

**Polyinosinic acid enhances delivery of adenovirus vectors *in vivo* by preventing sequestration in liver macrophages**

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Adenovirus is among the preferred vectors for gene therapy because of its superior *in vivo* gene-transfer efficiency. However, upon systemic administration, adenovirus is preferentially sequestered by the liver, resulting in reduced adenovirus-mediated transgene expression in targeted tissues. In the liver, Kupffer cells are responsible for adenovirus degradation and contribute to the inflammatory response. As scavenger receptors present on Kupffer cells are responsible for the elimination of blood-borne pathogens, we investigated the possible implication of those receptors in the clearance of the adenovirus vector. Polyinosinic acid [poly(I)], a scavenger receptor A ligand, was analysed for its capability to inhibit adenovirus uptake specifically in macrophages. In *in vitro* studies, the addition of poly(I) before virus infection resulted in a specific inhibition of adenovirus-induced gene expression in a J774 macrophage cell line and in primary Kupffer cells. In *in vivo* experiments, pre-administration of poly(I) caused a 10-fold transient increase in the number of adenovirus particles circulating in the blood. As a consequence, transgene expression levels measured in different tissues were enhanced (by 5- to 15-fold) compared with those in animals that did not receive poly(I). Finally, necrosis of Kupffer cells, which normally occurs as a consequence of systemic adenovirus administration, was prevented by the use of poly(I). No toxicity, as measured by liver-enzyme levels, was observed after poly(I) treatment. From our data, we conclude that poly(I) can prevent adenovirus sequestration by liver macrophages. These results imply that, by inhibiting adenovirus uptake by Kupffer cells, it is possible to reduce the dose of the viral vector to diminish the liver-toxicity effect and to improve the level of transgene expression in target tissues. In systemic gene-therapy applications, this will have great impact on the development of targeted adenoviral vectors.

## INTRODUCTION

One of the main challenges in gene therapy is to engineer vectors that are targeted systemically to specific receptors. Among the different gene-therapy vectors available, adenovirus is preferred because of its superior *in vivo* gene-transfer efficiency to a wide spectrum of both dividing and non-dividing cell types. However, its broad tropism also represents an important limitation for its use in therapeutic applications where specific gene transfer is required. Upon systemic administration, the majority of the adenovirus accumulates rapidly in the liver. This is accompanied by the induction of acute-phase responses, including elevation of cytokines (tumour necrosis factor alpha, interleukin-6, etc.) and chemokines (Mip-2, Ip-10, etc.) and expression of genes involved in leukocyte trafficking (Cotter & Muruve, 2005; Muruve, 2004; Muruve *et al.*, 1999; Zhang *et al.*, 2001). Hepatic Kupffer cells (KCs) have been shown to contribute to the inflammatory response, and to be responsible for the short half-life of the circulating viral particles and indirectly for the lower transgene expression in other cell types (Alemany *et al.*, 2000; Lieber *et al.*, 1997; Smith *et al.*, 2008; Wolff *et al.*, 1997). Elimination of viruses by KCs results in a non-linear correlation between low doses of injected viruses and transgene expression (Fechner *et al.*, 1999; Tao *et al.*, 2001; Ziegler *et al.*, 2002). Administration of sufficient numbers of viral particles to exceed a threshold level necessary to saturate the uptake by KCs can produce a linear correlation with transgene expression (Fechner *et al.*, 1999; Tao *et al.*, 2001; Ziegler *et al.*, 2002).

The development of systemically deliverable adenoviral vectors requires two main conditions: targeting of the virus to selected cells and detargeting of the virus from native receptors, especially those present in the liver, which sequesters the majority of intravenously injected virus via KCs, sinusoidal liver endothelial cells and hepatocytes. From *in vitro* studies, it was found that adenovirus infection starts by a high-affinity interaction of the viral fiber knob with the coxsackievirus–adenovirus receptor (CAR) (Bergelson *et al.*, 1997; Tomko *et al.*, 1997). Subsequently, internalization of the virus occurs via binding of the RGD motif in the penton base protein to cellular  $\alpha_3\beta_3$  and  $\alpha_5\beta_5$  integrins (Wickham *et al.*, 1993). Furthermore, heparan sulfate glycosaminoglycans have been shown to provide a third cell-surface interaction with adenovirus particles (Dechecchi *et al.*, 2000; Smith *et al.*, 2003a).

A number of strategies to alter adenovirus tropism have been used to eliminate adenovirus–receptor interactions (Bangari & Mittal, 2006; Glasgow *et al.*, 2006; Mizuguchi & Hayakawa, 2004; Nicklin *et al.*, 2005). Initial attempts to detarget the liver have been done by ablating the CAR- and integrin-based interactions (Akiyama *et al.*, 2004; Koizumi *et al.*, 2003; Martin *et al.*, 2003; Nakamura *et al.*, 2003). Also, fiber-shaft modifications have been introduced to eliminate virus interactions with native receptors (Bayo-Puxan *et al.*, 2006; Breidenbach *et al.*, 2004; Nicol *et al.*, 2004; Shayakhmetov *et al.*, 2004; Smith *et al.*, 2006).

*al.*, 2003b; Vigne *et al.*, 2003). However, the results are variable. Some animal studies showed reduced transduction of the liver, whilst in others, liver uptake was not affected by these modifications. Smith *et al.* (2003b) examined the role of heparan sulfate proteoglycans (HSPGs) in liver adenovirus uptake *in vivo* by replacing a putative HSPG-binding motif (KKTK) in the fiber shaft with an irrelevant peptide sequence, and observed a reduction in liver transgene expression of 90 %. However, Di Paolo *et al.* (2007) demonstrated recently that the KKTK motif plays only a minimal role in hepatic infectivity *in vivo*.

Chemical methods have also been used to detarget adenovirus. These methods include PEGylation (Croyle *et al.*, 2000, 2002; Fisher *et al.*, 2001; Lanciotti *et al.*, 2003; Ogawara *et al.*, 2004; O'Riordan *et al.*, 1999) or polymer coating of the adenovirus (Green *et al.*, 2004), which resulted in decreased toxicity and increased plasma half-life of adenovirus, allowing site-specific targeting.

Specific strategies aimed at preventing the interaction of adenovirus with KCs have been devised based on the selective depletion of KCs or pre-dosing of animals with transcriptionally inactive adenovirus (Alemany *et al.*, 2000; Lieber *et al.*, 1997; Schiedner *et al.*, 2003b; Wolff *et al.*, 1997). Scavenger receptors on KCs are responsible for the elimination of blood-borne pathogens and polyanionic macromolecules, such as modified lipoproteins and modified albumins (Gordon, 2002; Gough & Gordon, 2000; Krieger, 1997; Mukhopadhyay & Gordon, 2004; Peiser *et al.*, 2002; Platt & Gordon, 2001; Platt *et al.*, 2002; Shirai *et al.*, 1999; Swart *et al.*, 1999; Taylor *et al.*, 2005; Yamada *et al.*, 1998).

In this study, we hypothesized that the scavenger receptors present on KCs may serve as a receptor for adenovirus. To investigate this hypothesis, we isolated KCs and hepatocytes and infected them with an adenoviral vector expressing a reporter gene. Specific inhibition of gene expression was observed when a scavenger receptor A ligand, polyinosinic acid [poly(I)], was applied to the cells prior to adenovirus administration. *In vivo*, pre-administration of poly(I) in mice and rats resulted in a higher level of transgene expression with no effect on toxicity. Overall, these findings suggest that this approach will be useful for increasing the selectivity and efficiency of gene transfer in gene-therapy applications.

## METHODS

### Cells.

The murine macrophage-like J774 cell line, the hepatocellular carcinoma HepG2 cell line and the human embryonic kidney 293 cell line were obtained from the ATCC. The mouse endothelioma H5V cell line was kindly provided by Dr A. Vecchi (Mario Negri Institute for Pharmacological Research, Milan, Italy). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen) containing L-glutamine (Gibco-BRL), 10 % fetal bovine serum (FBS; BioWhittaker) and penicillin (100 IU ml<sup>-1</sup>)/streptomycin (100 µg ml<sup>-1</sup>) (Gibco-BRL) at 37 °C in a humidified 5 % CO<sub>2</sub>/95 % air atmosphere. The adenovirus-transformed human embryonic kidney 293 cell line was cultured in DMEM/F-12 (Gibco-BRL) containing 10 % FBS, 2 mM L-glutamine (Gibco-BRL) and penicillin (100 IU ml<sup>-1</sup>)/streptomycin (100 µg ml<sup>-1</sup>) (Gibco-BRL).

Rat hepatocytes and KCs were isolated from male Wag/Rij rats (200–250 g; Harlan CPB) after collagenase perfusion of the liver, followed by centrifugation and counterflow centrifugal elutriation as described previously (Kamps *et al.*, 1997). After isolation, hepatocytes were cultured in 24-well plates in Williams E medium (Gibco Invitrogen) supplemented with 10 % heat-inactivated FBS, 2 mM L-glutamine, 100 U penicillin ml<sup>-1</sup> and 100 µg streptomycin ml<sup>-1</sup>. KCs were grown in 24-well plates in RPMI 1640 medium (Gibco Invitrogen) supplemented with 20 % FBS, 2 mM L-glutamine, 100 U penicillin ml<sup>-1</sup> and 100 µg streptomycin ml<sup>-1</sup>. The purity of KCs was controlled by immunocytochemistry using the ED2 antibody and by staining for endogenous peroxidase activity, for which KCs are positive and endothelial cells are negative. The purity was >80 %.

### Inhibitors.

The following scavenger receptor-effective and -ineffective ligands were used: poly(I) and polycytidylic acid [poly(C)] potassium salts (both from Sigma) and plasmid DNA.

### Adenoviral vector.

AdTL is an E1- and E3-deleted recombinant serotype 5 adenovirus (He *et al.*, 1998) that contains a green fluorescent protein (GFP) and luciferase gene-expression cassette, each under the control of a cytomegalovirus promoter (Alemany & Curiel, 2001). The virus was grown in 293 cells and purified in HEPES/sucrose buffer (pH 8.0) according to a conventional double CsCl gradient centrifugation method (Becker *et al.*, 1994), and the number of viral particles (vp) was calculated from the OD<sub>260</sub>. The number of p.f.u. was determined by plaque-forming assay. The ratio vp : p.f.u. ratio was determined to be 1 : 18.7.

### ***In vitro* infection and reporter-gene expression analysis.**

J774 and HepG2 cells were seeded into 96-well culture plates at a density of 10 000 cells per well for viral transduction experiments. After 24 h, cells were first incubated for 1 h at 37 °C in the presence or absence of scavenger-receptor inhibitors [poly(I), poly(C) or plasmid DNA] as indicated in the experiments. After 1 h, AdTL was added at a concentration of 1000 vp per cell in DMEM containing 2 % FBS (infection medium). One hour after infection, the infection medium was replaced by normal culture medium and cells were incubated for 48 h before performing the luciferase assay. The cells were lysed with cell-culture lysis buffer (Promega) and the lysates were analysed with the luciferase assay system (Promega) on a LumiCount luminometer (Packard). All data are expressed as relative light units (RLU).

Adenovirus infection of freshly isolated hepatocytes and KCs was performed as described above in the presence or absence of poly(I). Forty-eight hours post-infection, cells were analysed for expression of the GFP reporter protein by using fluorescence microscopy.

### **Animal infection and transgene-expression analysis.**

The effect of scavenger receptor A blockade on liver transgene levels and adenovirus blood circulation times were determined in C57/Bl mice or WagRij rats (Harlan CPB). Animals were anaesthetized (isoflurane/N<sub>2</sub>O/O<sub>2</sub> inhalation) and the indicated amount of vp diluted in HEPES/sucrose buffer (pH 8.0) was injected intravenously via the orbital plexus (mice) or penis vein (rats). When indicated, 4.0 or 0.2 mg poly(I) in PBS was injected into rats or mice, respectively, 5 min prior to injection of the virus. An aliquot of blood was collected by orbital puncture 5, 30, 60 and 120 min after intravenous administration. The blood samples were then centrifuged to collect plasma and the numbers of vp in the plasma samples were subsequently measured by serial dilution and infection of 293 cells based on a conventional plaque-forming assay. In brief, each plasma sample was diluted serially in DMEM/F-12 supplemented with 2 % heat-inactivated FBS and added to 293 cells plated in 96-well tissue-culture plates (10 000 cells per well). After 14 days, cells were analysed for GFP expression and cytopathic effect. To estimate the number of vp in the plasma sample, this limiting-dilution assay was performed in parallel with the original solution of virus constructs injected into the animal.

For detection of transgene levels, animals were sacrificed 48 h after viral injection and organs were excised and frozen in liquid N<sub>2</sub>. Tissue samples were ground into a fine powder with a pestle and mortar in an ethanol/dry ice bath. The tissue powders were consequently lysed with cell-culture lysis buffer (Promega) and, after three cycles of freeze–thawing, centrifuged and the recovered supernatants were analysed with the luciferase assay system (Promega) on a LumiCount luminometer (Packard). Protein concentration was based on the assumption that one-third of the total tissue wet mass is protein, to normalize the RLU values.

The experiments were performed according to the European ethical board statement. Approval and description of the experiments are contained in the D2772 and D4314A documents at the University of Groningen.

### **Quantitative real-time PCR.**

DNA was isolated from liver tissue by using DNeasy Tissue kits (Qiagen) according to the manufacturer's protocol. DNA was purified by using minicolumns and dissolved in elution buffer, and its concentration was determined spectrophotometrically ( $A_{260}/A_{280}$ ). The concentration of adenovirus DNA was determined by real-time PCR using the Applied Biosystems Prism 7900HT sequence detection system with SDS 2.1 software. Amplification was carried out in a total volume of 20  $\mu$ l with SYBR Green PCR MasterMix (Applied Biosystems), forward (hexon; 5'-CTTCGATGATGCCGCAGTG-3') and reverse (5'-GGGCTCAGGTACTCCGAGG-3') primers and extracted DNA. The parameters used were one cycle of 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 56 °C for 15 s and 72 °C for 40 s. Adenovirus copy number was quantified by using a standard curve created from dilutions of adenovirus DNA from 1 500 000 copies to 15 copies in a background of mouse genomic DNA. Samples were amplified in duplicate and the mean total copy number was normalized to copies of viral DNA ( $\mu$ g DNA)<sup>-1</sup>.

### **Immunohistochemistry.**

Immunohistochemistry was performed on frozen liver sections to locate KCs with the F4/80 antibody (Serotec). Livers were frozen in liquid N<sub>2</sub> and 7  $\mu$ m sections were cut, fixed with acetone, air-dried and rehydrated in PBS. Primary antibody was used at a 1 : 50 dilution, followed by rabbit anti-rat-biotin at a 1 : 100 dilution. Colour development was performed with 3-amino-9-ethylcarbazole (AEC; Sigma) dissolved in N,N-dimethylformamide (Merck)/0.5 M acetate buffer, pH 4.9. Slides were counterstained with Mayer's haematoxylin and mounted in Kaiser's glycerin.

### **Statistical analysis.**

Differences in gene expression were analysed by using a one-sided paired Student's *t*-test, assuming equal variance. Differences were considered to be significant when  $P < 0.05$ .

## RESULTS

### ***In vitro* evaluation of scavenger-receptor inhibitors**

Macrophage scavenger-receptor ligands are polyanionic molecules or macromolecular complexes (Gordon, 2002). We used a panel of these ligands to determine the role of scavenger receptors in adenovirus transduction. First, we compared the effect of these ligands on virus transduction in the J774 cell line (scavenger receptor A-positive and CAR-negative) and a cancer cell line, HepG2 (scavenger receptor A-negative and CAR-positive) (Choi *et al.*, 2004; Hamblin *et al.*, 2000; Rhainds *et al.*, 1999) (Fig. 1). For infection, a basic adenoviral vector (AdTL) containing reporter genes was used. In J774 cells, poly(I), a scavenger receptor-effective ligand (Krieger & Herz, 1994; Platt & Gordon, 2001), reduced the transgene levels by 83 % when used at  $25 \mu\text{g ml}^{-1}$ , whereas poly(C) ( $25 \mu\text{g ml}^{-1}$ ) or plasmid DNA ( $10 \mu\text{g ml}^{-1}$ ), scavenger receptor-ineffective ligands (Krieger & Herz, 1994; Platt & Gordon, 2001), did not. In HepG2 cells, no reduction in transgene levels was measured when the cells were infected in the presence of the ligands, including poly(I). Poly(I) did not reduce GFP expression in freshly isolated rat hepatocytes when infected with adenovirus (Fig. 2), whereas expression in KCs was blocked almost completely (Fig. 2). As poly(I) is a selective ligand of scavenger receptors, these data suggest that scavenger receptors are involved in adenovirus uptake in macrophages.

### **Effects of poly(I) on gene expression *in vivo***

Based on these results, we carried out *in vivo* experiments in rats to determine whether inhibition of the scavenger receptors would indeed result in a reduction of virus sequestration by KCs and thus an increment of virus transfer to tissues. Animals were injected with poly(I) or PBS prior to administration of AdTL. Transgene expression was analysed 48 h post-infection. In frozen liver sections of rats, GFP expression increased when poly(I) was administered (Fig. 3a) and distribution changed from a patchy pattern to a regular diffuse pattern. Together, these results can be attributed to diminished uptake of virus by liver macrophages and an increase in infection and transduction in surrounding liver tissue, through the CAR receptor and/or HSPGs on hepatocytes (Smith *et al.*, 2003a). Higher values for luciferase expression, measured in different organs, also resulted when poly(I) was injected prior to adenovirus administration (Fig. 3b). The higher luciferase activity, registered as a consequence of the use of poly(I), can be explained by a reduction of viral particles degraded by liver macrophages. This results in more viral particles being available to infect tissues expressing the virus native receptor.

### **Effects of poly(I) on adenovirus clearance from blood**

To evaluate whether blockade of the scavenger receptors by poly(I) results in reduced clearance of adenovirus from the circulation, blood samples from mice were analysed for adenovirus levels by plaque-forming assays. At the low dose of adenovirus,  $5 \times 10^9$  vp per

mouse, which was selected to be below the threshold of KC saturation (Tao *et al.*, 2001; Ziegler *et al.*, 2002), we observed a rapid clearance of virus from the blood, with a maximum level of virus present in the blood of approximately  $5 \times 10^3$  p.f.u.  $\text{ml}^{-1}$  at 5 min after virus administration. Pre-administration of poly(I) increased the maximum level by more than 10-fold. At the high dose of virus administration,  $5 \times 10^{10}$  vp per mouse, a similar increase in the maximum level of circulating virus was measured 30 min after the virus injection (Fig. 4). These data demonstrate that pre-administration of poly(I) to animals increased blood levels of adenovirus.

### **Effects of poly(I) on adenovirus levels in the liver**

In mice injected with the low and high doses of adenovirus,  $5 \times 10^9$  or  $5 \times 10^{10}$  particles, luciferase activity and adenovirus content in the liver were compared. At both doses, the viral copy number of liver tissue, as determined by RT-PCR 48 h after administration, was significantly higher when poly(I) was administered prior to AdTL, compared with AdTL alone (Fig. 5a). This resulted in an accompanying higher gene-expression level, as determined by luciferase measurements (Fig. 5b). The pre-virus injection of poly(I) results in a reduced clearance of adenovirus, with a consequent enhancement of viral particles and an increase in transgene levels in the liver.

### **Poly(I) toxicity in combination with adenovirus**

Adenovirus administration can induce liver toxicity, reduction of KC numbers and elevated liver-enzyme levels (Liu *et al.*, 2003; Manickan *et al.*, 2006; Schiedner *et al.*, 2003a; Shayakhmetov *et al.*, 2004). Mouse liver sections were stained for KCs 48 h after administration of AdTL and KC numbers were quantified. With the low dose of adenovirus ( $5 \times 10^9$  vp), KC numbers remained unchanged (data not shown). At the high dose of virus ( $5 \times 10^{10}$  vp), a reduction in KC count was observed in animals injected with AdTL only, but not in mice pre-injected with poly(I) (Fig. 6a, b). The levels of the liver enzymes aspartate aminotransferase and alanine aminotransferase in serum obtained 48 h after injection of AdTL only or AdTL in combination with poly(I) are low and very similar, indicating no toxicity from treatment with either adenovirus or virus combined with poly(I) (Fig. 7) at this time point. However, to study the direct effect of poly(I) on the toxicity caused by uptake by KCs, a similar experiment should be performed within 24 h of adenovirus administration.

## DISCUSSION

We present a new method to detarget adenoviral vectors from KCs, leading to an increase in adenovirus-mediated transgene expression in tissues and reduced liver toxicity. In various studies, detargeting of the liver of systemically administered adenoviral vectors has been addressed in different manners. Although the early steps of adenovirus infection have been studied extensively *in vitro*, much is still unknown about the mechanisms governing adenovirus infection *in vivo*. To date, attempts to modify adenovirus liver tropism by abolishing CAR and/or integrin interactions have not been successful in all animal models analysed (Brunetti-Pierri *et al.*, 2004; Hodges *et al.*, 2005; Lievens *et al.*, 2004; Nicol *et al.*, 2004; Park *et al.*, 2005; Smith *et al.*, 2002).

Shayakhmetov *et al.* (2004) demonstrated that coagulation factor IX and the complement component C4-binding protein can bind the adenovirus fiber-knob domain and provide a bridge for virus uptake through cell-surface HSPGs and low-density lipoprotein receptor-related protein. An adenoviral vector that contained mutations in the fiber-knob domain that ablated blood-factor binding demonstrated significantly reduced infection of liver cells and liver toxicity *in vivo* (Shayakhmetov *et al.*, 2005). Intravenous delivery of adenovirus results in interaction of the virus with circulating platelets (Stone *et al.*, 2007). Virus-platelet aggregates are then taken up by KCs and degraded. Depletion of platelets prior to virus infection could improve levels of target-cell transduction at a lower vector dose (Stone *et al.*, 2007).

The ability of KCs to take up large particles, such as adenovirus, is well established (Smith *et al.*, 2008). In these studies, we show that poly(I), a scavenger receptor A inhibitor, has a profound effect on the sequestration of adenovirus by KCs.

We demonstrated previously (Kamps *et al.*, 1997) that poly(I) inhibited the uptake of negatively charged liposomes by KCs efficiently, indicating that scavenger receptors are involved in the uptake of these liposomes. Scavenger-receptor binding is thought to result from the intrinsic negative charge of the binding ligand. Although the fiber of Ad5 is positively charged to promote binding to target cells, the rest of the capsid displays predominantly negatively charged residues (Mei & Wadell, 1995). Also, serum proteins associated on the surface may play an important role in hepatic uptake of negatively charged particles via scavenger receptors (Furumoto *et al.*, 2004). Here, we show that binding of adenovirus to KCs can be abolished by pre-treatment with poly(I). The reduced liver sequestration of adenovirus observed after pre-treatment with poly(I) allows lower doses to be used with similar transgene-expression levels.

In our study, we did not attempt to detarget the adenovirus from its natural receptor (CAR) and, thus, we observed increased hepatic expression after pre-treatment with poly(I). This may be a feasible approach for treating liver or metabolic diseases, but may not be desirable for other gene-therapy applications. Detargeting hepatocytes may be

accomplished by genetic CAR ablation of adenovirus, with concomitant introduction of targeting ligands into the adenovirus capsid by chemical modification (Ogawara *et al.*, 2004) or by using bifunctional ligands, such as bispecific antibodies (Haisma *et al.*, 2000).

The results indicate that blocking of scavenger receptor A by administration of poly(I) prior to adenovirus injection can be used to reduce the clearance of virus by KCs and is a feasible approach to increase the availability of adenovirus without affecting toxicity. Higher availability of adenoviral particles for specific targeting will increase the efficiency of gene therapy. Poly(I) combined with targeted viral vector is a powerful tool for gene-therapy applications.

## **ACKNOWLEDGEMENTS**

This work has been supported by European Community 'Anti-Tumour Targeting' grant no. LSHC-CT-2005-518178. We thank Dr R. Alemany (Institut d'Investigació Biomèdica de Bellvitge–Institut Català d'Oncologia, L'Hospitalet de Llobregat, Barcelona, Spain) for generously providing us with the AdTL virus, and Bert Dontje (Department of Pathology and Laboratory Medicine, Medical Biology section, UMCG, University of Groningen) for technical assistance during animal experiments.

## REFERENCES

- Akiyama, M., Thorne, S., Kirn, D., Roelvink, P. W., Einfeld, D. A., King, C. R. & Wickham, T. J. (2004).** Ablating CAR and integrin binding in adenovirus vectors reduces nontarget organ transduction and permits sustained bloodstream persistence following intraperitoneal administration. *Mol Ther* **9**, 218–230. [Medline](#)
- Alemaný, R. & Curiel, D. T. (2001).** CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. *Gene Ther* **8**, 1347–1353. [Medline](#)
- Alemaný, R., Suzuki, K. & Curiel, D. T. (2000).** Blood clearance rates of adenovirus type 5 in mice. *J Gen Virol* **81**, 2605–2609. [Medline](#)
- Bangari, D. S. & Mittal, S. K. (2006).** Current strategies and future directions for eluding adenoviral vector immunity. *Curr Gene Ther* **6**, 215–226. [Medline](#)
- Bayo-Puxan, N., Cascallo, M., Gros, A., Huch, M., Fillat, C. & Alemaný, R. (2006).** Role of the putative heparan sulfate glycosaminoglycan-binding site of the adenovirus type 5 fiber shaft on liver detargeting and knob-mediated retargeting. *J Gen Virol* **87**, 2487–2495. [Medline](#)
- Becker, T. C., Noel, R. J., Coats, W. S., Gomez-Foix, A. M., Alam, T., Gerard, R. D. & Newgard, C. B. (1994).** Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol* **43**, 161–189. [Medline](#)
- Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L. & Finberg, R. W. (1997).** Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* **275**, 1320–1323. [Medline](#)
- Breidenbach, M., Rein, D. T., Wang, M., Nettelbeck, D. M., Hemminki, A., Ulasov, I., Rivera, A. R., Everts, M., Alvarez, R. D. & other authors (2004).** Genetic replacement of the adenovirus shaft fiber reduces liver tropism in ovarian cancer gene therapy. *Hum Gene Ther* **15**, 509–518. [Medline](#)
- Brunetti-Pierri, N., Palmer, D. J., Beaudet, A. L., Carey, K. D., Finegold, M. & Ng, P. (2004).** Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. *Hum Gene Ther* **15**, 35–46. [Medline](#)
- Choi, C. F., Tsang, P. T., Huang, J. D., Chan, E. Y., Ko, W. H., Fong, W. P. & Ng, D. K. (2004).** Synthesis and in vitro photodynamic activity of new hexadeca-carboxy phthalocyanines. *Chem Commun (Camb)* 2236–2237. [Medline](#)
- Cotter, M. J. & Muruve, D. A. (2005).** The induction of inflammation by adenovirus vectors used for gene therapy. *Front Biosci* **10**, 1098–1105. [Medline](#)
- Croyle, M. A., Yu, Q. C. & Wilson, J. M. (2000).** Development of a rapid method for the PEGylation of adenoviruses with enhanced transduction and improved stability under harsh storage conditions. *Hum Gene Ther* **11**, 1713–1722. [Medline](#)

**Croyle, M. A., Chirmule, N., Zhang, Y. & Wilson, J. M. (2002).** PEGylation of E1-deleted adenovirus vectors allows significant gene expression on readministration to liver. *Hum Gene Ther* **13**, 1887–1900.

[Medline](#)

**Dehecchi, M. C., Tamanini, A., Bonizzato, A. & Cabrini, G. (2000).** Heparan sulfate glycosaminoglycans are involved in adenovirus type 5 and 2-host cell interactions. *Virology* **268**, 382–390.

[Medline](#)

**Di Paolo, N. C., Kalyuzhniy, O. & Shayakhmetov, D. M. (2007).** Fiber shaft-chimeric adenovirus vectors lacking the KKTK motif efficiently infect liver cells in vivo. *J Virol* **81**, 12249–12259. [Medline](#)

**Fechner, H., Haack, A., Wang, H., Wang, X., Eizema, K., Pauschinger, M., Schoemaker, R., Veghel, R., Houtsmuller, A. & other authors (1999).** Expression of coxsackie adenovirus receptor and alphav-integrin does not correlate with adenovector targeting in vivo indicating anatomical vector barriers.

*Gene Ther* **6**, 1520–1535. [Medline](#)

**Fisher, K. D., Stallwood, Y., Green, N. K., Ulbrich, K., Mautner, V. & Seymour, L. W. (2001).** Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. *Gene Ther* **8**,

341–348. [Medline](#)

**Furumoto, K., Nagayama, S., Ogawara, K., Takakura, Y., Hashida, M., Higaki, K. & Kimura, T. (2004).** Hepatic uptake of negatively charged particles in rats: possible involvement of serum proteins in recognition by scavenger receptor. *J Control Release* **97**, 133–141. [Medline](#)

**Glasgow, J. N., Everts, M. & Curiel, D. T. (2006).** Transductional targeting of adenovirus vectors for gene therapy. *Cancer Gene Ther* **13**, 830–844. [Medline](#)

**Gordon, S. (2002).** Pattern recognition receptors: doubling up for the innate immune response. *Cell* **111**, 927–930. [Medline](#)

**Gough, P. J. & Gordon, S. (2000).** The role of scavenger receptors in the innate immune system. *Microbes Infect* **2**, 305–311. [Medline](#)

**Green, N. K., Herbert, C. W., Hale, S. J., Hale, A. B., Mautner, V., Harkins, R., Hermiston, T., Ulbrich, K., Fisher, K. D. & Seymour, L. W. (2004).** Extended plasma circulation time and decreased toxicity of polymer-coated adenovirus. *Gene Ther* **11**, 1256–1263. [Medline](#)

**Haisma, H. J., Grill, J., Curiel, D. T., Hoogeland, S., van Beusechem, V. W., Pinedo, H. M. & Gerritsen, W. R. (2000).** Targeting of adenoviral vectors through a bispecific single-chain antibody. *Cancer Gene Ther* **7**, 901–904. [Medline](#)

**Hamblin, M. R., Miller, J. L. & Ortel, B. (2000).** Scavenger-receptor targeted photodynamic therapy. *Photochem Photobiol* **72**, 533–540. [Medline](#)

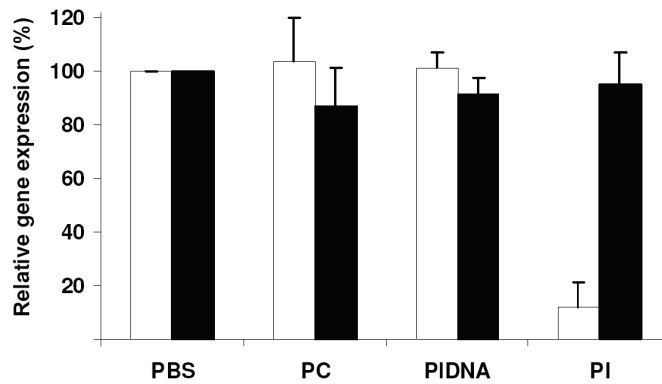
**He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W. & Vogelstein, B. (1998).** A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* **95**, 2509–2514. [Medline](#)

- Hodges, B. L., Taylor, K. M., Chu, Q., Scull, S. E., Serriello, R. G., Anderson, S. C., Wang, F. & Scheule, R. K. (2005).** Local delivery of a viral vector mitigates neutralization by antiviral antibodies and results in efficient transduction of rabbit liver. *Mol Ther* **12**, 1043–1051. [Medline](#)
- Kamps, J. A., Morselt, H. W., Swart, P. J., Meijer, D. K. & Scherphof, G. L. (1997).** Massive targeting of liposomes, surface-modified with anionized albumins, to hepatic endothelial cells. *Proc Natl Acad Sci U S A* **94**, 11681–11685. [Medline](#)
- Koizumi, N., Mizuguchi, H., Sakurai, F., Yamaguchi, T., Watanabe, Y. & Hayakawa, T. (2003).** Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and alphav integrin-binding ablation. *J Virol* **77**, 13062–13072. [Medline](#)
- Krieger, M. (1997).** The other side of scavenger receptors: pattern recognition for host defense. *Curr Opin Lipidol* **8**, 275–280. [Medline](#)
- Krieger, M. & Herz, J. (1994).** Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu Rev Biochem* **63**, 601–637. [Medline](#)
- Lanciotti, J., Song, A., Doukas, J., Sosnowski, B., Pierce, G., Gregory, R., Wadsworth, S. & O’Riordan, C. (2003).** Targeting adenoviral vectors using heterofunctional polyethylene glycol FGF2 conjugates. *Mol Ther* **8**, 99–107. [Medline](#)
- Lieber, A., He, C. Y., Meuse, L., Schowalter, D., Kirillova, I., Winther, B. & Kay, M. A. (1997).** The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. *J Virol* **71**, 8798–8807. [Medline](#)
- Lievens, J., Snoeys, J., Vekemans, K., Van Linthout, S., de Zanger, R., Collen, D., Wisse, E. & De Geest, B. (2004).** The size of sinusoidal fenestrae is a critical determinant of hepatocyte transduction after adenoviral gene transfer. *Gene Ther* **11**, 1523–1531. [Medline](#)
- Liu, Q., Zaiss, A. K., Colarusso, P., Patel, K., Haljan, G., Wickham, T. J. & Muruve, D. A. (2003).** The role of capsid-endothelial interactions in the innate immune response to adenovirus vectors. *Hum Gene Ther* **14**, 627–643. [Medline](#)
- Manickan, E., Smith, J. S., Tian, J., Eggerman, T. L., Lozier, J. N., Muller, J. & Byrnes, A. P. (2006).** Rapid Kupffer cell death after intravenous injection of adenovirus vectors. *Mol Ther* **13**, 108–117. [Medline](#)
- Martin, K., Brie, A., Saulnier, P., Perricaudet, M., Yeh, P. & Vigne, E. (2003).** Simultaneous CAR- and alpha V integrin-binding ablation fails to reduce Ad5 liver tropism. *Mol Ther* **8**, 485–494. [Medline](#)
- Mei, Y. F. & Wadell, G. (1995).** Molecular determinants of adenovirus tropism. *Curr Top Microbiol Immunol* **199**, 213–228. [Medline](#)
- Mizuguchi, H. & Hayakawa, T. (2004).** Targeted adenovirus vectors. *Hum Gene Ther* **15**, 1034–1044. [Medline](#)
- Mukhopadhyay, S. & Gordon, S. (2004).** The role of scavenger receptors in pathogen recognition and innate immunity. *Immunobiology* **209**, 39–49. [Medline](#)

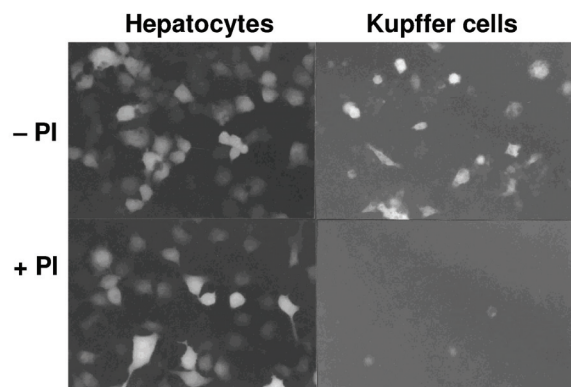
- Muruve, D. A. (2004).** The innate immune response to adenovirus vectors. *Hum Gene Ther* **15**, 1157–1166. [Medline](#)
- Muruve, D. A., Barnes, M. J., Stillman, I. E. & Libermann, T. A. (1999).** Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury in vivo. *Hum Gene Ther* **10**, 965–976. [Medline](#)
- Nakamura, T., Sato, K. & Hamada, H. (2003).** Reduction of natural adenovirus tropism to the liver by both ablation of fiber-coxsackievirus and adenovirus receptor interaction and use of replaceable short fiber. *J Virol* **77**, 2512–2521. [Medline](#)
- Nicklin, S. A., Wu, E., Nemerow, G. R. & Baker, A. H. (2005).** The influence of adenovirus fiber structure and function on vector development for gene therapy. *Mol Ther* **12**, 384–393. [Medline](#)
- Nicol, C. G., Graham, D., Miller, W. H., White, S. J., Smith, T. A., Nicklin, S. A., Stevenson, S. C. & Baker, A. H. (2004).** Effect of adenovirus serotype 5 fiber and penton modifications on in vivo tropism in rats. *Mol Ther* **10**, 344–354. [Medline](#)
- Ogawara, K., Rots, M. G., Kok, R. J., Moorlag, H. E., Van Loenen, A. M., Meijer, D. K., Haisma, H. J. & Molema, G. (2004).** A novel strategy to modify adenovirus tropism and enhance transgene delivery to activated vascular endothelial cells in vitro and in vivo. *Hum Gene Ther* **15**, 433–443. [Medline](#)
- O'Riordan, C. R., Lachapelle, A., Delgado, C., Parkes, V., Wadsworth, S. C., Smith, A. E. & Francis, G. E. (1999).** PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. *Hum Gene Ther* **10**, 1349–1358. [Medline](#)
- Park, B. H., Lee, J. H., Jeong, J. S., Rha, S. H., Kim, S. E., Kim, J. S., Kim, J. M. & Hwang, T. H. (2005).** Vascular administration of adenoviral vector soaked in absorbable gelatin sponge particles (GSP) prolongs the transgene expression in hepatocytes. *Cancer Gene Ther* **12**, 116–121. [Medline](#)
- Peiser, L., Mukhopadhyay, S. & Gordon, S. (2002).** Scavenger receptors in innate immunity. *Curr Opin Immunol* **14**, 123–128. [Medline](#)
- Platt, N. & Gordon, S. (2001).** Is the class A macrophage scavenger receptor (SR-A) multifunctional? – The mouse's tale. *J Clin Invest* **108**, 649–654. [Medline](#)
- Platt, N., Haworth, R., Darley, L. & Gordon, S. (2002).** The many roles of the class A macrophage scavenger receptor. *Int Rev Cytol* **212**, 1–40. [Medline](#)
- Rhains, D., Falstrault, L., Tremblay, C. & Brisette, L. (1999).** Uptake and fate of class B scavenger receptor ligands in HepG2 cells. *Eur J Biochem* **261**, 227–235. [Medline](#)
- Schiedner, G., Bloch, W., Hertel, S., Johnston, M., Molojavyi, A., Dries, V., Varga, G., van Rooijen, N. & Kochanek, S. (2003a).** A hemodynamic response to intravenous adenovirus vector particles is caused by systemic Kupffer cell-mediated activation of endothelial cells. *Hum Gene Ther* **14**, 1631–1641. [Medline](#)

- Schiedner, G., Hertel, S., Johnston, M., Dries, V., van Rooijen, N. & Kochanek, S. (2003b).** Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. *Mol Ther* **7**, 35–43. [Medline](#)
- Shayakhmetov, D. M., Li, Z. Y., Ni, S. & Lieber, A. (2004).** Analysis of adenovirus sequestration in the liver, transduction of hepatic cells, and innate toxicity after injection of fiber-modified vectors. *J Virol* **78**, 5368–5381. [Medline](#)
- Shayakhmetov, D. M., Gaggar, A., Ni, S., Li, Z. Y. & Lieber, A. (2005).** Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J Virol* **79**, 7478–7491. [Medline](#)
- Shirai, H., Murakami, T., Yamada, Y., Doi, T., Hamakubo, T. & Kodama, T. (1999).** Structure and function of type I and II macrophage scavenger receptors. *Mech Ageing Dev* **111**, 107–121. [Medline](#)
- Smith, T., Idamakanti, N., Kylefjord, H., Rollence, M., King, L., Kaloss, M., Kaleko, M. & Stevenson, S. C. (2002).** In vivo hepatic adenoviral gene delivery occurs independently of the coxsackievirus-adenovirus receptor. *Mol Ther* **5**, 770–779. [Medline](#)
- Smith, T. A., Idamakanti, N., Marshall-Neff, J., Rollence, M. L., Wright, P., Kaloss, M., King, L., Mech, C., Dinges, L. & other authors (2003a).** Receptor interactions involved in adenoviral-mediated gene delivery after systemic administration in non-human primates. *Hum Gene Ther* **14**, 1595–1604. [Medline](#)
- Smith, T. A., Idamakanti, N., Rollence, M. L., Marshall-Neff, J., Kim, J., Mulgrew, K., Nemerow, G. R., Kaleko, M. & Stevenson, S. C. (2003b).** Adenovirus serotype 5 fiber shaft influences in vivo gene transfer in mice. *Hum Gene Ther* **14**, 777–787. [Medline](#)
- Smith, J. S., Xu, Z. & Byrnes, A. P. (2008).** A quantitative assay for measuring clearance of adenovirus vectors by Kupffer cells. *J Virol Methods* **147**, 54–60. [Medline](#)
- Stone, D., Liu, Y., Shayakhmetov, D., Li, Z. Y., Ni, S. & Lieber, A. (2007).** Adenovirus-platelet interaction in blood causes virus sequestration to the reticuloendothelial system of the liver. *J Virol* **81**, 4866–4871. [Medline](#)
- Swart, P. J., Harmsen, M. C., Kuipers, M. E., Van Dijk, A. A., Van Der Strate, B. W., Van Berkel, P. H., Nuijens, J. H., Smit, C., Witvrouw, M. & other authors (1999).** Charge modification of plasma and milk proteins results in antiviral active compounds. *J Pept Sci* **5**, 563–576. [Medline](#)
- Tao, N., Gao, G. P., Parr, M., Johnston, J., Baradet, T., Wilson, J. M., Barsoum, J. & Fawell, S. E. (2001).** Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol Ther* **3**, 28–35. [Medline](#)
- Taylor, P. R., Martinez-Pomares, L., Stacey, M., Lin, H. H., Brown, G. D. & Gordon, S. (2005).** Macrophage receptors and immune recognition. *Annu Rev Immunol* **23**, 901–944. [Medline](#)
- Tomko, R. P., Xu, R. & Philipson, L. (1997).** HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc Natl Acad Sci U S A* **94**, 3352–3356. [Medline](#)

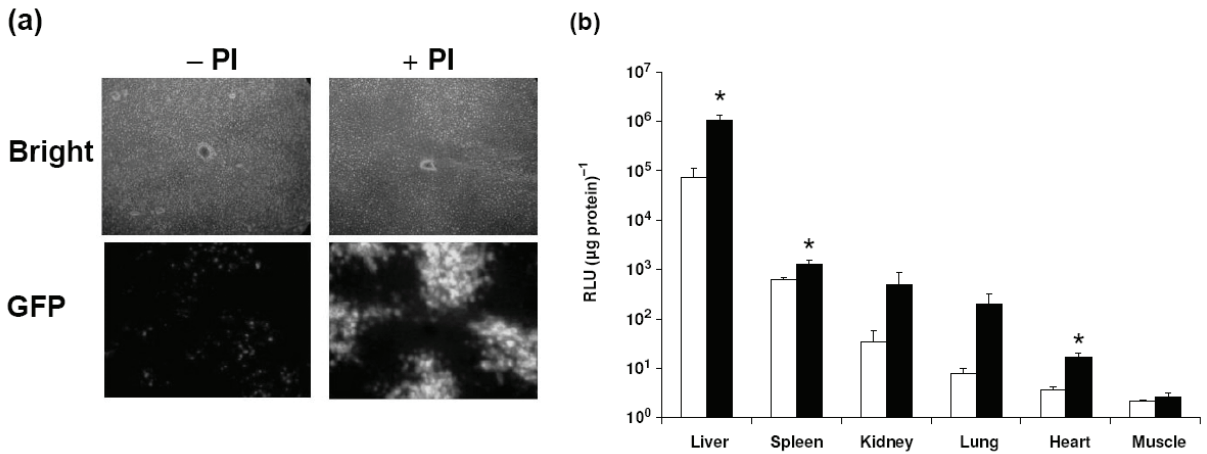
- Vigne, E., Dedieu, J. F., Brie, A., Gillardeaux, A., Briot, D., Benihoud, K., Latta-Mahieu, M., Saulnier, P., Perricaudet, M. & Yeh, P. (2003).** Genetic manipulations of adenovirus type 5 fiber resulting in liver tropism attenuation. *Gene Ther* **10**, 153–162. [Medline](#)
- Wickham, T. J., Mathias, P., Cheres, D. A. & Nemerow, G. R. (1993).** Integrins  $\alpha\beta3$  and  $\alpha\beta5$  promote adenovirus internalization but not virus attachment. *Cell* **73**, 309–319. [Medline](#)
- Wolff, G., Worgall, S., van Rooijen, N., Song, W. R., Harvey, B. G. & Crystal, R. G. (1997).** Enhancement of in vivo adenovirus-mediated gene transfer and expression by prior depletion of tissue macrophages in the target organ. *J Virol* **71**, 624–629. [Medline](#)
- Yamada, Y., Doi, T., Hamakubo, T. & Kodama, T. (1998).** Scavenger receptor family proteins: roles for atherosclerosis, host defence and disorders of the central nervous system. *Cell Mol Life Sci* **54**, 628–640. [Medline](#)
- Zhang, Y., Chirmule, N., Gao, G. P., Qian, R., Croyle, M., Joshi, B., Tazelaar, J. & Wilson, J. M. (2001).** Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. *Mol Ther* **3**, 697–707. [Medline](#)
- Ziegler, R. J., Li, C., Cherry, M., Zhu, Y., Hempel, D., van Rooijen, N., Ioannou, Y. A., Desnick, R. J., Goldberg, M. A., Yew, N. S. & Cheng, S. H. (2002).** Correction of the nonlinear dose response improves the viability of adenoviral vectors for gene therapy of Fabry disease. *Hum Gene Ther* **13**, 935–945. [Medline](#)



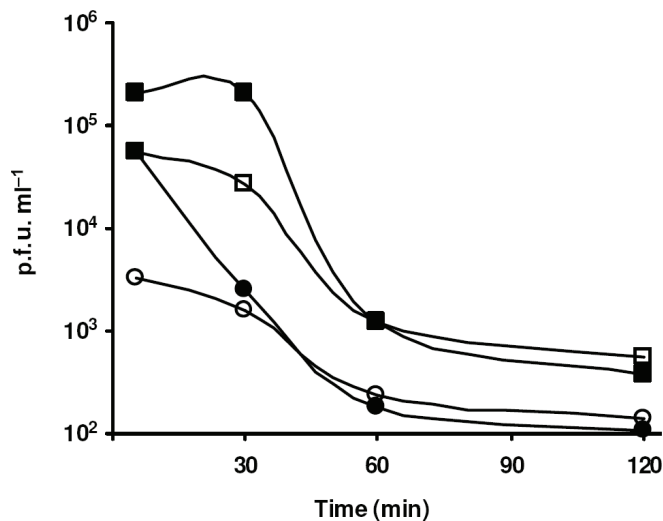
**Fig. 1.** Poly(I) prevents adenovirus infection of macrophages. Relative luciferase gene-expression levels of J774 (white bars) and HepG2 cells (black bars) infected with adenovirus particles in the presence of medium control (PBS), poly(C) (PC), plasmid DNA (PIDNA) or poly(I) (PI). To determine gene expression, luciferase activity was measured 48 h post-infection. Gene expression is presented as a percentage of that in cells infected with AdTL in the presence of medium control. Results are expressed as the mean $\pm$ SD relative light units (RLU) of at least three different experiments.



**Fig. 2.** Poly(I) blocks adenovirus infection of Kupffer cells. Hepatocytes and Kupffer cells were isolated from rats injected with PBS (-PI) or 4 mg poly(I) (+PI) per rat, 5 min prior to infection with  $5 \times 10^{11}$  adenovirus particles per animal. Expression of GFP was analysed 48 h after adenovirus infection and representative images are shown.



**Fig. 3.** Poly(I) alters the distribution of adenovirus transgene expression in rats. Transgene expression in rats injected with PBS (-PI) or with PBS containing 4 mg poly(I) (+PI) per rat, 5 min prior to injection of  $5 \times 10^{11}$  adenovirus particles per animal. (a) Rat liver sections were analysed by fluorescence microscopy 48 h after viral injection. (b) Luciferase measurements in tissue homogenates 48 h after injection of AdTL. White bars represent animals injected with PBS; black bars represent animals pre-injected with poly(I). Results are expressed as the mean  $\pm$  SD relative light units (RLU) of three rats. \* $P < 0.05$  compared with controls (animals injected with PBS).



**Fig. 4.** Poly(I) alters the blood clearance of adenovirus. Mice were injected with PBS or PBS containing 0.2 mg poly(I) per mouse, 5 min prior to injection with  $5 \times 10^9$  or  $5 \times 10^{10}$  adenoviral particles (resulting in estimated concentrations of  $1.3 \times 10^8$  and  $1.3 \times 10^9$  p.f.u. ml<sup>-1</sup>, respectively, at time 0). Blood levels of AdTL were determined by plaque-forming assays on 293 cells (CAR-positive) and expressed as p.f.u. ml<sup>-1</sup>. ,  $5 \times 10^9$  vp AdTL; , poly(I) +  $5 \times 10^9$  vp AdTL; ,  $5 \times 10^{10}$  vp AdTL; , poly(I) +  $5 \times 10^{10}$  vp AdTL. Results are expressed as the mean of results from three rats.



