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SR-A and SREC-I binding peptides increase HDAd-mediated liver transduction

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Abstract

Helper-dependent adenoviral (HDAd) vectors can mediate long-term, high-level transgene expression from transduced hepatocytes without inducing chronic toxicity. However, vector therapeutic index is narrow because of a toxic acute response with potentially lethal consequences elicited by high vector doses. Kupffer cells and liver sinusoidal endothelial cells (LSECs) are major barriers to efficient hepatocyte transduction. We investigated two small peptides (PP1 and PP2) developed by phage display to block scavenger receptor type A (SR-A) and scavenger receptor expressed on endothelial cells type I (SREC-I) respectively, for enhancement of HDAd-mediated hepatocyte transduction efficiency. Pre-incubation of J774A.1 macrophages with either PP1 or PP2 prior to HDAd infection significantly reduced viral vector uptake. *In vivo*, fluorochrome-conjugated PP1 and PP2 injected intravenously into mice co-localized with both CD68 and CD31 on Kupffer cells and LSECs, respectively. Compared to saline pre-treated animals, intravenous injections of both peptides prior to the injection of an HDAd resulted in up to 3.7- and 2.9-fold increase of hepatic transgene expression with PP1 and PP2, respectively. In addition to hepatocyte transduction, compared to control saline injected mice, pre-treatment with either peptide resulted in no increased levels of serum interleukin-6 (IL-6), the major marker of adenoviral vector acute toxicity. In summary, we developed small peptides that significantly increase hepatocyte transduction efficacy and improve HDAd therapeutic index with potential for clinical applications.

Keywords

SR-A; SREC-I; Helper-dependent adenoviral vectors

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INTRODUCTION

Helper-dependent adenoviral (HDAd) vectors hold tremendous potential for a large number of therapeutic applications ranging from inherited disorders to cancer and vaccination. HDAd can infect a large variety of cell types to mediate long-term, high level expression without chronic toxicity[1]. However, a major disadvantage of HDAd is the induction of a potent and potentially lethal acute reaction following systemic injection of high vector doses[2]. HDAd derived from human adenovirus serotype 5 (Ad5) exhibit a strong liver tropism. However, there are various obstacles preventing hepatocyte transduction following systemic administration. Kupffer cells (KCs) and fenestrated liver sinusoidal endothelial cells (LSECs) have been recognized as major barriers for efficient adenovirus (Ad)-mediated liver transduction. KCs are liver resident macrophages protruding into the vascular space that rapidly and avidly remove blood-borne Ad particles[3-7]. As a consequence of vector uptake by cells that are not relevant for disease treatment, higher and potentially toxic doses are required to transduce the target cells that are the hepatocytes. In addition, for cancer gene therapy, vector sequestration by macrophages reduces the efficacy of intravenously injected Ad by reducing the number of vector particles available for targeting metastatic cancer cells[8-10]. We and others recently identified scavenger receptor type A (SR-A) and scavenger receptor expressed on endothelial cells type I (SREC-I) as HDAd receptors on KCs and LSECs that play an important role in vector uptake[11,12]. In this work, we investigated small peptides that bind SR-A and SREC-I and increase efficiency of HDAd-mediated hepatocyte transduction.

RESULTS

Scavenger receptors and HDAd-mediated hepatocyte transduction

Scavenger receptors (SRs) are expressed on multiple cell types and to evaluate the contribution of non-KC dependent SR-mediated HDAd particle uptake, we depleted KCs in C57BL/6 wild-type mice by clodronate liposomes[13]. Following 48 hours from clodronate injection, when KC number is greatly diminished[13], mice were injected with polyinosine (poly[I]) that bind and inhibit SR-mediated uptake[14], prior to the injection of 1×10^{12} viral particles (vp)/kg of an HDAd bearing the baboon α-fetoprotein (bAFP) secreted reporter gene under the control of a hepatocyte specific promoter (HDAd-AFP)[15]. As previously reported[14, 16], clodronate or poly[I] pre-treatment resulted in significantly higher liver transduction compared to control mice pre-injected with saline (one way ANOVA and post-hoc Tukey's test: $p<0.01$) (Fig. 1). Interestingly, compared to mice injected with clodronate or poly[I] alone, poly[I] increased hepatic transduction of 1.8- and 1.9-fold ($P<0.01$) respectively, in mice that were depleted of KCs by clodronate (Figure 1). These results suggest that poly[I] prevents HDAd uptake uptake in cell types other than macrophages. Endothelial cells appear to be the more likely candidate cells involved in KC independent vector uptake and SREC-I, that is mainly expressed on endothelial cells[17-19], is a good receptor candidate. In contrast, SR-A is predominantly expressed on KCs and macrophages[20]. Nevertheless, in the subsequent studies we aimed at binding both SRs to achieve preferential hepatocyte transduction.

SR-A and SREC-I binding peptides reduce HDAd viral particle uptake

While PP1, a SR-A specific binding peptide was previously reported[21], no binding peptides were yet available for SREC-I. By phage display, we screened three different libraries searching for amino acid motifs binding SREC-I. Phages underwent three panning cycles against recombinant human SREC-I protein and sixteen clones per library were isolated and sequenced. Six different consensus sequences were built and assayed by competitive ELISA for binding affinity against the recombinant protein (Supplementary Table 1). The peptide sequence ITPPGMWSQSQR, herein named as PP2, resulted in the highest binding affinity and was used for further experiments.

To investigate the efficacy of PP1 and PP2 at blocking vector uptake by SR-A and SREC-I, respectively, J774A.1 macrophages were incubated with various concentrations of PP1, PP2, or a combination of the two peptides before infection with an HDAd-LacZ vector[22]. Control cells were incubated with poly[I] or PBS. At the tested concentrations, pre-treatment with PP1 or PP2 resulted in significantly lower HDAd vector uptake (one way ANOVA and post-hoc Tukey's test: $p<0.01$) (Fig. 2a and 2b). Co-incubation with both PP1 and PP2 did not result in further reduction of vector uptake compared to incubations with each single peptide (Fig. 2b). Owing to the lack of an additive effect on inhibition of vector uptake, we hypothesized that PP2 is not entirely specific for its targets and it cross-react with SR-A. To test this hypothesis, J774A.1 cells knocked-down for SR-A expression (Supplementary Fig. 1) were incubated with tetramethylrhodamine (TAMRA)-labeled PP1 or PP2 to measure peptide uptake by cytofluorimetry. As expected, SR-A silenced cells showed a decrease of PP1 uptake compared to control cells (*t*-test: $p<0.01$) (Fig. 2c and 2d). A reduction of PP2 uptake ($p<0.01$) was observed in SR-A knocked-down cells, thus showing that SR-A expression affects PP2 uptake (Fig. 2c and 2d). The lower uptake of PP2 compared to PP1 might be explained by the significantly higher SR-A expression in J774A.1 cells compared to SREC-I[23]. Finally, we investigated whether intravenously injected PP1 and PP2 target macrophages and endothelial cells *in vivo*. Wild-type C57BL/6 mice were injected with TAMRA labeled-PP1 or -PP2 and livers and spleens were harvested for confocal microscopy analysis of co-localization of each peptide with CD68 or CD31, as markers of macrophages and endothelial cells, respectively. In liver, PP1 and PP2 signals were detected in both $CD68^+$ and $CD31^+$cells (Fig. 3a); in spleen both peptides localized mostly in the marginal zone, both in $CD68^+$ macrophages and in $CD31^+$ stromal endothelial cells (Fig. 3b).

PP1 and PP2 pre-treatments increase HDAd-mediated hepatocyte transduction

Having shown that PP1 and PP2 target macrophage and endothelial cells *in vivo*, we next investigated whether the two peptides enhance hepatocyte transduction efficiency by HDAd vectors. To this end, C57BL/6 mice were administered intravenously with increasing doses (3.3, 33, and 330 nmol/kg) of PP1, PP2, or with saline 5 minutes prior to the injection of 1×10^{11}vp/kg of HDAd-AFP. Compared to saline injected animals, mice pre-injected with 33 and 330 nmol/kg of PP1 showed a 2.3-fold (one way ANOVA and post-hoc Tukey's test: $p<0.05$) and 3.7-fold ($p<0.01$) increase at four weeks post-injection (Fig. 4a).

Mice pre-injected with 33 and 330 nmol/kg of PP2 showed a significant 2.5- ($p<0.05$) and 2.9-fold ($p<0.01$) increase in transduction efficiency at four weeks post-injection compared to saline controls (Fig. 4b). Co-administration of PP1 and PP2, each at the doses of 33 or 330 nmol/kg, resulted in 2.8-fold higher levels of serum AFP compared to saline treated mice that was not higher compared to each peptide alone (Supplementary Fig. 2). Increased liver transduction was confirmed by X-gal histochemistry and β-galactosidase (β-gal) activity in mice injected intravenously with 330 nmol/kg of PP1 or PP2 prior to the injection of 5×10^{11}vp/kg of HDAd-LacZ (Figure 4e). Control poly[I] pre-injected mice showed higher percentage of β-gal-positive cells and 5.1-fold higher ($p<0.01$) β-gal activity compared to saline pre-treated mice (Fig. 4c and 4d). Compared to saline pre-injected animals, PP1 and PP2 pre-injected mice showed a higher percentage of β-gal-positive cells and a significant 3.6- ($p<0.01$) and 2.8-fold ($p<0.05$) higher levels of β-gal activity, respectively (Fig. 4c and 4d).Vector genome copies measured by qPCR were slightly higher but not statistically different in livers of mice pre-treated with 330 nmol/kg of PP1 and PP2 compared to control animals at 72 hours after the injection of 5×10^{11}vp/kg of HDAd-CMV-LacZ (Fig. 4e). However, at this early time point, livers contain vector genome copies derived from bloodborne viral particles that are taken up by KCs that are not transduced. In contrast, at 3-months post-injection, hepatic vector genome copy numbers were 3.9- and 2.7-fold higher than saline controls in mice injected with 330 nmol/kg of PP1 and PP2, respectively, prior to the injection of HDAd-AFP vector at the dose of 1×10^{11} vp/kg (*t*-test: $p<0.05$) (Fig. 4f). Peptide pre-treatment did not affect significantly vector uptake by spleen, lung, and kidney at both time point analyzed (Fig. 4e and 4f).

Acute toxicity in mice pre-treated with PP1 and PP2

Intravenously injected Ad vectors elicit an acute innate immune response with increased serum levels of pro-inflammatory cytokines[2, 7]. To investigate the effect of PP1 and PP2 on the acute response elicited by HDAd vectors, we measured serum interleukin-6 (IL-6) at 6 hours after the injection of 3×10^{12} vp/Kg of HDAd-AFP or saline in mice pre-injected with the minimum effective dose of PP1 (33 nmol/kg) or PP2 (33 nmol/kg). As controls, mice were pre-injected with poly[I] or saline. As previously described[11], at high vector doses poly[I] pre-treatment resulted in a marked increase of serum IL-6 levels compared to saline pre-injected animals (one way ANOVA and post-hoc Tukey's test; $p<0.05$). Although resulting in higher transduction efficiency, both pre-injections of PP1 or PP2 resulted in a modest decrease of serum IL-6 that was not statistically significant compared to mice pre-injected with saline (Fig. 5). Moreover, injections of PP1 or PP2 alone without vector or prior to the administration of the more clinically relevant dose of 1×10^{11} vp/kg did not result in a significant increase of serum IL-6 levels compared to control saline pre-injected mice (Supplementary Fig. 3a).

Ad vectors are also known to induce rapid KC death[24] associated with an increase of serum lactic dehydrogenase (LDH)[25]. To test whether SR binding peptides affect KC necrosis induced by Ad vectors, C57BL/6 mice were injected intravenously with PP1 (33 nmol/kg), PP2 (33 nmol/kg) or saline prior to the injection of 1×10^{12} vp/kg or 5×10^{12} vp/Kg of HDAd-AFP, or saline. After 24 hours, mice were sacrificed and livers were stained for KC marker CD68. At the lower vector dose only a moderate but not statistically significant 15%

decrease in KC number was detected whereas at the higher dose a significant 45% decrease in KCs was observed (one way ANOVA and post-hoc Tukey's test; $p<0.05$) (Supplementary Fig. 3b). Injections of PP1 and PP2 alone did not result in decreased KC number (data not shown) and no significant differences were observed between HDAd injected mice pre-treated with PP1 or PP2 compared to control mice injected with saline prior to HDAd injections (Supplementary Fig. 3b). Serum LDH levels at 30 minutes post-vector injection[25] were not affected by peptide pre-injections (Supplementary Fig. 3c). Taken together, these results show that SR binding peptides do not prevent KC death.

DISCUSSION

HDAd acute toxicity is a major limitation preventing broader applications in the clinic of these vectors. During the last decade, physical method aimed at confining HDAd to the liver[15, 26-28], capsid modification by synthetic polymers, or genetic engineering for Ad vectors de-targeting from KCs[29, 30] have been investigated to overcome this problem. Based on our previous finding that SR-A and SRECI are involved in vector uptake by macrophages and endothelial cells[11], we have herein investigated SR binding peptides for inhibition of vector particle uptake by these cell types to increase efficiency of hepatocyte gene transfer. Both small inhibiting peptides were generated by phage display, a powerful technology that since its development[31] has brought to human applications several antibody and peptides[32]. While SR-A binding peptide PP1 has been previously developed for atherosclerosis[21], the SREC-I binding peptide PP2 has been generated in this study. Both peptides were effective at reducing HDAd vector uptake by macrophages *in vitro* and at increasing hepatocyte transduction *in vivo*. Moreover, both peptides resulted in higher efficiency of hepatocyte transduction compared to antigen-binding fragments (Fab) previously used to block uptake by the two receptors[11]. Both peptides were designed against human receptors and thus, the efficacy on enhancement of liver transduction might be underestimated in mice. PP1 was in fact found to avidly bind to human-derived THP-1 from humans and to a significantly lesser extent to murine RAW264.7 cells[21].

As previously shown, Ad vectors activate an innate immune response immediately after systemic injection of high vector doses. This acute response results in high serum levels of proinflammatory cytokines and chemokines in animal models[7, 33] and humans[34] and is associated to rapid KC death[25]. Despite an up to 3.9-fold increase in liver transduction, no significant increase in IL-6 serum levels was observed in mice pre-treated with the SR binding peptides, thus suggesting that these peptides do not result in higher toxicity and instead might have a protective role. Moreover, both peptides result in reduced activation of the inflammatory response and thus appear to be safer than poly[I].

Ad vectors have been widely used for cancer gene therapy with over 15,000 patients enrolled in clinical trials with oncolytic Ad vectors (http://www.wiley.com/legacy/wileychi/genmed/clinical/). Nevertheless, vector sequestration by liver resident macrophages remains a major limitation. The development of pharmaceutical tools based on SR binding peptides for Ad vector de-targeting from KCs has potential for improving the efficacy of this promising therapeutic modality, particularly for metastatic cancer.

SR-A-mediated uptake by macrophages and Kupffer cells has also been involved in uptake of other viral vectors, namely vectors derived from adeno-associated virus serotype 8 (AAV8) that have been demonstrated to be effective for liver-directed gene therapy in humans[35, 36]. As for HDAd vectors, uptake by macrophages result in loss of viral vector particles for hepatocyte transduction and reduction of efficacy of gene therapy. Therefore, binding peptides against SR-A and SREC-I have also potential applications for increasing the efficacy of AAV vectors.

SRs have multiple functions in binding of modified low density lipoproteins (LDL) as well as in recognition and uptake of pathogens. SREC-I on endothelial cells mediates binding and degradation of acetylated and oxidized LDL. Therefore, SREC-I binding peptides have potential to reduce uptake of modified LDL by macrophages and endothelial cells, which would in turn reduce the risks of atherosclerosis[37].

Interaction and uptake of Tamm-Horsfall protein (THP) by SR-A and SREC-I have been proposed as an important mechanism in local host defense and could contribute to inflammatory kidney diseases associated with THP-specific antibody responses[38]. Moreover, SREC-I mediates internalization of heat shock protein 90 (HSP90) and antigen cross-presentation[39]. Therefore, binding of SRs might have a therapeutic role in preventing the deleterious consequences of inflammatory and immunological diseases.

In conclusion, SR-A and SREC-I binding peptides are effective in enhancing HDAd-mediated hepatocyte transduction and have potential for increasing the vector therapeutic index. Besides, applications for hepatocyte gene therapy and cancer gene therapy, these peptides have potential for broader applications based on the diverse functions of SRs in atherosclerosis, inflammation, and immunity.

MATERIALS AND METHODS

Phage display and peptide synthesis

Phage display was performed for screening of SREC-I binding peptides (Rx Biosciences, Rockville, MD, USA). 12-mer, 7-mer, and 7C7 cyclic peptides libraries in M13KE phages (New England Biolabs, Ipswich, MA, USA) were screened on immobilized recombinant human SRECI/SCARF1 Fc Chimera (R&D, Minneapolis, MN, USA) using ER2738 cells (New England Biolabs) for amplification passages. Panning was performed as per Rx Biosciences' proprietary protocol. Briefly, three different panning experiments were performed. Libraries were titered and 2×10^{11} phages from each library were transferred onto SREC-I coated wells and incubated for 1 hour at room temperature. Plates were washed in Tris-buffered saline (TBS) with 0.1%, 0.25%, or 0.5% [v/v] Tween-20 and bound phages were eluted using low pH glycine. Eluates were titered and amplified in ER2738 cells. To isolate phagic DNA, phages were mixed with ER2738 host cells and spread on LB-X-Gal, Isopropyl β-D-1-thiogalactopyranoside (IPTG) plates and 20 well isolated colonies per library were picked and amplified. Phages were precipitated with NaCl, Poly(ethylene glycol) (PEG) and re-dissolved in 4M NaI buffer. Sixteen clones for each library were sequenced.

Affinities of peptides were assessed by ELISA. ELISA plates were coated overnight at 4°C with 100 μg/mL recombinant human SREC-I/SCARF1 Fc Chimera (R&D, Minneapolis, MN, USA) in 0.1 M NaHCO3 (pH 8.6) and blocked for 2 hours at 4°C with PBS, 1% BSA. Washing was performed in TBS, 0.5% Tween-20 as in the third panning round. Positive phages were diluted and incubated at room temperature for 1 hour. After washing, horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal antibody (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) was added at 1:5000 dilution and incubated for 1 hour at room temperature. Plates were washed in TBS, 0.1% Tween-20. Substrate solution was added and after 10 minutes of incubation samples were read at 450 nm. Each sample was assayed in triplicate and affinity was expressed as absorbance at 450 nm.

Peptides were synthesized by PRIMM (Milano, Italy) at a purity >95% by HPLC and solubilized in water or DMSO. Fluorescent peptides were made by conjugation with carboxytetramethylrhodamine (TAMRA). TAMRA conjugation to the N-Terminus of peptides was obtained in solid phase by reaction of TAMRA with resin-bound peptides in the presence of 4 equivalents of TAMRA, 4 equivalents of (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate and 8 equivalents of N,N-Diisopropylethylamine in dimethylformamide for 12 hours at room temperature.

HDAd vectors

HDAd-AFP and HDAd-LacZ vectors bear the PEPCK-WL-bAFP and the MCMV-LacZ expression cassettes respectively, as described in details elsewhere[15, 40]. HDAd was produced in 116 cells with the helper virus AdNG163 as described previously[40, 41]. Helper virus contamination was determined on vector preparations and were found to be <0.05%. DNA analyses of HDAd genomic structure was confirmed as described elsewhere[40].

Cells studies

J774A.1 cells were purchased from European Collection of Cell Culture (Salisbury, UK) and cultured according to supplier's instructions. Cells were pre-treated with PP1, PP2, a combination of PP1 and PP2, control peptides, polyinosine (poly[I]), or phosphate buffer solution (PBS) 30 minutes prior to HDAd infection. Cells were infected with HDAd-LacZ at a multiplicity of infection (m.o.i.) of 100 viral particle (vp)/cell for 1 hour and the infection media were then removed and replaced with fresh regular maintenance medium. Cells were harvested after 24 hours from the infection. Three independent experiments were performed for each condition.

Silencing experiments were performed in J774A.1 cells in 6-well plates with Msr1 siRNA (5′-GUGAGAGUAUUGAACAACATT-3′) and scrambled sequence siRNA as negative control (Invitrogen Life Technologies, Grand Island, NY, USA) using 25 pmol/well of each siRNA and using RNAiMAX transfection reagent (Invitrogen Life Technologies) according to manufacturer's instructions. Each treatment was performed in duplicate. After 48 hours, cells were incubated for 2 hours with fluorescent PP1 and PP2, washed, and collected in PBS, 0.1% Bovine Serum Albumin (BSA). Msr-1 silencing was verified by real time PCR and Western blotting. Following siRNA transfection, J774A.1 cells were harvested and underwent total RNA extraction using RNAeasy kit (Qiagen, Hilden, Germany) according to

manufacturer's instructions. RNA was reverse transcribed using a first-strand complementary deoxyribonucleic acid kit with random primers according to manufacturer's protocol (Invitrogen Life Technologies). The qPCR reactions were performed with SYBR Green Master Mix (Roche, Indianapolis, IN, USA) using Roche Light Cycler 480 system (Roche, Indianapolis, IN, USA). PCR conditions were as follows: preheating, 5 minutes at 95°C; 40 cycles of 15 seconds at 95°C, 15 seconds at 60°C, and 25 seconds at 72°C. Quantification results were expressed in terms of cycle threshold (Ct). The Ct values were averaged for each technical duplicate. For expression analysis β2-microglobulin housekeeping gene was used as endogenous control (reference marker) using LightCycler 480 software version 1.5 (Roche). Differences between mean Ct values of tested genes and those of the reference gene were calculated as ΔCt gene = Ct gene - Ct reference. Untreated samples were used as calibrator and relative fold increase in expression levels was determined as $E^{-\Delta\Delta Ct}$, E being primer efficiency. The primers used for Msr1 gene were: Msr1-for 5′-CTCAGACTGAAGGACTGGGAACACTCAC-3′, Msr1-rev 5′-TCACCTTTAACACCTGGAATACCTCTTA-3′.

Protein extraction was performed in RIPA buffer according to standard procedures. Anti-human SR-A (Santa Cruz Biotechnology, Dallas, TX, USA) and anti-human β-actin (Novus Biological, Cambridge, UK) were used as primary antibody; secondary antibody were ECL anti-goat and anti-mouse HRP, respectively (GE Healthcare, Waukesha, WI, USA). Analysis of band intensities was performed using Quantity One basic software (Bio-Rad Laboratories, Hercules, CA, USA). Cytofluorimetric analysis was performed on BD FACSCanto (Becton Dickinson, San Jose, CA, USA) using BD FACSDiva software (Becton Dickinson, San Jose, CA, USA).

Mice and injections

Six- to nine-week-old male wild-type C57BL/6 mice (Charles River Laboratories, Calco, Italy) were used for all experiments. Dilutions of vectors and peptides were made in saline solutions. Mouse injections were performed retro-orbitally in a volume of 200 μL. Polyinosinic acid potassium salt was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in PBS, and injected at the dose of 0.05 mg/mouse[11]. For KC depletion, mice were injected with clodronate liposomes (clodronateliposomes.org, Haarlem, The Netherlands) 48 hours before vector administration[13]. Serum samples were collected at various times post-injection by retro-orbital bleedings. Determinations of serum baboon AFP and mouse IL-6 were performed by ELISA (R&D, Minneapolis, MN, USA), according to manufacturer's instructions. Total proteins were extracted from livers and β-galactosidase activity was determined using β-galactosidase enzyme assay system with reporter lysis buffer (Promega, Madison, WI, USA) according to manufacturer's instructions. Serum LDH was measured by colorimetric method (Gentaur, Kampenhout, Belgium) according to manufacturer's instructions.

HDAd vector genome copies

HDAd vector genome copies were determined in cells and in tissues. Total DNA was extracted from cells and mouse tissues using standard phenol-chloroform extraction and quantitated by absorbance at 260 nm. Three different specimens per organ per mouse were

processed. Quantitative real-time PCR was performed in duplicate for each sample using the LightCycler FastStart DNA Master SYBR Green I (Roche, Indianapolis, IN, USA) in a total volume of 20 μl using 1 mM of each HDAd-specific primers (5′-TCTGAATAATTTTGTGTTACTCATAGCGCG-3′ and 5′-CCCATAAGCTCCTTTTAACTTGTTAAAGTC-3′). Cycling conditions consisted of 95° C for 10 minutes followed by 45 cycles at 95° C for 10 seconds, 60° C for 7 seconds and 72° C for 20 seconds. Serial dilutions of a plasmid bearing the PCR target sequence were used as a control to determine the amounts of HDAd. Results were analyzed with Light Cycler software version 3.5 (Roche, Indianapolis, IN, USA).

Immunofluorescence and X-gal staining

For immunofluorence, at sacrifice animals were perfused with PBS pH 7.4 and harvested livers were fixed with 1% paraformaldehyde, PBS pH 7.4 for 5 minutes. After immersion, post-fixation in 1% paraformaldehyde and 0.5% glutaraldehyde solution for 3 hours followed by overnight immersion in 30% sucrose, livers were included in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA). Embedded livers and spleens were cryo-sectioned at 4 μm and fixed in 4% paraformaldehyde. At least two sections per mouse were assayed in each experiment. Sections were permeabilized in PBS, 0.2% Triton and blocked in 5% BSA. Primary antibodies used were: anti-CD31 (550274-BD; Pharmingen, Oxford, UK) and anti-CD68 (MCA1957; BD Serotec, Kidlington, UK). Secondary antibody was anti-rat AlexaFluor-647 (Invitrogen Life Technologies). Confocal microscopy images were obtained using LSM 710 microscope (Plan-Apochromat 63X/1.40 oil DIC M27 objective; zoom x0.6) and ZEN 2008 software (Carl Zeiss, Oberkochen, Germany). At least five images per slide per animal were analyzed for each staining. ImageJ software (NIH, Bethesda, MD) was used for $CD68^+$ cell count.

X-gal histochemistry was performed on liver specimens as previously described[2] in mice sacrificed at 72 hours post-injection. Three images per section from three sections per animal were analyzed.

Statistical analyses

Student’s t-test and one-way ANOVA followed by post hoc multi-comparison Tukey’s test were performed as statistical analysis. Error bars in the figures represent standard errors of the mean (s.e.m).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Brunetti-Pierri N, Ng P. Helper-dependent adenoviral vectors for liver-directed gene therapy. Hum Mol Genet. 2011; 20(R1):R7–13.
2. Brunetti-Pierri N, Palmer DJ, Beaudet AL, Carey KD, Finegold M, Ng P. Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. Hum Gene Ther. 2004; 15(1):35–46.
3. Ganesan LP, Mohanty S, Kim J, Clark KR, Robinson JM, Anderson CL. Rapid and efficient clearance of blood-borne virus by liver sinusoidal endothelium. PLoS Pathog. 2011; 7(9):e1002281. [PubMed: 21980295]
4. Wolff G, Worgall S, van Rooijen N, Song WR, Harvey BG, Crystal RG. Enhancement of in vivo adenovirus-mediated gene transfer and expression by prior depletion of tissue macrophages in the target organ. J Virol. 1997; 71(1):624–9. [PubMed: 8985392]
5. Alemany R, Suzuki K, Curiel DT. Blood clearance rates of adenovirus type 5 in mice. J Gen Virol. 2000; 81(Pt 11):2605–9. [PubMed: 11038370]
6. Tao N, Gao GP, Parr M, Johnston J, Baradet T, Wilson JM, et al. Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. Mol Ther. 2001; 3(1): 28–35. [PubMed: 11162308]
7. Zhang Y, Chirmule N, Gao GP, Qian R, Croyle M, Joshi B, et al. Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. Mol Ther. 2001; 3(5 Pt 1):697–707. [PubMed: 11356075]
8. Nemunaitis J, Cunningham C, Buchanan A, Blackburn A, Edelman G, Maples P, et al. Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: safety, feasibility and biological activity. Gene Ther. 2001; 8(10):746–59. [PubMed: 11420638]
9. Small EJ, Carducci MA, Burke JM, Rodriguez R, Fong L, van Ummersen L, et al. A phase I trial of intravenous CG7870, a replication-selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer. Mol Ther. 2006; 14(1):107–17.
10. Nemunaitis J, Senzer N, Sarmiento S, Zhang YA, Arzaga R, Sands B, et al. A phase I trial of intravenous infusion of ONYX-015 and enbrel in solid tumor patients. Cancer Gene Ther. 2007; 14(11):885–93. [PubMed: 17704755]
11. Piccolo P, Vetrini F, Mithbaokar P, Grove NC, Bertin T, Palmer D, et al. SR-A and SREC-I Are Kupffer and Endothelial Cell Receptors for Helper-dependent Adenoviral Vectors. Mol Ther. 2013
12. Khare R, Reddy VS, Nemerow GR, Barry MA. Identification of adenovirus serotype 5 hexon regions that interact with scavenger receptors. J Virol. 2012; 86(4):2293–301. [PubMed: 22156515]
13. van Rooijen N, van Kesteren-Hendrikx E. “In vivo” depletion of macrophages by liposome-mediated “suicide”. Methods Enzymol. 2003; 373:3–16. [PubMed: 14714393]
14. Haisma HJ, Kamps JA, Kamps GK, Plantinga JA, Rots MG, Bellu AR. Polyinosinic acid enhances delivery of adenovirus vectors in vivo by preventing sequestration in liver macrophages. J Gen Virol. 2008; 89(Pt 5):1097–105. [PubMed: 18420786]
15. Brunetti-Pierri N, Ng T, Iannitti DA, Palmer DJ, Beaudet AL, Finegold MJ, et al. Improved hepatic transduction, reduced systemic vector dissemination, and long-term transgene expression by delivering helper-dependent adenoviral vectors into the surgically isolated liver of nonhuman primates. Hum Gene Ther. 2006; 17(4):391–404. [PubMed: 16610927]
16. Kuzmin AI, Finegold MJ, Eisensmith RC. Macrophage depletion increases the safety, efficacy and persistence of adenovirus-mediated gene transfer in vivo. Gene Ther. 1997; 4(4):309–16. [PubMed: 9176516]
17. Holzl MA, Hofer J, Kovarik JJ, Roggenbuck D, Reinhold D, Goihl A, et al. The zymogen granule protein 2 (GP2) binds to scavenger receptor expressed on endothelial cells I (SREC-I). Cell Immunol. 2011; 267(2):88–93. [PubMed: 21190681]
18. Matsumoto K, Sano H, Nagai R, Suzuki H, Kodama T, Yoshida M, et al. Endocytic uptake of advanced glycation end products by mouse liver sinusoidal endothelial cells is mediated by a

scavenger receptor distinct from the macrophage scavenger receptor class A. The Biochemical journal. 2000; 352(Pt 1):233–40. [PubMed: 11062078]

19. Naito M, Suzuki H, Mori T, Matsumoto A, Kodama T, Takahashi K. Coexpression of type I and type II human macrophage scavenger receptors in macrophages of various organs and foam cells in atherosclerotic lesions. The American journal of pathology. 1992; 141(3):591–9. [PubMed: 1519666]
20. Lougheed M, Lum CM, Ling W, Suzuki H, Kodama T, Steinbrecher U. High affinity saturable uptake of oxidized low density lipoprotein by macrophages from mice lacking the scavenger receptor class A type I/II. J Biol Chem. 1997; 272(20):12938–44. [PubMed: 9148899]
21. Segers FM, Yu H, Molenaar TJ, Prince P, Tanaka T, van Berkel TJ, et al. Design and validation of a specific scavenger receptor class AI binding peptide for targeting the inflammatory atherosclerotic plaque. Arterioscler Thromb Vasc Biol. 2012; 32(4):971–8. [PubMed: 22282357]
22. Brunetti-Pierri N, Palmer DJ, Mane V, Finegold M, Beaudet AL, Ng P. Increased hepatic transduction with reduced systemic dissemination and proinflammatory cytokines following hydrodynamic injection of helper-dependent adenoviral vectors. Mol Ther. 2005; 12(1):99–106. [PubMed: 15963925]
23. Tamura Y, Osuga J, Adachi H, Tozawa R, Takanezawa Y, Ohashi K, et al. Scavenger receptor expressed by endothelial cells I (SREC-I) mediates the uptake of acetylated low density lipoproteins by macrophages stimulated with lipopolysaccharide. J Biol Chem. 2004; 279(30): 30938–44. [PubMed: 15145948]
24. Schiedner G, Hertel S, Johnston M, Dries V, van Rooijen N, Kochanek S. Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. Mol Ther. 2003; 7(1):35–43. [PubMed: 12573616]
25. Manickan E, Smith JS, Tian J, Eggerman TL, Lozier JN, Muller J, et al. Rapid Kupffer cell death after intravenous injection of adenovirus vectors. Mol Ther. 2006; 13(1):108–17. [PubMed: 16198149]
26. Brunetti-Pierri N, Stapleton GE, Palmer DJ, Zuo Y, Mane VP, Finegold MJ, et al. Pseudo-hydrodynamic delivery of helper-dependent adenoviral vectors into non-human primates for liver-directed gene therapy. Mol Ther. 2007; 15(4):732–40. [PubMed: 17285138]
27. Brunetti-Pierri N, Stapleton GE, Law M, Breinholt J, Palmer DJ, Zuo Y, et al. Efficient, long-term hepatic gene transfer using clinically relevant HDAd doses by balloon occlusion catheter delivery in nonhuman primates. Mol Ther. 2009; 17(2):327–33.
28. Brunetti-Pierri N, Liou A, Patel P, Palmer D, Grove N, Finegold M, et al. Balloon catheter delivery of helper-dependent adenoviral vector results in sustained, therapeutic hFIX expression in rhesus macaques. Molecular therapy. 2012; 20(10):1863–70. [PubMed: 22828499]
29. Kim J, Kim PH, Kim SW, Yun CO. Enhancing the therapeutic efficacy of adenovirus in combination with biomaterials. Biomaterials. 2012; 33(6):1838–50. [PubMed: 22142769]
30. Waehler R, Russell SJ, Curiel DT. Engineering targeted viral vectors for gene therapy. Nat Rev Genet. 2007; 8(8):573–87.
31. Smith GP. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science. 1985; 228(4705):1315–7. [PubMed: 4001944]
32. Nixon AE, Sexton DJ, Ladner RC. Drugs derived from phage display: From candidate identification to clinical practice. mAbs. 2013; 6(1)
33. Schnell MA, Zhang Y, Tazelaar J, Gao GP, Yu QC, Qian R, et al. Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. Mol Ther. 2001; 3(5 Pt 1):708–22.
34. Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol Genet Metab. 2003; 80(1-2):148–58. [PubMed: 14567964]
35. van Dijk R, Montenegro-Miranda PS, Riviere C, Schilderink R, ten Bloemendaal L, van Gorp J, et al. Polyinosinic acid blocks adeno-associated virus macrophage endocytosis in vitro and enhances adeno-associated virus liver-directed gene therapy in vivo. Hum Gene Ther. 2013; 24(9):807–13. [PubMed: 24010701]

36. Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N Engl J Med. 2011; 365(25):2357–65. [PubMed: 22149959]

37. Brown MS, Goldstein JL. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Annu Rev Biochem. 1983; 52:223–61.

38. Pfistershammer K, Klauser C, Leitner J, Stockl J, Majdic O, Weichhart T, et al. Identification of the scavenger receptors SREC-I, Cla-1 (SR-BI), and SR-AI as cellular receptors for Tamm-Horsfall protein. J Leukoc Biol. 2008; 83(1):131–8. [PubMed: 17928461]

39. Murshid A, Gong J, Calderwood SK. Heat shock protein 90 mediates efficient antigen cross presentation through the scavenger receptor expressed by endothelial cells-I. J Immunol. 2010; 185(5):2903–17. [PubMed: 20686127]

40. Palmer D, Ng P. Improved system for helper-dependent adenoviral vector production. Mol Ther. 2003; 8(5):846–52. [PubMed: 14599819]

41. Suzuki M, Cela R, Clarke C, Bertin TK, Mourino S, Lee B. Large-scale production of high-quality helper-dependent adenoviral vectors using adherent cells in cell factories. Hum Gene Ther. 2010; 21(1):120–6. [PubMed: 19719388]

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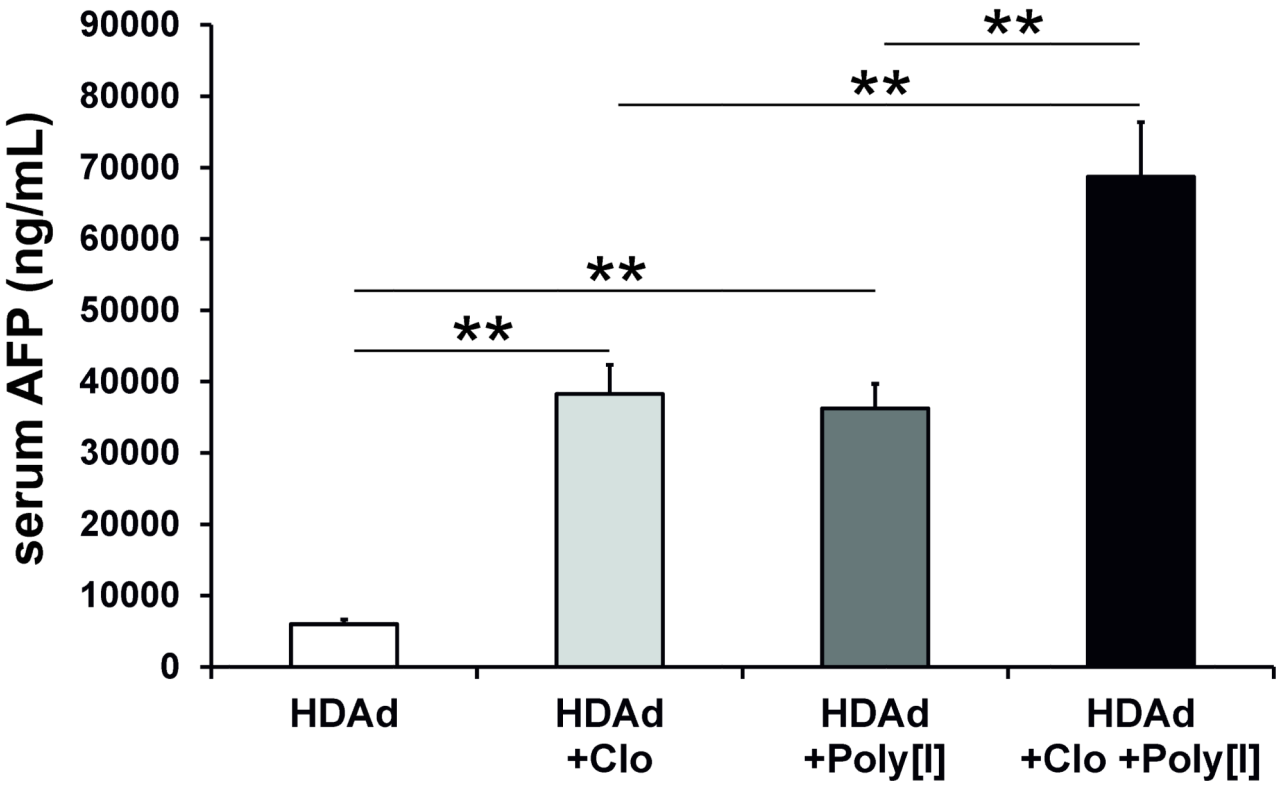

Figure 1. Polyinosine (poly[I]) increases HDAd-mediated hepatocyte transduction in Kupffer cell depleted livers.

Two days prior to HDAd-AFP injections, C57BL/6 mice were injected with PBS liposomes or clodronate (Clo). Poly[I] or saline injections were performed 5 minutes before vector injections. Serum AFP levels at 1 week post-injection are shown (n=5 per group; one way ANOVA and post-hoc Tukey's test: $^{**}p<0.01$).

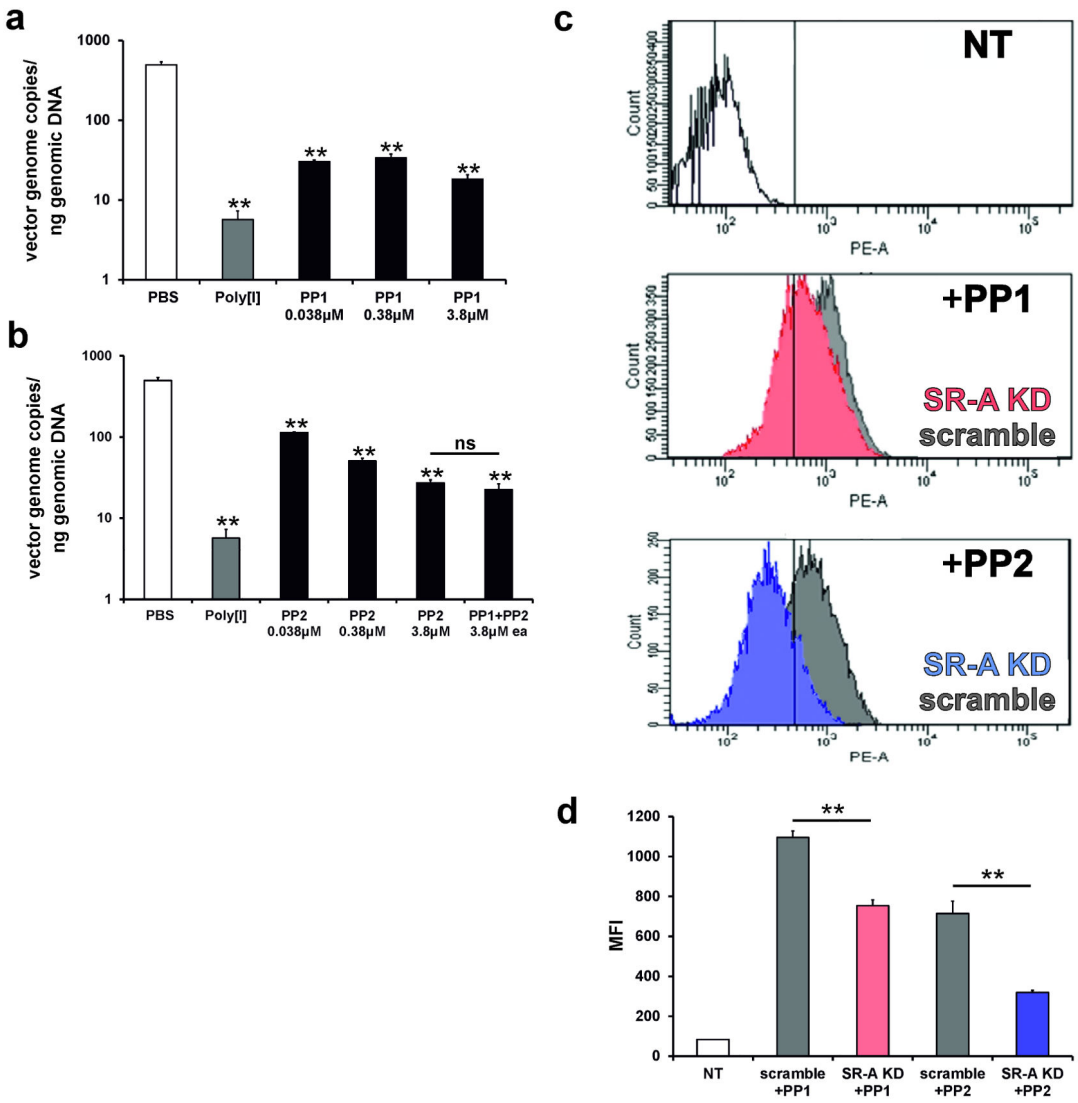

Figure 2. Inhibition of HDAd uptake by PP1 and PP2 in macrophages.

J774A.1 cells were incubated with PBS, polyinosine (poly[I]) or increasing concentrations of PP1 (**a**) or PP2 (**b**) prior to the infection with HDAd-LacZ vector. Vector genome copies were determined by qPCR (n=3 per group; one way ANOVA and post-hoc Tukey's test: $^{**}p<0.01$). (**c**) SR-A silencing (SR-A KD) reduces tetramethylrhodamine (TAMRA)-PP1 or -PP2 uptake in J774A.1 cells. (**d**) Fluorescence quantification showed reduction of PP1 and PP2 in SR-A knock-down cells compared to scramble transfected cells (n=2 per treatment; *t*-test: $^{**}p<0.01$). Abbreviations: PE-A, phycoerythrin; MFI, mean of fluorescence intensity; NT, not treated cells; ns, not statistically different.

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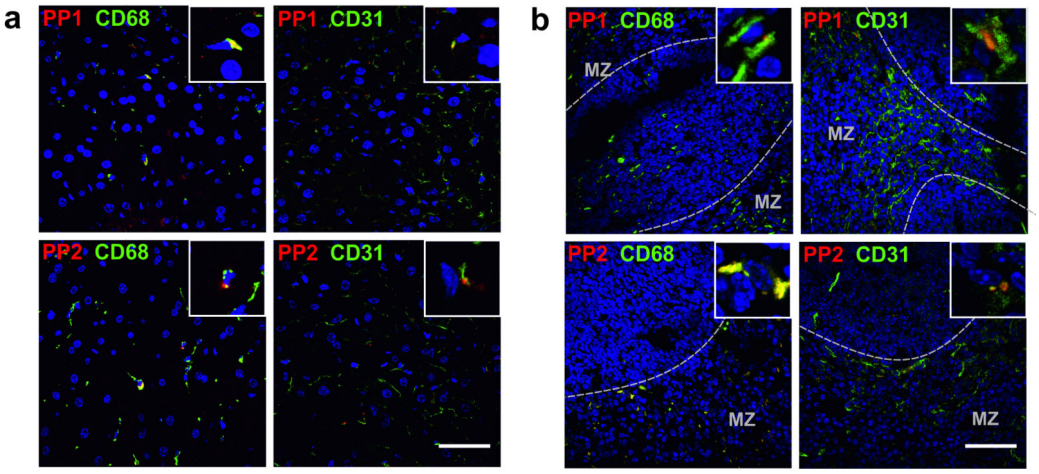

Figure 3. PP1 and PP2 target macrophages and endothelial cells in both liver and spleen.

(**a**) Liver confocal analysis showed co-localization of macrophage (CD68, green) and endothelial (CD31, green) specific antibodies with PP1 (red) and PP2 (red). (**b**) Spleen confocal analysis showed co-localization of PP1 (red) and PP2 (red) in marginal zone (MZ) in both $CD68^+$ macrophages and $CD31^+$ stromal endothelial cells (63x magnification; scale bar: 50μm).

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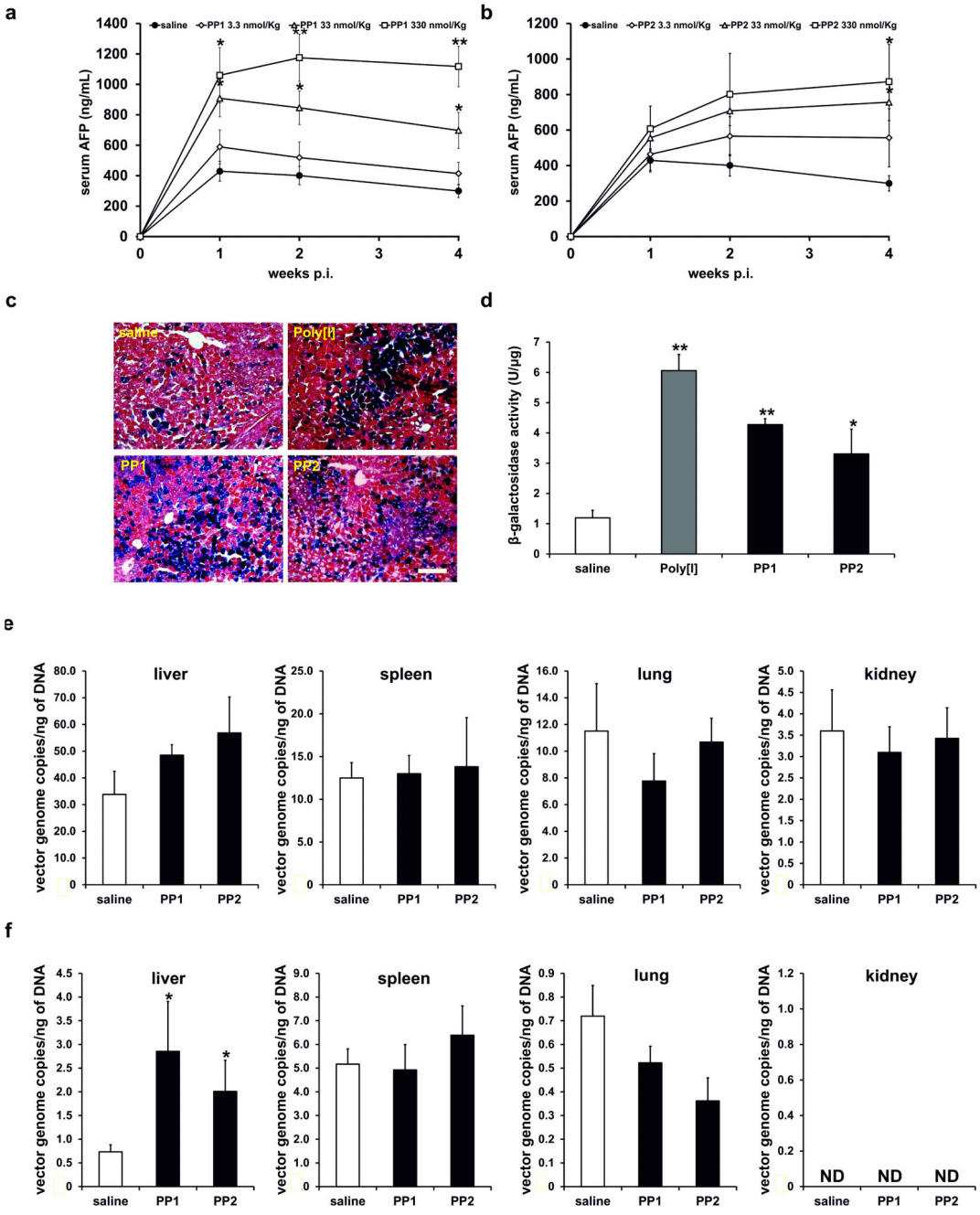

Figure 4. Pre-injections with PP1 or PP2 increases HDAd-mediated hepatocyte transduction.

(**a**) Serum AFP levels at various times post-injection (p.i.) in PP1 or saline pre-injected mice (at least n=5 per group; one way ANOVA and post-hoc Tukey's test: $^{**}p<0.01$, $^{*}p<0.05$ compared to saline). (**b**) Serum AFP levels at various times post-injection in PP2 or saline pre-injected mice (at least n=5 per group; one way ANOVA and post-hoc Tukey's test: $^{*}p<0.05$ compared to saline). Saline group is the same as in panel (**a**). (**c**) Representative images from liver X-gal histochemistry of mice receiving 5×10^{11} vp/kg of HDAd-LacZ after injections with saline, poly[I], PP1 (330 nmol/kg), or PP2 (330 nmol/kg) at 72 hours post-

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vector injection (20x magnification; scale bar: 500μm) (**d**) β-galactosidase activity in liver extracts from saline, polyinosine (poly[I]), PP1, or PP2 pre-treated animals at 72 hours post-injection (at least n=3 per group; *t*-test: $^{**}p<0.01$, $^{*}p<0.05$ compared to saline). (**e**) HDAd vector biodistribution at 72 hours after the injection of 5×10^{11} vp/kg of HDAd-CMVLacZ in mice pre-treated with PP1 (330 nmol/Kg) or PP2 (330 nmol/Kg) (at least n=3 per group). (**f**) HDAd vector biodistribution at 3 months post-injection in mice pre-treated with PP1 (330 nmol/Kg) or PP2 (330 nmol/Kg) prior to the injection of 1×10^{11} vp/kg of HDAd-AFP (at least n=5 per group; *t*-test: $^{*}p<0.05$ compared to saline). Abbreviations: ND, undetectable.

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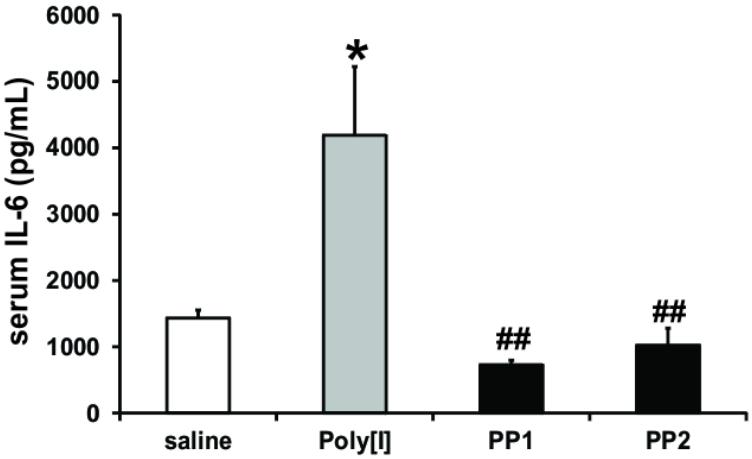

Figure 5. Serum IL-6 levels in mice injected with saline, polyinosine (poly[I]), PP1 (33 nmol/Kg), or PP2 (33 nmol/Kg) prior to the injection of 3×10^{12} vp/kg of HDAd-AFP.

Poly[I] pre-treatment resulted in marked increase of serum IL-6 at 6 hours post-injection compared to saline, PP1 and PP2 pre-injected animals (n=5 per group; one way ANOVA and post-hoc Tukey's test: *$p<0.05$ vs. saline; $^{\#\#}p<0.01$ vs. poly[I]).