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

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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 (KCs) 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 KCs and LSECs, respectively. Compared with 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 greater hepatocyte transduction, compared with control saline injected mice, pre-treatment with either peptide resulted in no increased levels of serum interleukin-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.

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 (Ad) serotype 5 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 have been recognized as major barriers for efficient 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 liver sinusoidal endothelial cells 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 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 h from clodronate injection, when KC number is greatly diminished,13 mice were injected with polyinosine (poly[I]) that binds and inhibits SR-mediated uptake.14 prior to the injection of 1 × 1012 vp kg−1 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 with control mice pre-injected with saline (one way analysis of variance (ANOVA) and post hoc Tukey’s test: P < 0.01) (Figure 1). Interestingly, compared with 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 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 scavenger receptors to achieve preferential hepatocyte transduction.

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SR-A and SREC-I binding peptides reduce HDAd vp uptake

Although 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 16 clones per library were isolated and sequenced. Six different consensus sequences were built and assayed by competitive enzyme-linked immunosorbtent assay (ELISA) for binding affinity against the recombinant protein (Supplementary Table 1). The peptide sequence ITTPGMWSQSQR, 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 phosphate buffer solution (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) (Figures 2a and b). Co-incubation with both PP1 and PP2 did not result in further reduction of vector uptake compared with incubations with each single peptide (Figure 2b). Owing to the lack of an additive effect on inhibition of vector uptake, we hypothesized that PP2 is not entirely specific for its target and it cross-reacts with SR-A. To test this hypothesis, J774A.1 cells knocked-down for SR-A expression (Supplementary Fig. 1) were incubated with tetracythylrhodamine (TAMRA)-labeled PP1 or PP2 to measure peptide uptake by cytometry. As expected, SR-A-silenced cells showed a decrease of PP1 uptake compared with control cells (t-test: P < 0.01) (Figures 2c and d). 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 (Figures 2c and d). The lower uptake of PP2 compared with PP1 might be explained by the significantly higher SR-A expression in J774A.1 cells compared with 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 (Figure 3a); in spleen, both peptides localized mostly in the marginal zone, both in CD68+ macrophages and in CD31+ stromal endothelial cells (Figure 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, 33, and 330 nmol kg⁻¹) of PP1, PP2 or with saline 5 min prior to the injection of 1 × 10¹¹ vp kg⁻¹ of HDAd-AFP. Compared with saline-injected animals, mice pre-injected with 33 and 330 nmol kg⁻¹ 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 4 weeks post injection (Figure 4a).

Mice pre-injected with 33 and 330 nmol kg⁻¹ of PP2 showed a significant 2.5- (P < 0.05) and 2.9-fold (P < 0.01) increase in transduction efficiency at 4 weeks post injection compared with saline controls (Figure 4b). Co-administration of PP1 and PP2, each at the doses of 33 or 330 nmol kg⁻¹, resulted in 2.8-fold higher levels of serum AFP compared with saline-treated mice that was not higher compared with 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⁻¹ of PP1 or PP2 prior to the injection of 5 × 10¹ⁱ vp kg⁻¹ of HDAd-LacZ (Figures 4c and d). 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 with saline-pre-treated mice (Figures 4c and d). Compared with 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 (Figures 4c and d). Vector genome copies measured by qPCR were slightly higher but not statistically different in livers of mice pre-treated with 330 nmol kg⁻¹ of PP1 and PP2 compared with control animals at 72 h after the injection of 5 × 10¹¹ vp kg⁻¹ of HDAd-LacZ (Figure 4e). However, at this early time point, livers contain vector genome copies derived from bloodborne vector 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⁻¹ of PP1 and PP2, respectively, prior to the injection of HDAd-AFP vector at the dose of 1 × 10¹¹ vp kg⁻¹ (t-test: P < 0.05) (Figure 4f). Peptide pre-treatment did not significantly affect vector uptake by spleen, lung and kidney at both time points analyzed (Figures 4e and f).

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.27 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 h after the injection of 3 × 10¹² vp kg⁻¹ of HDAd-AFP or saline in mice pre-injected with the minimum effective dose of PP1 (33 nmol kg⁻¹) or PP2 (33 nmol kg⁻¹). 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 with 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 with mice pre-injected with saline (Figure 5). Moreover, injections of PP1 or PP2 alone without vector or prior to the administration of the more clinically relevant dose of 1 × 10¹¹ vp kg⁻¹ did not result in a significant increase of serum

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IL-6 levels compared with control saline-pre-injected mice (Supplementary Figure 3a). Ad vectors are also known to induce rapid KC death associated with an increase of serum lactic dehydrogenase. To test whether SR binding peptides affect KC necrosis induced by Ad vectors, C57BL/6 mice were injected intravenously with PP1 (33 nmol kg$^{-1}$), PP2 (33 nmol kg$^{-1}$) or saline prior to the injection of 1 × 10^{12} or 5 × 10^{12} vp kg$^{-1}$ of HDAd-AFP, or saline. After 24 h, mice were killed 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; **$P < 0.01$). (c) SR-A silencing (SR-A KD) reduces 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 with 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.

**Figure 2.** Inhibition of HDAd uptake by PP1 and PP2 in macrophages. J774A.1 cells were incubated with PBS, poly[I] or increasing concentrations of PP1 (a) or PP2 (b) or a combination of PP1 and 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 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 with 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.
antigen-binding fragments (Fab) previously used to block uptake in higher efficiency of hepatocyte transduction remains a major limitation. The development of pharmaceutical vectors (http://www.wiley.com/legacy/wileychi/genmed/clinical/).

Nevertheless, vector sequestration by liver-resident macrophages and endothelial cells,11 we have herein investigated that SR-A and SREC-I 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 the 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,33 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 models7,33 and humans34 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 KCs has also been involved in uptake of other viral vectors, namely vectors derived from adeno-associated virus serotype 8 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 also have potential applications for increasing the efficacy of AAV vectors.

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

Interaction and uptake of Tamm-Horsfall protein by SR-A and SREC-I have been proposed as important mechanisms in local host defense and could contribute to inflammatory kidney diseases associated with Tamm-Horsfall protein-specific antibody responses.38 Moreover, SREC-I mediates internalization of heat shock protein 90 (HSP90) and antigen cross-presentation.39 Therefore, binding of scavenger receptors 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

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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 with saline). (b) Serum AFP levels at various times p.i. in PP2 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 with saline). Saline group is the same as in panel (a). (c) Representative images from liver X-gal histochemistry of mice receiving \( 5 \times 10^{11} \) vp kg\(^{-1}\) of HDAd-LacZ after injections with saline, poly[I], PP1 (330 nmol kg\(^{-1}\)) or PP2 (330 nmol kg\(^{-1}\)) at 72 h post vector injection (×20 magnification; scale bar: 500 \( \mu \)m) (d) β-galactosidase activity in liver extracts from saline, poly[I], PP1 or PP2 pre-treated animals at 72 h post injection (at least \( n = 3 \) per group; \( t \)-test: \(* * P < 0.01, * P < 0.05\) compared with saline). (e) HDAd vector biodistribution at 72 h after the injection of \( 5 \times 10^{11} \) vp kg\(^{-1}\) of HDAd-LacZ in mice pre-treated with PP1 (330 nmol kg\(^{-1}\)) or PP2 (330 nmol kg\(^{-1}\)) (at least \( n = 3 \) per group). (f) HDAd vector biodistribution at 3 months p.i. in mice pre-treated with PP1 (330 nmol kg\(^{-1}\)) or PP2 (330 nmol kg\(^{-1}\)) prior to the injection of \( 1 \times 10^{11} \) vp kg\(^{-1}\) of HDAd-AFP (at least \( n = 5 \) per group; \( t \)-test: \(* P < 0.05\) compared with saline). Abbreviation: ND, undetectable.
Three independent experiments were performed for each condition. Silencing experiments were performed in J774A.1 cells in 6-well plates with Msr1 siRNA (5'-GUGAGAGAUGUUGAACCATT-3') and scrambled siRNA as negative control (Innvitrogen Life Technologies, Grand Island, NY, USA) using 25 pmol per well of each siRNA. Vector transfection reagent (Innvitrogen Life Technologies) according to manufacturer's instructions. Each treatment was performed in duplicate. After 48 h, cells were incubated for 2 h with fluorescent PP1 and PP2, washed, and collected in PBS, 0.1% bovine serum albumin. Msr1 silencing was verified by real time PCR and western blotting. Following siRNA transfection, J774A.1 cells were harvested and underwent total DNA 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 (Innvitrogen Life Technologies). The qPCR reactions were performed with SYBR Green Master Mix (Roche, Indianapolis, IN, USA) using Roche Light Cycler 480 system (Roche). PCR conditions were as follows: preheating, 5 min at 95 °C for 40 cycles of 15 s at 95 °C, 5 s at 60 °C, and 25 s 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 ΔΔCt. Ct gene – Ct reference. Untreated samples were used as calibrator and relative fold increase in expression levels was determined as EΔΔCt, E being primer efficiency. The primers used for Msr1 gene were: Msr1-for 5'-CTCAGACTGAAGACTGGAACACTAC3'; Msr1-rev 5'-TCACCTTTAACACTGGAATCTTTA-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 antibodies; secondary antibodies were ECL anti-goat and anti-mouse horseradish peroxidase, 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 FACSanto (Becton Dickinson, San Jose, CA, USA) using BD FACSdiva software (Becton Dickinson).

Mice and injections
Six- to 9-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. Polyniosinic acid potassium salt was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in PBS and injected at the dose of 0.05 mg per mouse. For KC depletion, mice were injected with clodronate liposomes (clodronateliposomes.org, Haarlem, The Netherlands) 48 h before vector administration. 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), according to manufacturer's instructions. Total proteins were extracted from livers and β-gal activity was determined using β-gal enzyme assay system with reporter lysis buffer (Promega, Madison, WI, USA) according to manufacturer's instructions. Serum lactate dehydrogenase was measured by colorimetric method (Gentaur, Kempenhout, Belgium) according to manufacturer's instructions.

HDAv vector genome copies
HDAv 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) in a total volume of 20 μl using 1 μl of each HDAv-specific primers (5′-TCTGAAATTTTTGTGTTACTCATAGCGCG-3′ and 5′-CCCTAAGCTCCTTTTAACTTGTTAAAGTC-3′) and SYBR Green I. Cycling conditions were 95 °C for 10 min followed by 45 cycles at 95 °C for 10 s, 60 °C for 7 s and 72 °C for 20 s. Serial dilutions of a plasmid bearing the PCR target sequence were used as a control to determine the amounts of HDAv. Results were analyzed with Light Cycler software version 3.5 (Roche).

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Figure 5. Serum IL-6 levels in mice injected with saline, poly(I), PP1 (33 nmol kg⁻¹) or PP2 (33 nmol kg⁻¹) prior to the injection of 3 × 10⁷ vp kg⁻¹ of HDAv-AFP. Poly(I) pre-treatment resulted in marked increase of serum IL-6 at 6 h post injection compared with 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)).
Immunofluorescence and X-gal staining
For immunofluorescence, at the time of killing, animals were perfused with PBS pH 7.4 and harvested livers were fixed with 1% paraformaldehyde, PBS pH 7.4 for 5 min. After immersion, post fixation in 1% paraformaldehyde and 0.5% glutaraldehyde solution for 3 h followed by overnight immersion in 30% sucrose, livers were included in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA, USA). 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% bovine serum albumin. 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) (Carl Zeiss, Oberkochen, Germany) and ZEN 2008 software (Carl Zeiss). At least five images per slide per animal were analyzed for each staining. ImageJ software (NIH, Bethesda, MD, USA) was used for CD68 cell count.
X-gal histochemistry was performed on liver specimens as previously described in mice killed at 72 h 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 multiple-comparison Tukey’s test were performed as statistical analysis. Error bars in the figures represent standard errors of the mean.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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