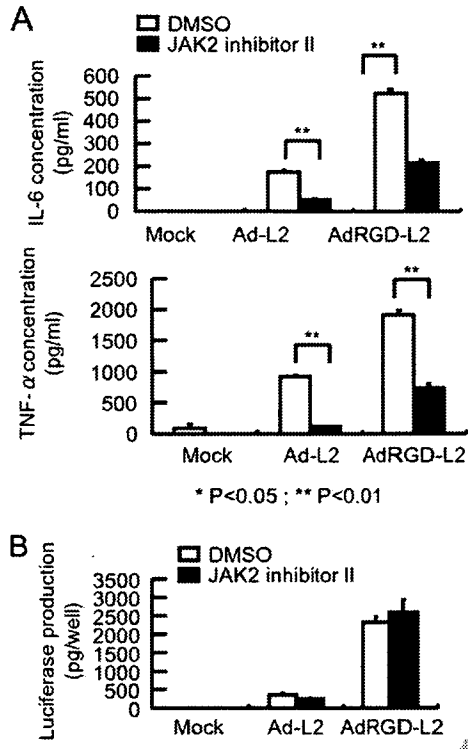

FIGURE 6. JAK2 inhibitor II suppresses the Ad vector-mediated cytokine production in RAW264.7 cells. RAW264.7 cells pretreated with IFN-γ were incubated with JAK2 inhibitor II (50 μM) for 1 h and the cells were stimulated by Ad vectors for 24 h. *A*. The concentrations of IL-6 and TNF-α in the cultured 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).

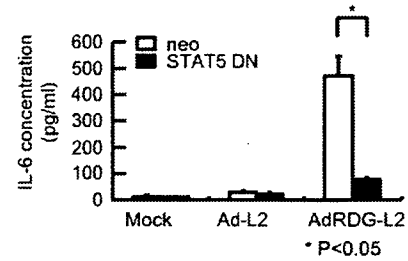

FIGURE 7. Ad vector-mediated induction of cytokine production and luciferase production in RAW264.7-STAT5DN cells. IFN-γ-activated RAW-neo cells and RAW-STAT5DN cells were stimulated by Ad-L2 or AdRGD-L2 (10.000 VP/cell) for 24 h. The concentration of IL-6 in the cultured supernatants was measured by 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.

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-inejected 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

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.

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Disclosures

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Mini review

Innate immune response induced by gene delivery vectors

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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.

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,

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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

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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.

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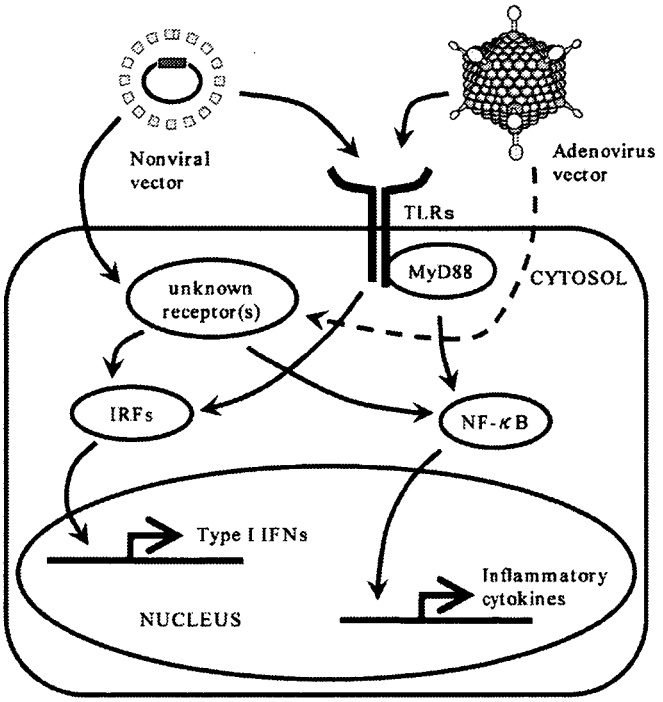

Fig. 1. General scheme of Ad vector- or nonviral vector-induced inflammatory gene expression in immune cells. TLR: toll-like receptor; MyD88: myeloid differentiation primary response gene 88; IRF: interferon regulatory factor; NF-κB: nuclear factor kappa B; IFN: interferon.

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

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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.

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

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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

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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 μ*M* 2-mercaptoethanol, and human Flt3 ligand (Flt3L, 100 ng/ml; PeproTech, Rocky Hill, NJ) or murine granulocyte-macrophage colony-

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

Stimulation of cells and measurement of cytokine production

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

Flow cytometric analysis of costimulatory molecule expression

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

Reverse transcription-polymerase chain reaction analysis

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

In vivo *innate immune response to adenoviral vectors*

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

RESULTS

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

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

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

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

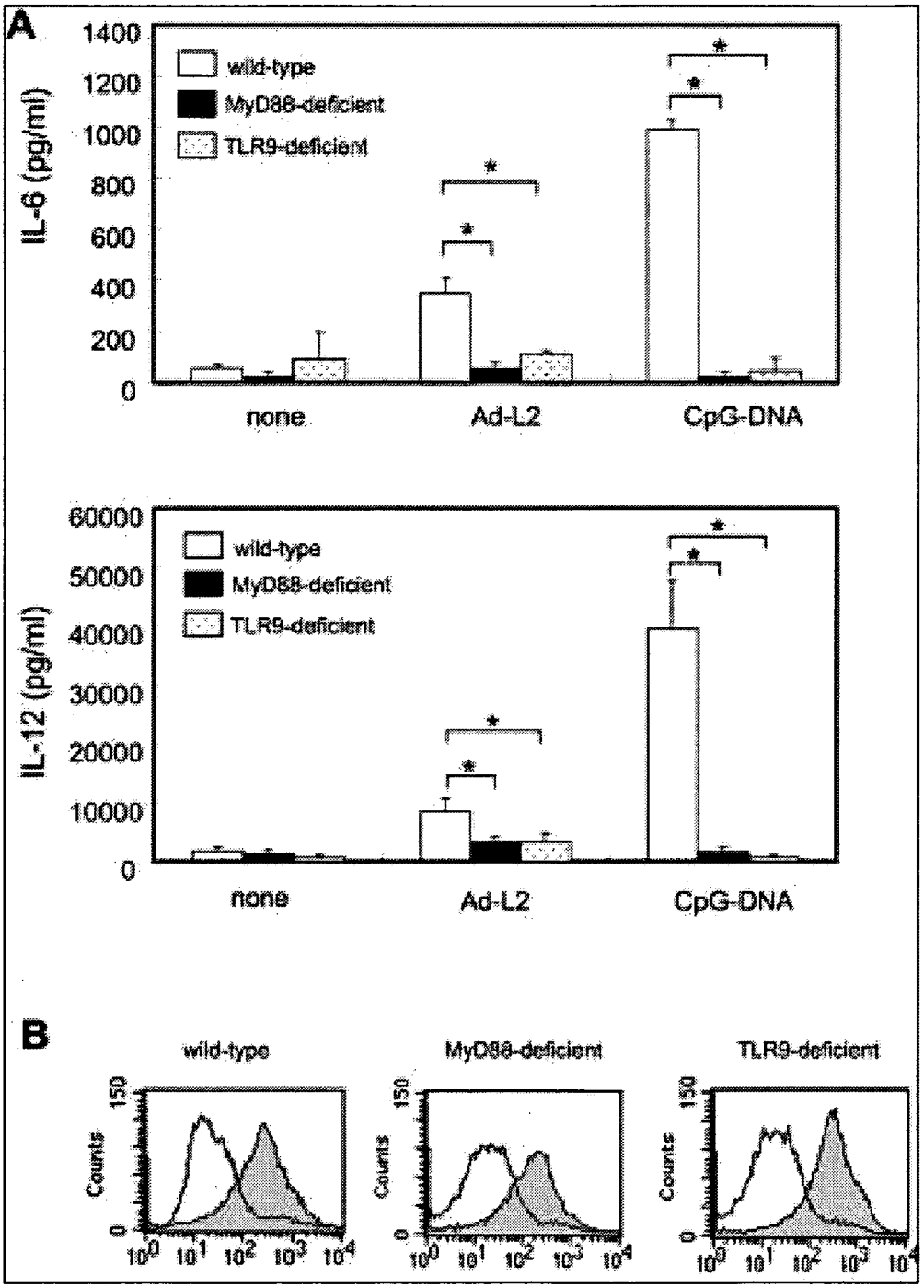

FIG. 1. MyD88- and TLR9-dependent activation of GM-CSF-DCs after Ad infection. (**A**) Ad vector-induced production of IL-6 (*top*) and IL-12 (*bottom*). GM-CSF-DCs from wild-type, MyD88-deficient, and TLR9-deficient mice were stimulated with Ad-L2 (10,000 VP/cell) or CpG-DNA (2.5 μM) for 48 hr, and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

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

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

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

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

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

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

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

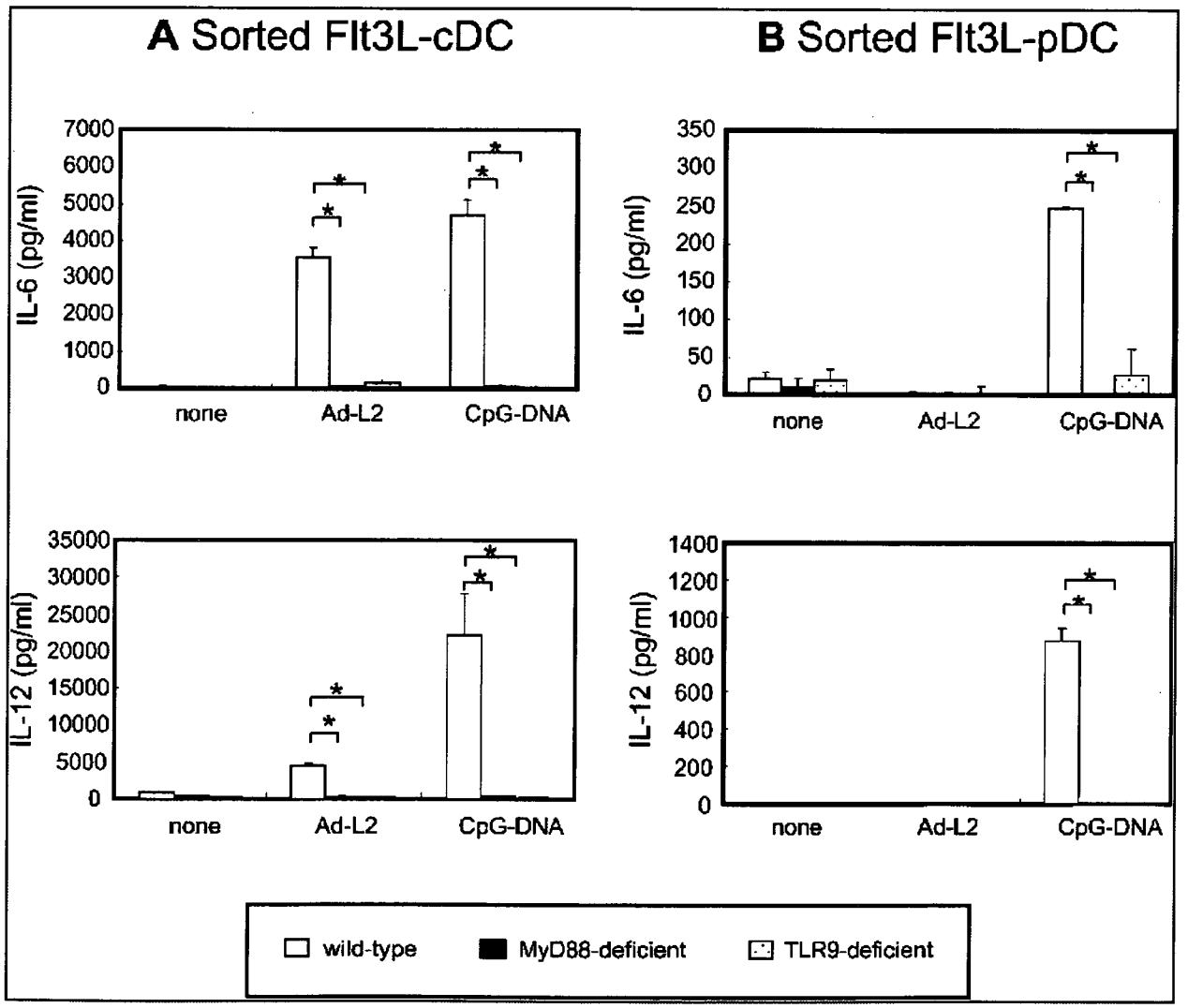

FIG. 2. Ad vector-infected MyD88- and TLR9-dependent production of IL-6 and IL-12 in Flt3L-DCs. cDCs (**A**) and pDCs (**B**) were isolated from Flt3L-stimulated bone marrow cells and purified by cell sorting. The cells were stimulated with Ad-L2 (10,000 VP/cell) or CpG-DNA (2.5 μM) for 48 hr, and then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

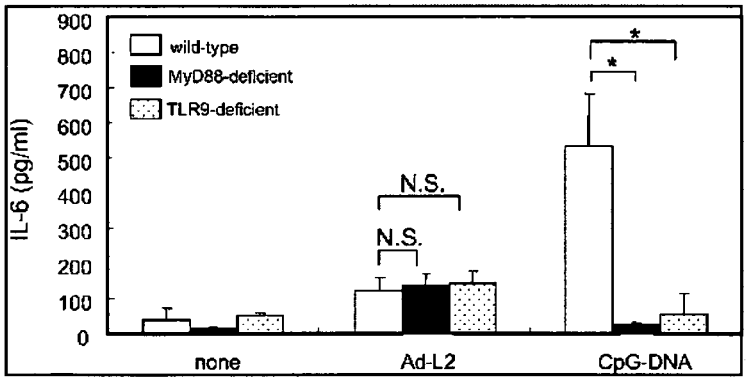

FIG. 3. MyD88- and TLR9-independent IL-6 production in peritoneal macrophages stimulated with Ad vectors. IFN-γ-activated peritoneal macrophages from wild-type, MyD88-deficient, and TLR9-deficient mice were incubated with Ad-L2 (10,000 VP/cell) or CpG-DNA (2.5 μ*M*) for 48 hr, and the amounts of IL-6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

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

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

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

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

DISCUSSION

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

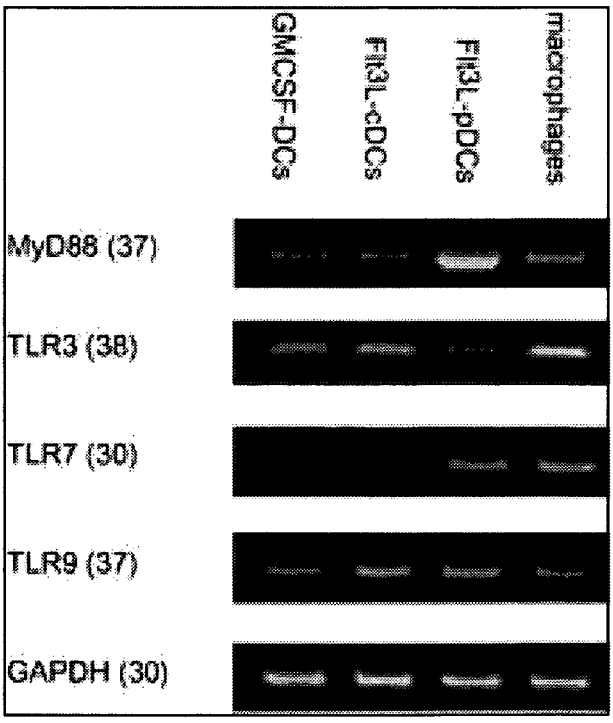

FIG. 4. MyD88, TLR3, TLR7, and TLR9 mRNA expression in immune cells. Total RNA samples were isolated from GM-CSF-DCs, Flt3L-cDCs, Flt3L-pDCs, and peritoneal macrophages. The expression of MyD88, TLR3, TLR7, and TLR9 mRNAs was tested by RT-PCR. From left to right: Lane 1, GM-CSF-DCs; lane 2, Flt3L-cDCs;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

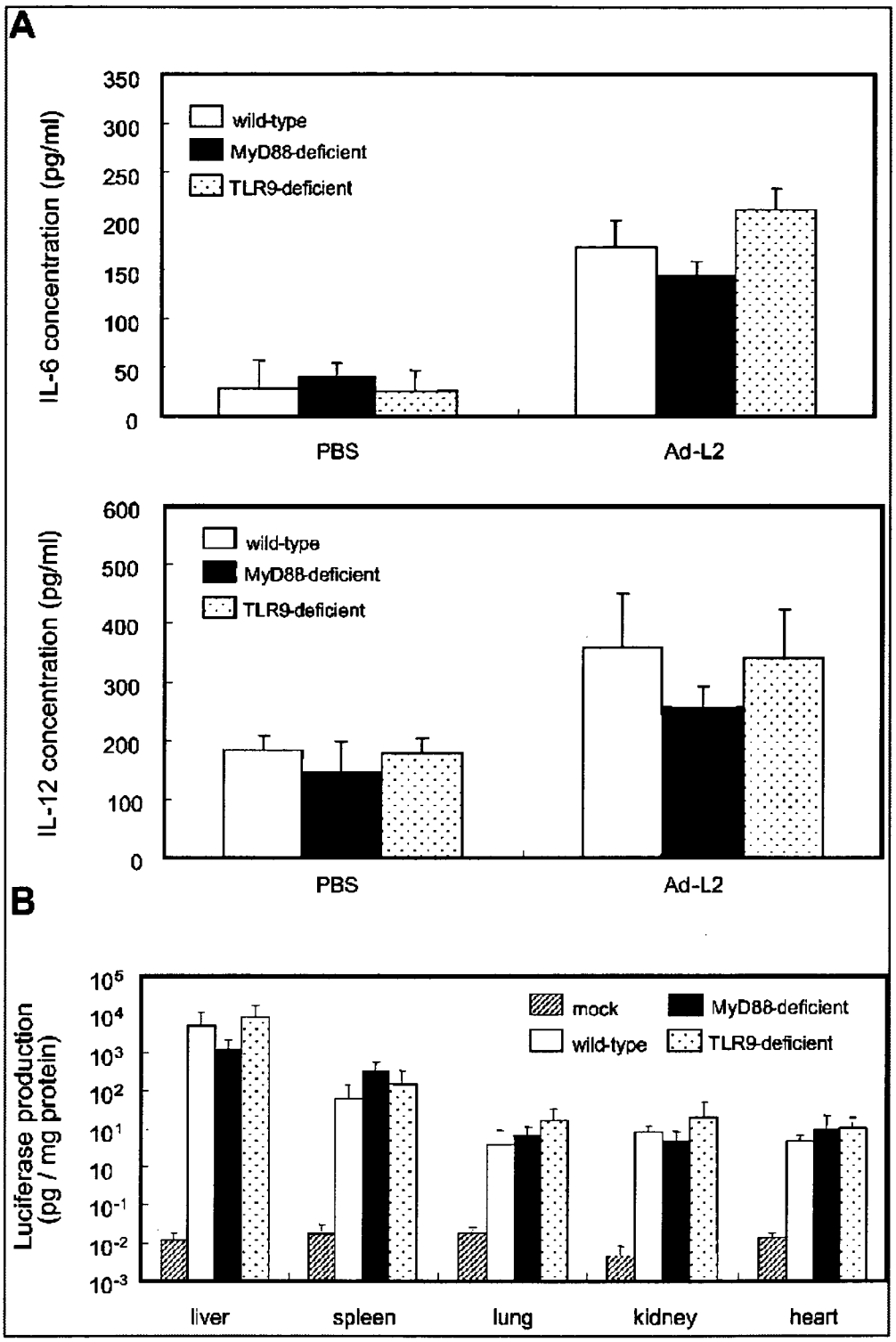

FIG. 5. (**A**) IL-6 (*top*) and IL-12 (*bottom*) concentration in serum and (**B**) luciferase production in organs after systemic administration of Ad-L2 to mice. Six hours postadministration, the blood and organs, including the heart, lung, liver, kidney, and spleen, were recovered. IL-6 and IL-12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

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

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

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

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

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

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

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

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AUTHOR DISCLOSURE STATEMENT

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