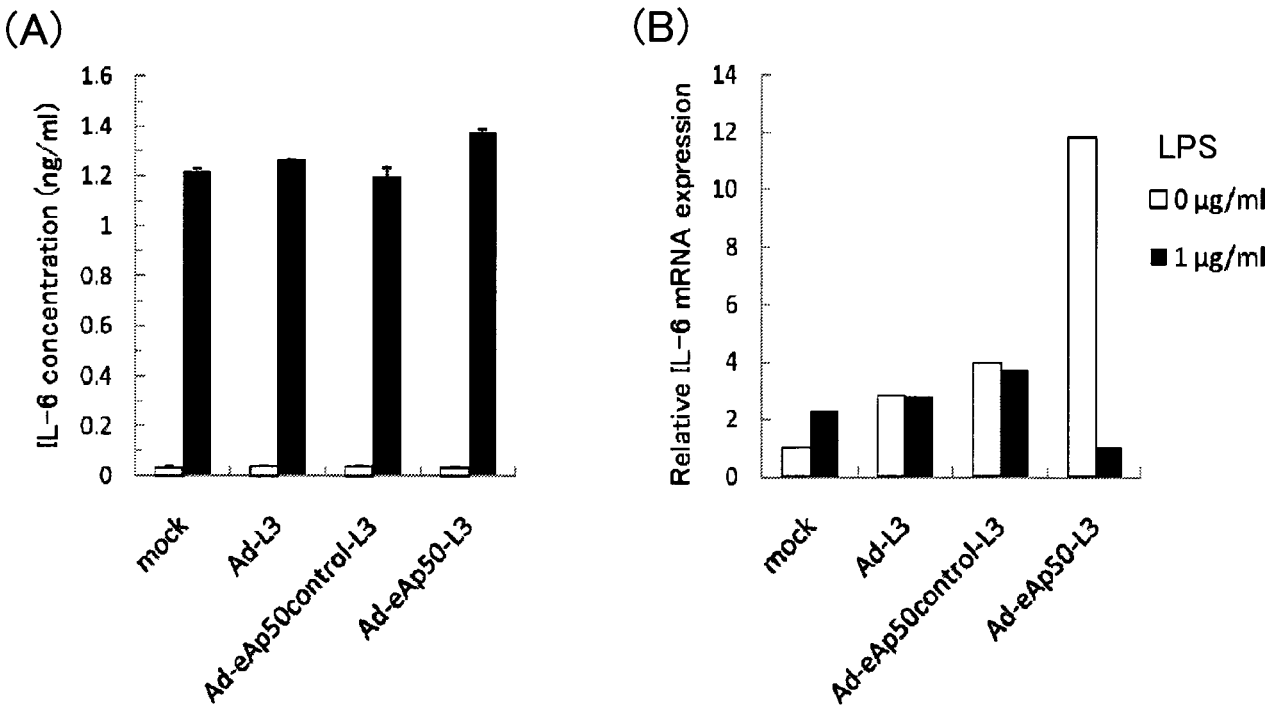

Fig. 13 Ad-eAp50 vector-mediated suppression of cytokines on the LPS-stimulated MS-1 cells.
The MS-1 cells were transduced with 3000 vp/cell of Ad-L3, Ad-eAp50-L3, or Ad-eAp50control-L3 for 1.5 h. After 24 hr-cultivation, cells were stimulated with LPS (1 mg/ml) in DMEM containing 10% FCS for 24 h. IL-6 levels in their supernatants were measured by ELISA (A). Total mRNA samples of stimulated cells were isolated, and IL-6 cDNA were measured using the quantitative TaqMan PCR assay after the RT reaction (B). Data are expressed as means ± SD of triplicate culture.

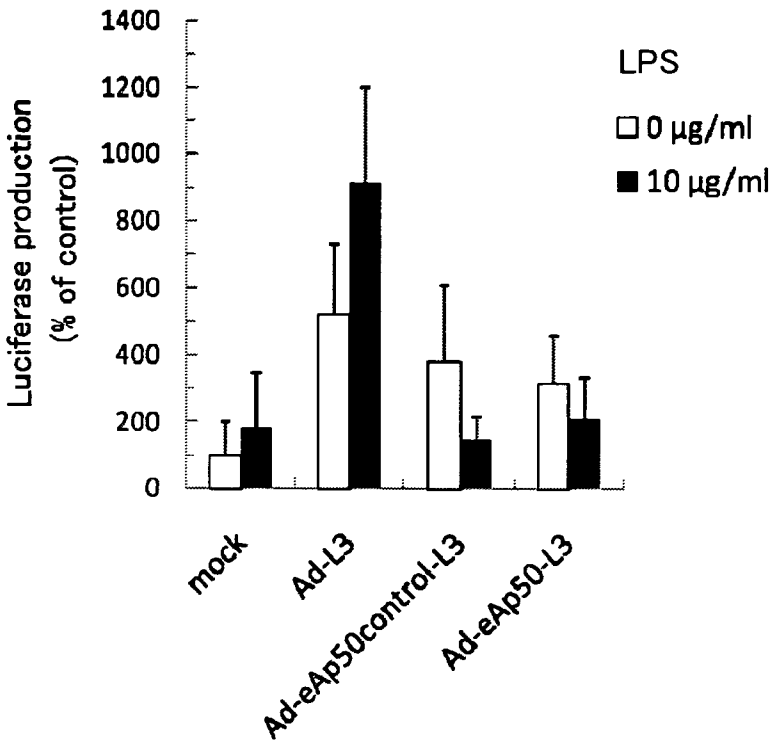

Fig. 14 Ad-eAp50 vector-mediated suppression of NF-κB in A549 cells.
The cells, which were transfected with the renilla reniformis luciferase reporter plasmid (p-ELAM-RL), were transduced with 3000 vp/cell Ad-L3, Ad-eAp50-L3, Ad-eAp50control-L3 for 1.5 h. After 24 hr-cultivation, cells were stimulated with LPS (10 mg/ml) in MEM containing 10% FCS for 1 h, and renilla reniformis luciferase activity was measured using the dual-luciferase reporter assay system. Renilla reniformis luciferase activity was normalized by the amount of proteins of the cell lysate. Data are presented as the means ± SE of triplicate culture.

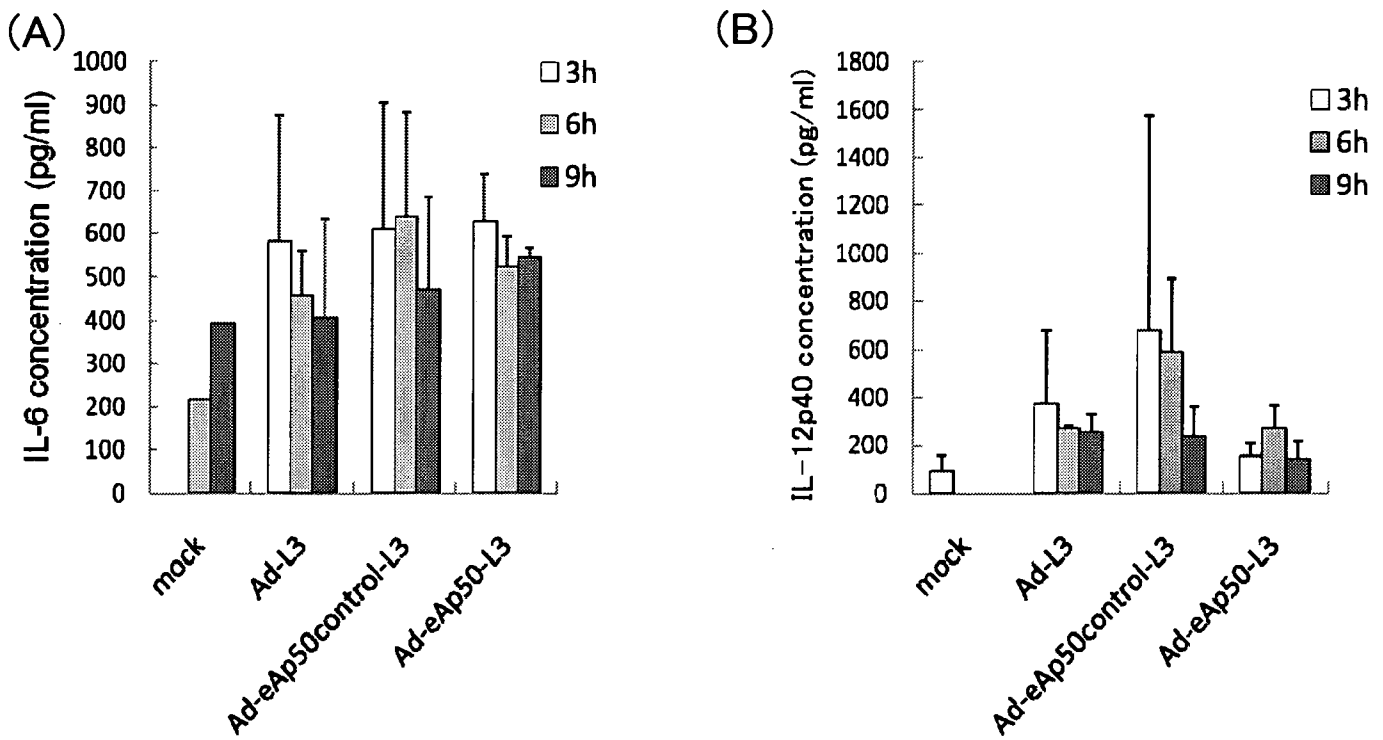

Fig. 15 Induction of various inflammatory cytokines by intravenous injection of Ad vectors.
A final volume of 200 ml of Ad vectors (5×10^{10} VP/mouse) was injected intravenously into each mouse. After the indicated time had passed following the Ad injection, peripheral blood serum was collected, and the concentration of IL-6 (A) and IL-12 (B) were measured by ELISA. All data are expressed as mean ±S.D. of 3 mice per group.

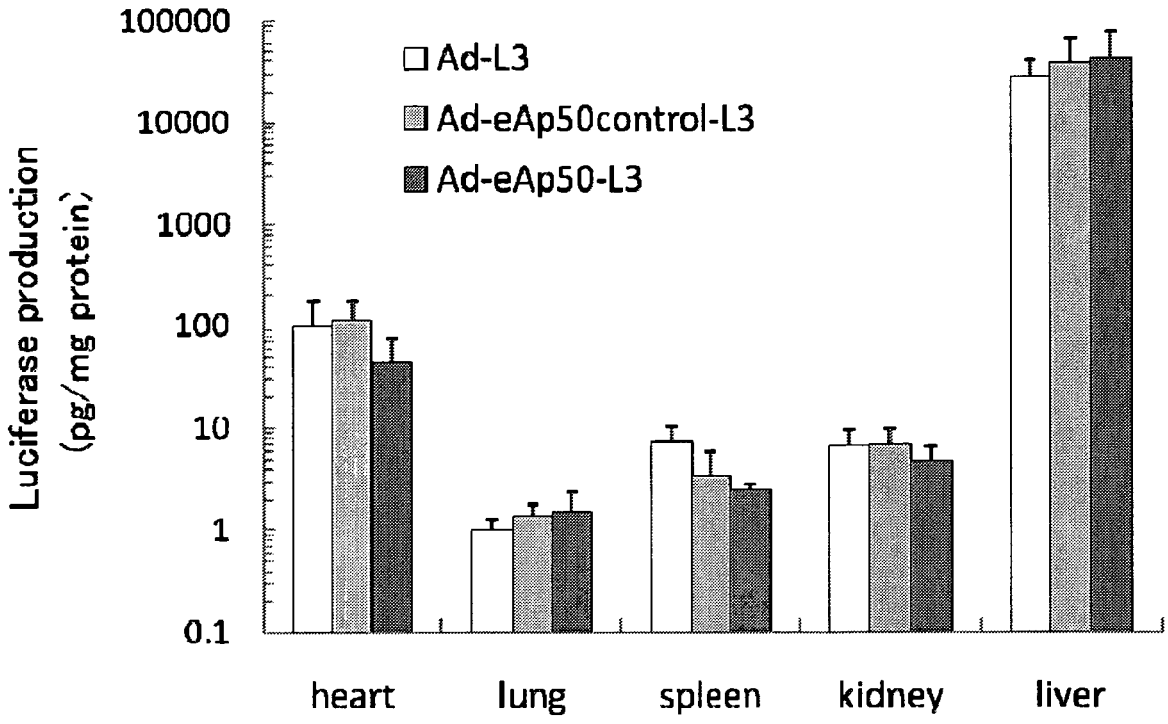

Fig. 16 Luciferase production in mice after intravenous administration of Ad vectors.
A final volume of 200 ml of Ad vectors (5×10^{10} VP/mouse) was injected intravenously into each mouse. After 48 h, each organs were harvested, and luciferase production and protein concentration were measured. All data are expressed as mean ±S.D. of 3 mice per group.

Table 1 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
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Sakurai H., Kawabata K., Sakurai F., Nakagawa S., Mizuguchi H.	Innate immune response induced by gene delivery vectors.	Int. J. Pharm.	in press		
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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
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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

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The Journal of Immunology

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

AQ: B

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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

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[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.

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.

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.

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.

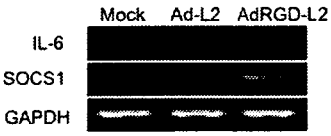

FIGURE 1. SOCS1 expression in Ad vector-transduced RAW264.7 cells. Total mRNA samples were isolated from RAW264.7 cells 6 h after the transduction of Ad-L2 or AdRGD-L2 (10,000 VP/cell). After the reverse transcriptase reaction, SOCS1 and IL-6 mRNA levels were measured by semiquantitative RT-PCR assay.

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,

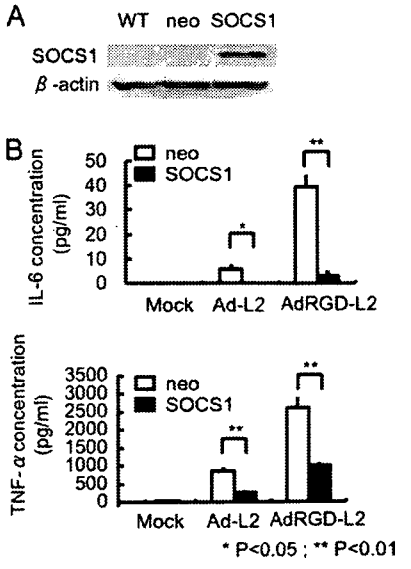

FIGURE 2. Ad vector-mediated induction of cytokine production in RAW264.7-SOCS1 cells. The expression of SOCS1 in RAW264.7-SOCS1 cells was detected by Western blot analysis (*A*). IFN-γ-activated RAW264.7-neo cells and RAW264.7-SOCS1 cells were stimulated by Ad-L2 or AdRGD-L2 (10,000 VP/cell) for 24 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).

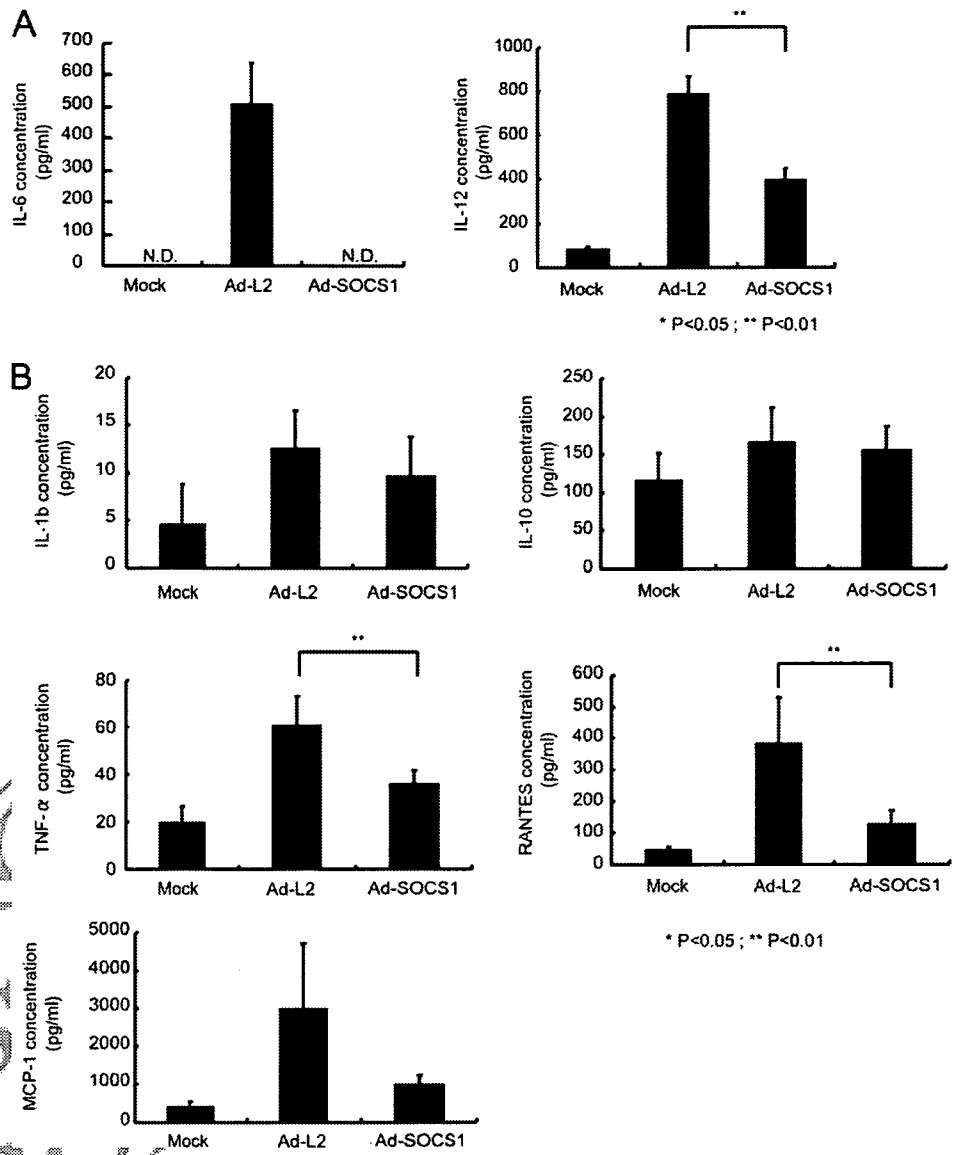

FIGURE 3. Inflammatory cytokine production after the systemic administration of Ad-L2 or Ad-SOCS1 into mice. Ad-L2 or Ad-SOCS1 (5×10^{10} VP/mouse) was i.v. injected into mice. Six hours later, blood samples were collected from the inferior vena cava, and the concentrations of IL-6 and IL-12 in the serum 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. 2*A*) and observed Ad vector-mediated cytokine production in these cells (Fig. 2*B*). 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. 2*B*). 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. 3*A*). The Ad vector-mediated induction

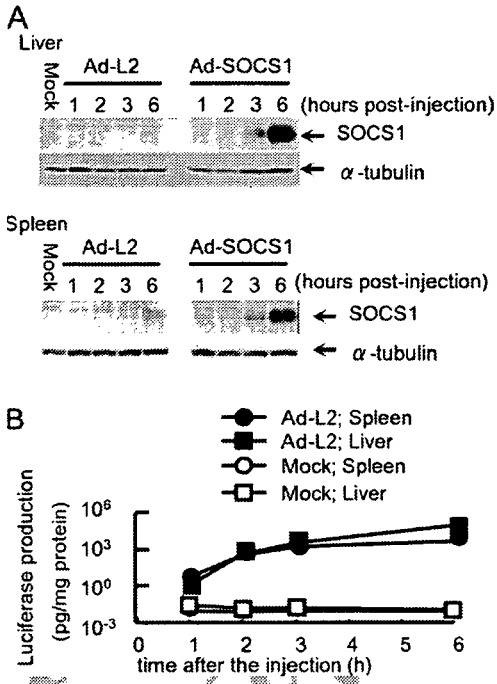

FIGURE 4. Transgene expression in the liver and spleen by the systemic administration of Ad vectors. Ad-SOCS1-mediated SOCS1 expression in the liver and the spleen was examined at 1, 2, 3, and 6 h after i.v. injection of Ad vectors, as determined by Western blotting (*A*). Luciferase production in the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

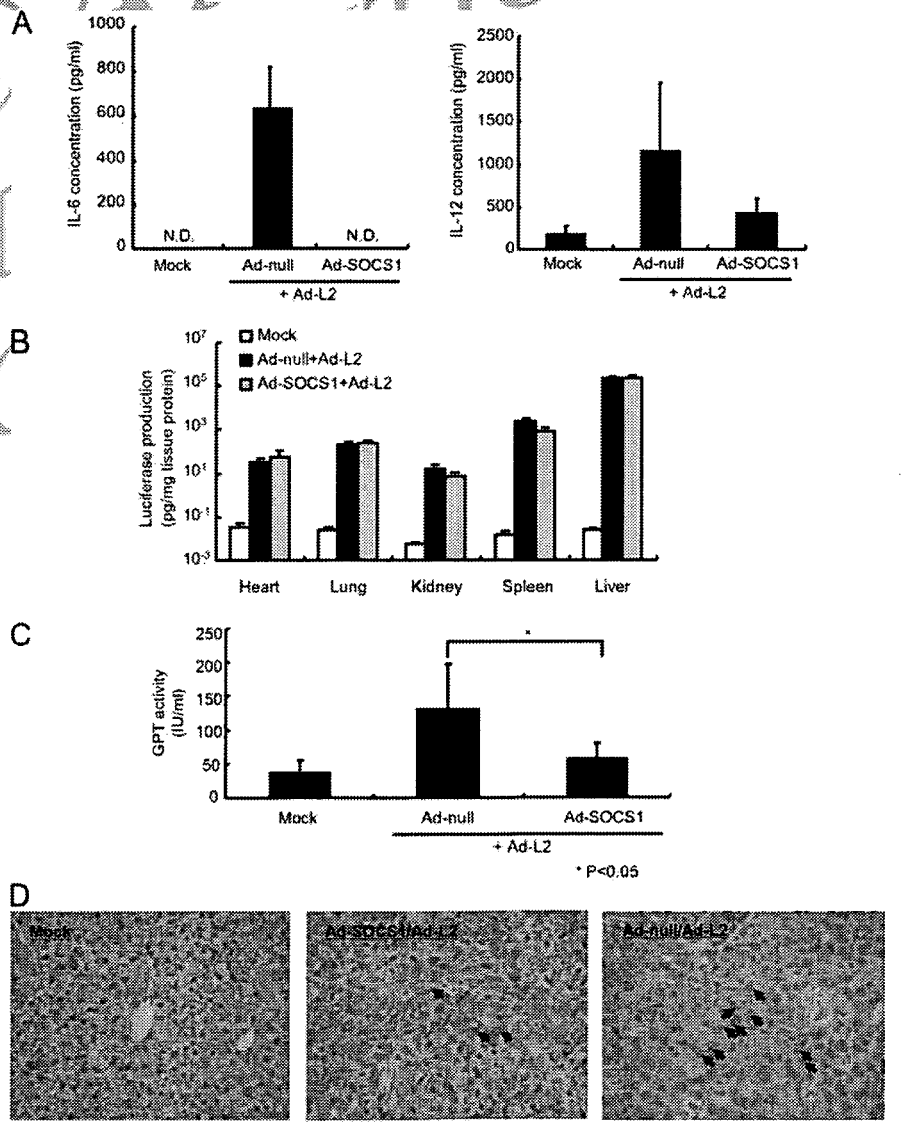

FIGURE 5. Inflammatory cytokine production, luciferase production, and liver toxicity after the systemic coinjection of Ad-L2 and Ad-SOCS1 into mice. Ad-L2/Ad-null or Ad-L2/Ad-SOCS1 were i.v. coinjected into mice (Ad-L2: Ad-SOCS1 = 1:9; total 5×10^{10} VP/mouse). Blood samples were collected from the 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 ($n = 3$).