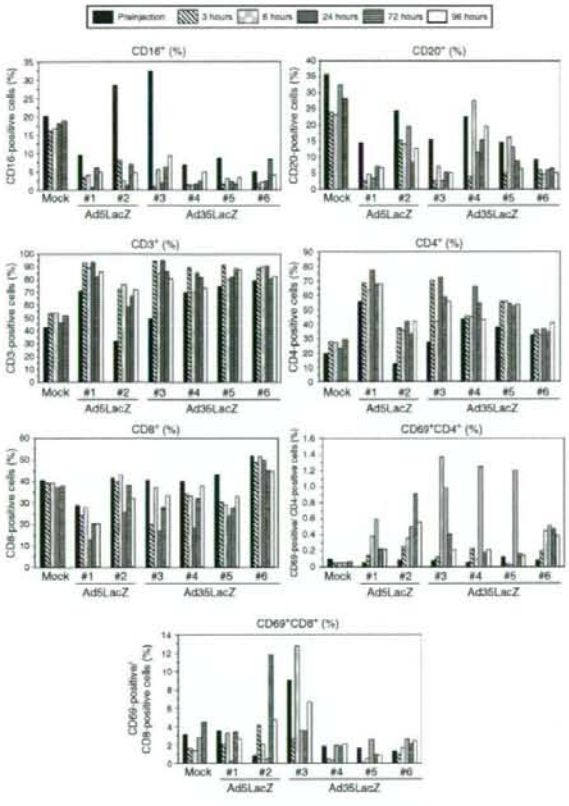

Figure 6 Profiles of peripheral blood lymphocyte subsets after systemic administration of adenoviral (Ad) vectors in cynomolgus monkeys. Ad35LacZ or Ad5LacZ was intravenously injected into cynomolgus monkeys and blood was collected as described for **Figure 2**. Peripheral blood mononuclear cells were stained with monoclonal antibodies following hemolysis, and fluorescence-activated cell sorting analysis was performed for evaluation of profiles of lymphocyte subsets.

elevation in the white blood cells was observed in the Ad vector–injected monkeys. The elevated white blood cells level returned to normal at 24 hours after the injection.

Next, we examined which types of blood cells were increased or decreased after Ad vector injection (**Figure 6**). The Ad vector injection induced a rapid decline in the percentages of $CD16^+$ cells (natural killer cells, granulocytes, and monocytes). Monkeys #2 and #3 showed sharp decreases of 71 and 97% of $CD16^+$ cells, respectively, at 3 hours after the injection. The percentages of $CD20^+$ cells (B cells) quickly dropped in all the monkeys, including a mock-infected monkey. In contrast, the $CD3^+$ cell (T-cell) levels were sharply elevated in the animals receiving the Ad vectors. We found a 1.1- to 2.3-fold increase in $CD3^+$ cell levels at 3 hours after the injection. $CD8^+$ cells did not increase, but rather decreased after the injection; however, increases in $CD4^+$ cells were found in the Ad vector–injected monkeys. The $CD4^+$ cell levels were 1.1- to 3.4-fold elevated compared with the preinjection levels, with a peak at 24 hours after the injection, in most of the animals. The administration of Ad vectors also increased

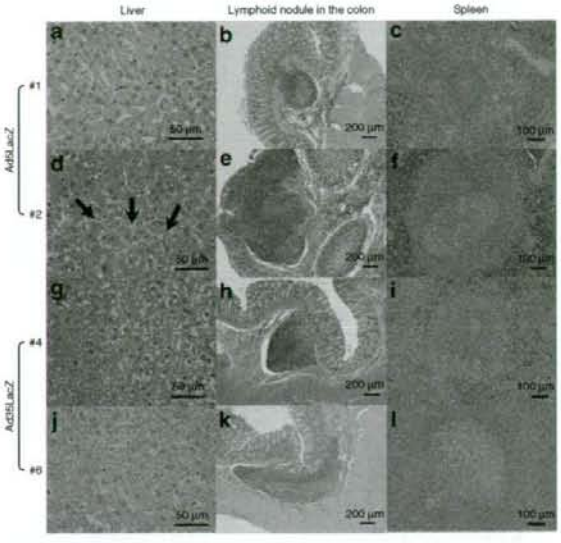

Figure 7 Histopathological analysis of liver, lymphoid nodules in the colon, and spleen. Representative histological sections of the liver (**a**, **d**, **g**, **j**), lymphoid nodules in the colon (**b**, **e**, **h**, **k**), and spleen (**c**, **f**, **i**, **l**) from animals killed 4 days after systemic injection of a low or high dose of Ad35LacZ (monkeys #4 and #6) or Ad5LacZ (#1 and #2). The arrows indicate necrosis of hepatocytes.

$CD69^+CD4^+$ cells (activated $CD4^+$ cells) more predominantly than $CD69^+CD8^+$ cells. Both $CD29^+CD4^+$ cells (memory helper T cells) and $CD29^-CD4^+$ cells (naive helper T cells) increased in the Ad vector–injected animals (data not shown). These results indicate that, overall, both Ad35 and Ad5 vectors cause similar changes in hematological profiles after systemic infusion.

Clinical observation and histopathological examinations

In order to perform a safety assessment of the Ad vectors, the health condition of the animals was monitored until necropsy. None of the Ad vector–infused monkeys showed any apparent abnormalities in appetite, body weight, body temperature, or heart rate. However, the low dose of Ad35LacZ (#3) induced vomiting 3 hours after the injection, and a skin rash was observed in monkey #2 on day 2.

In order to further evaluate the safety profiles of Ad vectors, organ histopathology was examined during necropsy. There were no obvious changes in the spleens of monkeys #1 and #3–#6, or in the livers in any of the animals. However, splenomegaly was found in monkey #2. The whitish nodules at the cut surface of the spleen in monkey #2 were the largest among those of all the monkeys examined. Marked swelling of the lymph nodules, especially in the colon and mesentery, was also found in monkey #2.

Microscopic analysis of tissue sections revealed that no apparent damage and inflammation were found in the liver of monkey #1 (**Figure 7a**). Although slight hyperplasia in the spleen white pulp occurred in this monkey (**Figure 7c**), no obvious changes were found in the lymphatic nodules of the colon (**Figure 7b**). In contrast, severe damage and inflammation, including necrosis of hepatocytes (**Figure 7d**, arrows) and infiltration of lymphocytes

into the Glisson's sheath (data not shown) were found in monkey #2. Furthermore, apparent severe hyperplasia in the lymphoid nodules of the colon (**Figure 7e**) and spleen white pulp (**Figure 7f**) had been induced in monkey #2. On the other hand, the livers of Ad35LacZ-treated monkeys exhibited almost no damage or inflammation (**Figure 7g** and **j**). In addition, Ad35LacZ induced hyperplasia in lymphoid nodules of the colon (**Figure 7h** and **k**) was only slightly developed and less serious than that induced by the high dose of Ad5LacZ. These results suggest that Ad5 vectors may cause more severe damage and/or inflammation in the liver and lymphoid nodules of the colon than Ad35 vectors. The spleen white pulp developed only slight hyperplasia in monkey #4 (**Figure 7i**), in contrast, the high dose of Ad35LacZ induced severe hyperplasia in the spleen white pulp (**Figure 7l**). The level of hyperplasia in spleen white pulp of monkey #6 appeared to be slightly more severe than that of monkey #2. The monkeys #3 and #5 did not show apparent abnormalities in the spleen or colon, although slight vacuolation in hepatocytes and infiltration of lymphocytes in the Glisson's sheath was found (data not shown). Hyperplasia in spleen white pulp and lymphatic follicles in the mesenteric, axillary, and inguinal lymph nodes (data not shown) occurred dose-dependently in the Ad35-injected animals as well as in the Ad5-injected ones.

DISCUSSION

In this study, subgroup B Ad35 vectors were intravenously infused into cynomolgus monkeys in order to evaluate the *in vivo* fundamental transduction properties of Ad35 vectors more thoroughly. Cynomolgus monkey CD46 and the CD46 of other nonhuman primates, have significant homology with human CD46 (ref. 19). In particular, short consensus repeats 1 and 2 (which are crucial for Ad35 binding to CD46),[22–24] of the CD46 of the cynomolgus monkey show high homology (85%) with those in human CD46. In addition, we confirmed that the monkey cells used in this study were highly stained with anti-human CD46 monoclonal antibody M177, which is specific for short consensus repeat 2, and that the antibody M177 significantly inhibited Ad35 vector–mediated transduction in the cynomolgus monkey cells (data not shown). The amino acid sequences important for Ad35 binding to CD46 (refs. 23,24) are also well conserved in cynomolgus monkey CD46. These results indicate that cynomolgus monkey CD46 serves as a cellular receptor for Ad35, at least in the context of *in vitro* transduction.

In this study, four and two cynomolgus monkeys were intravenously injected with the Ad35 and Ad5 vectors, respectively. We must exercise caution in interpreting the results because the sample size is small, as is natural in nonhuman primate studies. Overall, there are no dose responses in several transduction profiles of both Ad35 and Ad5 vectors, including blood concentration of Ad vectors and inflammatory cytokine production. The variations in the transduction profiles suggest that these profiles may depend largely on the specific Ad vector batch and on the differences between individuals, such as health conditions and genetic backgrounds, as well as on Ad vector doses. In the clinical trials using Ad vectors, inflammatory responses were dramatically different between patients receiving the same vector dose.[10] Gene therapy studies, both preclinical and clinical, should be performed with considerable caution in view of these individual differences. Further studies, including toxicogeomics, would be necessary in order to clarify which parameters play the most crucial roles in this entire process of transduction. Such studies would enable prediction of profiles of Ad vector–mediated transduction, and associated toxicities.

Although efficient transduction was achieved using Ad35 vectors *in vitro*, transduction of Ad35 vectors in the organs *in vivo* was hardly detectable after systemic infusion (**Figure 4**). In addition, the levels of Ad35 vector genome in the organs were one to five orders lower than those of the Ad5 vector genome (**Figure 3**). Previous studies demonstrated that, after systemic injection, Ad35 vectors were poor at transducing CD46-transgenic (CD46TG) mice, which ubiquitously express human CD46 in all the organs.[25,26] Chimeric Ad5 vectors containing Ad35 fiber protein also mediated much lower transgene expression in baboons than conventional Ad5 vectors did.[27] These results indicate that Ad35 vectors cannot transduce organs efficiently when introduced into the blood stream. There are two possible explanations for the poor transduction activity of Ad35 vectors after systemic administration. First, Ad35 vectors might be more susceptible than Ad5 vectors to degradation in the blood or in intracellular compartments such as endosomes/lysosomes after internalization. Fiber-substituted Ad5 vectors containing a fiber protein of Ad35 remain for a longer time in late endosome/lysosomal compartments after internalization than Ad5 vectors do.[28] Ad35 vectors might exhibit similar intracellular trafficking to the fiber-substituted Ad5 vectors, leading to high susceptibility to intracellular degradation. Second, Ad35 vectors might not be able to gain access to CD46 after systemic injection. CD46 is predominantly expressed on the basolateral sides of cells,[29,30] making it inaccessible to Ad35 vectors. Ad35 vectors which are not able to bind to CD46 on the cell surface would be phagocytosed into phagocytic cells, such as liver Kupffer cells, leading to degradation.

It is well known that erythrocytes of cynomolgus monkeys express CD46 (ref. 19) and that Ad35 causes hemagglutination of monkey erythrocytes.[31] Ad35 vectors might induce hemagglutination in the blood vessels after the injection, and this might lead to hemolysis and a decrease in the transduction efficiencies of Ad35 vectors. A large percentage of the Ad35 vectors recovered from the blood after the injection were associated with blood cells (**Figure 2c**). However, lactate dehydrogenase (a marker of hemolysis) levels in the sera of Ad35LacZ-injected animals at most of the time points were within normal levels and comparable with those in the sera of animals injected with Ad5LacZ, which does not induce hemagglutination of monkey erythrocytes. These results suggest that hemagglutination by Ad35 vectors would have, at most, a minimal influence on the transduction profiles of Ad35 vectors.

As mentioned earlier, CD46TG mice as well as cynomolgus monkeys were only poorly transduced with Ad35 vectors after intravenous administration, thereby suggesting that the transduction profiles of Ad35 vectors in CD46TG mice would correspond to those in primates and that CD46TG mice might be suitable as a small animal model for the study of Ad35 vectors. The profiles of inflammatory cytokine production by Ad35 vectors in cynomolgus monkeys were also approximately similar to those in CD46TG mice. Intravenous

infusion of Ad35 vectors resulted in levels of inflammatory cytokine production comparable to those induced by Ad5 vectors in the monkeys (**Figure 5b**) as well as in CD46TG mice.[32]

Histopathological analysis demonstrated that tissue damage and inflammatory responses, including hepatocyte necrosis, were less severe in all the Ad35 vector–infused monkeys than in the Ad5 vector–injected ones (**Figure 7**). Previous studies also demonstrated that Ad35 vectors are less immunogenic than Ad5 vectors in mice,[33,34] and this may result in the higher safety profiles of Ad35 vectors as compared to Ad5 vectors. It remains to be elucidated why Ad35 vectors produce less severe side effects than Ad5 vectors. Ad5 vectors were more widely distributed in most organs than Ad35 vectors, suggesting that Ad5 vectors may cause tissue damage and inflammatory responses throughout the whole body. On the other hand, Ad35LacZ induced much higher levels of IL-6 and interferon-γ in monkeys #5 and #3, respectively, than in the other Ad35LacZ-infused monkeys (**Figure 5b**), although no severe damage or inflammation was observed in these two animals. It remains unclear why such high levels of inflammatory cytokines were induced by Ad35 vectors in these animals; however, previous studies have indicated that the high levels of inflammatory cytokine induction might be involved in tissue damage.[9,35] It is important to pay attention to Ad35 vector–induced innate immune responses.

The poor transduction efficiencies of Ad35 vectors in organs after systemic administration could constitute another potential advantage in their use, namely, that locally administered Ad35 vectors would not cause unwanted side effects in organs other than the targeted organs, when draining from injected sites into the blood stream. This is in contrast to Ad5 vectors which, after injection into local tissues, have been shown to drain into the blood stream in large quantities and cause unwanted side effects in the liver and other organs.[36,37] We previously demonstrated that intramuscular injection of Ad35 vectors led to efficient transduction at the injected sites,[12] and thus local injection of Ad35 vectors would be expected to mediate efficient transduction at the injected sites without side effects in other organs.

In summary, we have demonstrated the transduction properties of Ad35 vectors after intravenous administration in nonhuman primates. Systemic infusion of Ad35 vectors did not result in detectable levels of transgene expression in the organs. Also, the tissue damage was less severe in the animals receiving Ad35 vectors than in those receiving Ad5 vectors, although two monkeys produced marked inflammatory cytokines after receiving Ad35 vectors. Further studies are in progress, focusing on the local injection of Ad35 vectors, and the results of these studies may further clarify the potential utility of Ad35 vectors.

MATERIALS AND METHODS

Ad vectors. An Ad5 vector and an Ad35 vector containing a β-galactosidase expression cassette, Ad5LacZ and Ad35LacZ, respectively, were prepared using an improved *in vitro* ligation method.[38–40] Briefly, for preparation of Ad5LacZ, pHMCMV6-LacZ, which was constructed by insertion of the β-galactosidase gene derived from pCMVβ (Clontech, Palo Alto, CA) into pHMCMV6,[39] was digested with I-*Ceu*I and PI-*Sce*I, and then ligated with I-*Ceu*I- and PI-*Sce*I-digested Ad5 vector plasmid pAdHM4.[39] The resulting plasmid was digested with *Pac*I and transfected into 293 cells with Superfect (Qiagen, Valencia, CA). The vector plasmid for Ad35LacZ was constructed in a similar manner, but using pHMCMV6-LacZ and pAdMS18.[25] The resulting plasmid was digested with *Sbf*I and transfected into 293-E1B cells,[25] which are a 293 transformant stably expressing Ad35 E1B-55K protein. The viruses were prepared using a standard method, and purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation. Determination of virus particle titers was accomplished spectrophotometrically using the methods of Maizel *et al.*[41] Luciferase-expressing Ad5 and Ad35 vectors, Ad5L and Ad35L, were constructed as explained earlier.[11]

In vitro *transduction.* Lung and kidney primary cells, isolated from embryonic cynomolgus monkeys and cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum, antibiotics, and L-glutamine, were seeded in a 96-well dish at 1×10^4 cells/well. On the following day, they were transduced with Ad5L or Ad35L at 300 and 3,000 vector particles/cell for 1.5 hours. After a 48-hour culture period, luciferase production in the cells was measured using a luciferase assay system (PicaGene LT2.0; Toyo Inki, Tokyo, Japan).

Animals. Young male cynomolgus monkeys (*Macaca fascicularis*) were housed and handled in accordance with the rules for animal care and management of the Tsukuba Primate Center and the guiding principles for animal experiments using nonhuman primates formulated by the Primate Society of Japan. The animals (~3 years of age, 1.88–2.96 kg) were certified free of intestinal parasites and seronegative for simian type-D retrovirus, herpesvirus B, varicella-zoster-like virus, and measles virus. The protocol of the experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation (Osaka, Japan).

In vivo *transduction.* Cynomolgus monkeys were sedated with ketamine (5–10 mg/kg) and injected with phosphate-buffered saline (mock), or Ad5LacZ or Ad35LacZ at 2×10^{12} vector particles/kg (high dose), 1×10^{12} vector particles/kg (intermediate dose), or 0.4×10^{12} vector particles/kg (low dose) through the saphenous vein at a rate of ~2 ml/minutes. Blood was collected for analysis at 3, 6, 24, 48, 72, and 96 hours after injection. Four days after vector administration, the monkeys were killed and the tissues were collected. Tissue samples were subjected to analysis as described in the later text.

β-Galactosidase assay and X-gal staining. β-Galactosidase activity in the organs was measured using Galacto-Light Systems (Applied Biosystems, Foster City, CA) as earlier described.[42] Protein concentrations were determined with a Bio-Rad assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. X-gal staining of tissue sections was performed as earlier described.[43]

Blood clearance and tissue distribution of Ad vectors. Blood clearance analysis of Ad vectors was performed using a real-time polymerase chain reaction assay, as earlier described.[8,40] Briefly, total DNA, including the Ad vector DNA, was isolated from whole blood samples. After isolation, the total DNA concentrations were determined, and the Ad DNA contents were quantified using a TaqMan fluorogenic detection system (ABI Prism 7700 sequence detector; Perkin-Elmer Applied Biosystems, Foster City, CA).

The association of Ad35 vectors to blood cells circulating in the blood stream was evaluated using a real-time polymerase chain reaction assay. Blood samples collected at the indicated time points were washed two times with phosphate-buffered saline immediately after isolation to remove unbound Ad35 vectors. After washing, total DNA was extracted from blood cells and the Ad35 DNA contents were assessed as described earlier.

The Ad DNA contents in each organ were similarly quantified using a real-time polymerase chain reaction assay, as described earlier, after isolation of the total DNA from each organ using a Automatic Nucleic Acid Isolation System (NA-2000; KURABO, Osaka, Japan).

Histopathology. For routine histopathology, tissues were fixed in 10% formalin at the time of necropsy, and processed for paraffin embedding.

Sections of 4-μm thickness were cut and stained with hematoxylin and eosin. The tissue sections were examined under a microscope.

Analysis of inflammatory cytokines, serum chemistry profiles, and hematology parameters. Blood was drawn from the saphenous veins of all the monkeys prior to vector administration and at 3, 6, 24, 72, and 96 hours after vector administration. Blood samples were collected into separate tubes containing either EDTA or no anticoagulant, for hematology and for determination of inflammatory cytokines and serum chemistry, respectively. Serum samples for analysis of inflammatory cytokines and serum chemistry were separated by centrifugation (4 °C, 2,500 rpm, 15 minutes), stored in a freezer at −80 °C, and thawed at the time of measurement. The levels of inflammatory cytokines (IL-6 and interferon-γ) in serum samples were measured using enzyme-linked immunosorbent assay (BioSource, Camarillo, CA). The serum chemistry parameters, which were measured with an automated chemistry analyzer AU400 (OLYMPUS, Tokyo, Japan), included AST, alanine aminotransferase, CPK, alkaline phosphatase, lactate dehydrogenase, and C-reactive protein. The hematology parameters that were determined included white blood cells, red blood cells, hemoglobin, platelets, $CD3^+$ cells, $CD4^+$ cells, $CD8^+$ cells, $CD16^+$ cells, $CD20^+$ cells, $CD29^+$ cells, and $CD69^+$ cells.

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SUPPLEMENTARY MATERIAL

Figure S1. *In vivo* transduction efficiencies of Ad35 and Ad5 vectors in cultured cynomolgus monkey T-cell line H-SCF.

Table S1. Dosing of cynomolgus macaques with β-galactosidase-expressing Ad vectors in this study.

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