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ORIGINAL ARTICLE

rAAV9 combined with renal vein injection is optimal for kidney-targeted gene delivery: conclusion of a comparative study

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Effective gene therapy strategies for the treatment of kidney disorders remain elusive. We report an optimized kidney-targeted gene delivery strategy using recombinant adeno-associated virus (rAAV) administered via retrograde renal vein injection in mice. Renal vein injection of rAAV consistently resulted in superior kidney transduction compared with tail vein injection using as little as half the tail vein dose. We compared rAAV5, 6, 8 and 9, containing either green fluorescent protein (GFP) or luciferase reporter genes driven by the Cytomegalovirus promoter. We demonstrated that although rAAV6 and 8 injected via renal vein transduced the kidney, transgene expression was mainly restricted to the medulla. Transgene expression was systematically low after rAAV5 injection, attributed to T-cell immune response, which could be overcome by transient immunosuppression. However, rAAV9 was the only serotype that permitted high-transduction efficiency of both the cortex and medulla. Moreover, both the glomeruli and tubules were targeted, with a higher efficiency within the glomeruli. To improve the specificity of kidney-targeted gene delivery with rAAV9, we used the parathyroid hormone receptor 'kidney-specific' promoter. We obtained a more efficient transgene expression within the kidney, and a significant reduction in other tissues. Our work represents the first comprehensive and clinically relevant study for kidney gene delivery.

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INTRODUCTION

The current standard of care for nephropathies that progress to end-stage renal disease is dialysis and kidney transplantation. However, there are several drawbacks associated with renal allografts such as severe shortage of donor organs and average waiting time of 3–5 years.[1] Moreover, renal allografts only have an average lifetime of 10–15 years.[2] Significant morbidity and mortality are also associated with transplant rejection (hyperacute, acute or chronic), infections and sepsis, and post-transplant lymphoproliferative disorder resulting from life-long use of immune suppressants.[3] For monogenic hereditary nephropathies, gene therapy may prevent kidney transplantation. However, in comparison to other organs such as brain, eye or liver, kidney-targeted gene therapy has been minimally explored because of its complex anatomical structure. The kidney is composed of different cell types compartmentalized within isolated anatomic structures that limit their access.

Here, we report an optimized kidney-targeted gene delivery using recombinant adeno-associated virus (rAAV) vectors delivered via retrograde renal vein injection in mice. We modified a technique that has first been described by Maruyama et al.[4] in rats. The authors clamped the left renal vein and artery and injected naked DNA into the vein and re-established the blood flow immediately after the injection. The clinical equivalent of this strategy in humans, renal venography, is minimally invasive and readily performed as an outpatient procedure.[5] rAAV is currently the safest vector available and is already being used in multiple clinical trials.[6] rAAV is a non-integrating virus, that is, its genome stabilizes as a predominantly episomal form in the host cells.[7] Though rAAV vectors have a small packaging capacity (<4.5 kb), they present many advantages such as their lack of pathogenicity, their capacity to infect both dividing and non-dividing cells, their persistence after infection, and availability of different serotypes.[8–10]

To date, few studies have been performed using AAV for kidney gene delivery using different routes of injection. Parenchymal injection of rAAV2 resulted in low transgene expression in the tubular structures near the point of injection.[11] Renal arterial injection of rAAV2 into rat kidneys led to a limited transduction of the S3 segments of proximal tubular cells, straight segments of the proximal tubule descending into the outer medulla, for only 6 weeks.[12] Moreover, significant inflammation and renal injury were noted and attributed to the procedure. Takeda et al.[13] compared rAAV serotype 1–5 using a catheter inserted into the renal artery of rats and mice. They showed that only rAAV2 could transduce the kidney but only the tubular epithelial cells. Recently, rAAV2, 8 and 9 mutants were microinjected into the renal cortex and only rAAV2 mutant led to a robust transgene expression in the distal tubular cells.[14] Overall, the limited success obtained in these studies was achieved with rAAV serotype 2. However, while AAV2 could be used efficiently for local tissue delivery such as the retina,[15,16] the prevalence of neutralizing antibodies (NAb) against rAAV2 in the human population makes this serotype inappropriate for clinical applications using intravascular delivery.[17]

In our study, we tested rAAV serotypes 5, 6, 8 and 9 for kidney gene delivery. These commonly used serotypes were selected for

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their lower immunogenicity in humans.[18] Indeed, the prevalence of AAV2 in the healthy population is higher (72%) than the prevalence of anti-AAV5 (40%), anti-AAV6 (46%), anti-AAV8 (38%) and anti-AAV9 (47%). We are presenting the first comprehensive study for kidney-specific gene delivery using a procedure and vectors relevant for clinical application. The levels of kidney transduction were investigated in both the cortex and medulla using two different reporter genes analyzed by both qualitative and quantitative assays. Our results showed that retrograde renal vein injection of rAAV9 represents a promising strategy for the treatment of kidney diseases.

RESULTS

Tail vein injection of rAAV vectors is not suitable to target kidneys

Tail vein injection of rAAV is a successful approach to target certain organs such as liver, muscle or heart.[19-21] We investigated whether this route of injection could be appropriate for kidney gene delivery by infusing via tail vein 1×10^{11} particles of rAAV5, 6, 8 and 9 expressing luciferase under the control of a Cytomegalovirus (CMV) promoter (AAV–CMV–luc) in 2-month-old C57BL/6 mice ($n = 3$/serotype). At 3 months post injection, we visualized luminescence in live mice and explanted tissues using the IVIS imaging system (Figures 1a–c). With rAAV5, no luciferase expression was observed in any tissue in live mice and only faint expression was found in the explanted muscle. With serotypes 6, 8 and 9, luciferase expression was predominantly observed in liver, but also in muscle with serotypes 6 and 9. Weak expression of luciferase was also detected in the heart with serotype 6. However, no transgene expression was detected in kidneys in any of the treated animals. To confirm these data, we performed a more sensitive assay, the luciferase assay, from explanted kidneys. We found very low-levels of luciferase expression in two out of the three mice injected with rAAV9–CMV–luc (25.34 and 11.18 relative light units mg^{-1}, respectively). These results strongly support that a more specific approach than tail vein injection is necessary to deliver a transgene within the kidney using rAAV.

rAAV9 injected via retrograde renal vein is optimal for kidney gene delivery

We used hydrodynamic fluid as a method of delivery to improve the access and spreading of the viral particles in all the renal structures. Indeed, by impacting fluid pressures within thin and stretchable capillaries, this process increases the permeability of the capillary endothelium[22] and epithelial junctions,[23] and generates transient pores in plasma membranes facilitating the cellular internalization of macromolecules of interest.[24] Using trypan blue, we determined that a retrograde renal vein injection was an efficient way to reach the cortex (Figures 2a and b). Kidney histology performed 24h after retrograde renal vein injection of 100 μl phosphate-buffered saline (PBS) and 15-min clamping revealed that this procedure does not induce kidney injury (data not shown).

We injected into the left renal vein 5×10^{10} particles of rAAV5, 6, 8 and 9–CMV–luc ($n = 6$ for each serotype and $n = 3$ for control), representing half the dose of virus particles injected in the tail vein. Luciferase expression was quantified at different time points from 1 week to 6 months post injection in live mice using the IVIS imaging system (Figures 2c and d). Luciferase expression was detected specifically within the kidney with rAAV5 up to 3 weeks after injection and subsequently signal dropped. rAAV6 led to high, stable and specific expression of luciferase in the injected kidney. The same level of luciferase expression within the left kidney was obtained with rAAV8. However, luminescence was also detected in the right kidney and in other tissues. rAAV9 led to the strongest transgene expression within the left kidney but also in other tissues. Six months post injection, the brain, heart, liver,

spleen, muscle, left and right kidneys were explanted and subjected to IVIS analysis (Figures 2e and f). The results confirmed our data observed in live mice. Luciferase expression was specifically detected in the injected kidney only with rAAV5 and 6, but the efficiency was higher with rAAV6. High level of luminescence was observed in the left kidney with rAAV8 and 9 but signal was found in other organs as well.

Efficient kidney gene delivery implies transduction of both cortex and medulla. Therefore, we investigated luciferase expression within both of these compartments in the left kidney using the luciferase assay (Figure 2g). The medulla was better targeted than the cortex by most of the rAAV serotypes except for rAAV9. In particular, rAAV6, which seemed optimal because of its efficient and specific kidney transduction, was the most efficient to transduce the medulla but only led to a weak transduction of the cortex. Similar results were obtained with rAAV8. In contrast, luciferase expression was strong in both the medulla and cortex with rAAV9.

In parallel, we performed renal vein injection of rAAV5, 6, 8 and 9–CMV–green fluorescent protein (GFP) in 2-month-old mice and analyzed them 3 months later ($n = 6$ for each serotype). GFP-specific reverse-transcribed quantitative PCR analysis performed on the cortex and medulla of the left kidney confirmed our data obtained with rAAV–CMV–luc. rAAV6 led to the highest transduction of the medulla but only rAAV9 was capable of transducing both the cortex and medulla (Figure 3a). We also estimated transgene expression by qPCR within glomeruli and enriched tubular population from cortex biopsies using magnetic bead-mediated isolation,[25] and the medulla. While transgene expression was detected in all these compartments, glomeruli were the main transduced structures (Figure 3b). We confirmed these data by confocal microscopy by estimating the number of GFP-positive proximal tubules and glomeruli in rAAV9–GFP-injected kidneys. We found that approximately 18% of the glomeruli and 2.9% of the proximal tubules were transduced. In the glomeruli, GFP was detected in both podocytes and mesangial cells (Figure 3c). Altogether, these data demonstrated that rAAV9 injected via the renal vein is the most efficient serotype to target the different compartments of the kidney, which is a key feature for kidney gene therapy.

Transient immunosuppression can overcome the decrease of transgene expression

We showed that rAAV5 led to efficient transgene delivery, but the signal dropped drastically after 3 weeks post injection (Figure 2d). Sera from mice injected with rAAV5, 6, 8 and 9–CMV–GFP ($n = 6$/serotype) before and after vector infusion were tested for the presence of NAb by enzyme-linked immunosorbent assay. NAb titers at baseline before renal vein injection were almost null for all the serotypes (data not shown). However, at 3 months post injection, the highest level of NAb was found for rAAV5, suggesting an immune response induced specifically by this AAV serotype (Figure 4a). To test the presence of a T-cell response generated by AAV5, we isolated kidneys from mice injected with rAAV5–CMV–GFP ($n = 6$, $n = 1$ for control) 4 weeks post injection and performed histological and confocal analyses. Results showed that rAAV5–CMV–GFP-infected kidney present with mononuclear infiltrations that stained positively with a CD3 antibody, a marker for T-lymphocytes (Figure 4b).

To investigate whether rAAV5 could be an efficient vector to target the kidney in absence of immune response, we injected 5×10^{10} particles of rAAV5–CMV–luc in transiently immunosuppressed mice ($n = 6$) using a combination of non-depleting CD4 antibodies (NDCD4ab) and cyclosporine A as previously described.[26] Efficient, specific and stable luciferase expression was observed in the injected kidney compared with immunocompetent mice (Figures 4c–e).

In order to determine which serotype, rAAV5 or 9, was the most appropriate for kidney gene delivery, we compared luciferase
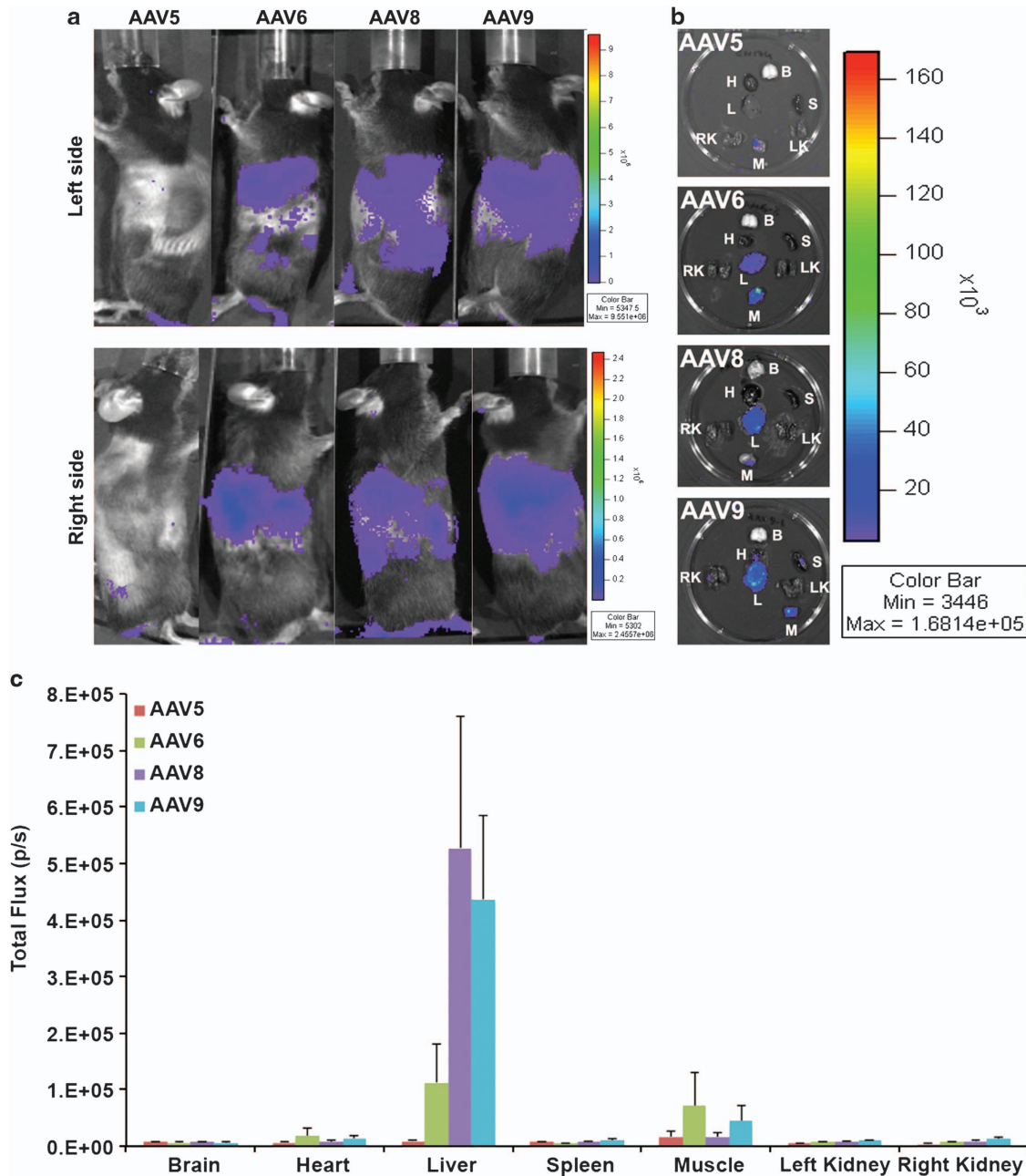
Figure 1. Expression of luciferase after tail vein injection of rAAV5, 6, 8 and 9–CMV–luc. (**a**) Representative pictures of the left (upper panel) and right (lower panel) sides of live mice at 12 weeks post injection of 1×10^{11} particles of rAAV5, 6, 8 and 9–CMV–luc ($n = 3$/serotype) via tail vein analyzed with the IVIS live imaging system. No luciferase expression was observed with rAAV5. For the other serotypes, luminescence was mainly seen in the upper level of the body. (**b**) The explanted organs, brain (B), heart (H), liver (L), spleen (S), muscle (M), left (LK) and right kidneys (RK), were then scanned with IVIS. Luciferase expression was mainly detected in the liver for rAAV6, 8 and 9 and in the muscle for rAAV6 and 9. (**c**) Histogram representing the quantification of luciferase expression in the different organs as an average of the three mice per serotype. Error bars represent s.e.m.

expression within the cortex and medulla of kidneys injected with either rAAV5–CMV–luc in presence of immunosuppressors or rAAV9–CMV–luc (Figure 4f). Results showed that serotype 9 remains the most efficient to transduce both the renal cortex and medulla.

Combining rAAV9 and the parathyroid hormone receptor 'kidney-specific' promoter P1 is optimal for kidney gene delivery

rAAV9 is the most efficient rAAV serotype for kidney gene delivery. However, transgene expression was also detected in other tissues. To overcome the non-specificity of rAAV9, we replaced the CMV

promoter by P1.[27] We chose P1 promoter because of its expression in a wide variety of renal cell types.[28,29] We injected 1×10^{11} particles of rAAV9–P1–luc via tail vein ($n = 3$) or 5×10^{10} particles via the left renal vein ($n = 6$) and analyzed the explanted tissues using the IVIS Imaging system at 3 months post injection. Transgene expression was decreased in most of the tissues from mice injected with rAAV9–P1–luc compared with mice injected with rAAV9–CMV–luc after both tail vein and renal vein injections (Figures 5a and b). Surprisingly, even if decreased, luciferase expression in the liver was still observed, suggesting that P1 is also
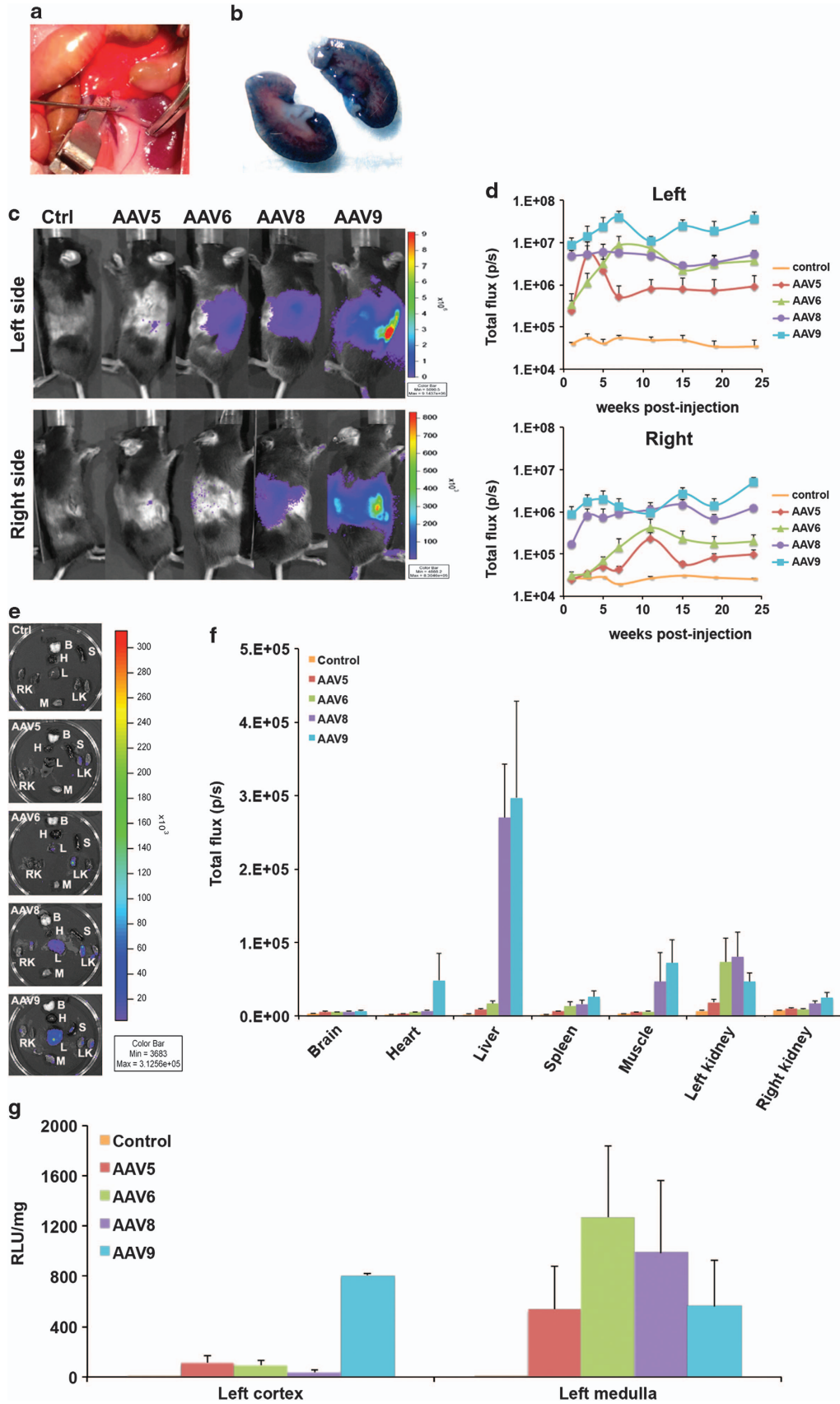
expressed in this tissue. However, after tail vein injection kidney transduction remained very low with rAAV9–P1–luc, supporting our previous observations that systemic injection of rAAV is not suitable for kidney gene delivery. In contrast, retrograde renal vein injection of rAAV9–P1–luc led to a strong expression of the luciferase within the kidney, even higher than with rAAV9–CMV–luc (Figure 5b). Confocal and qPCR analyses of the isolated left injected kidneys confirmed the presence of luciferase expression in both glomeruli and proximal tubules (Figure 5c and data not shown).

DISCUSSION

Kidney-targeted gene therapy may prevent dialysis and kidney transplantation for hereditary renal disorders. However, the complex structure of the kidney reduces the chance for successful gene transfer. Although both non-viral and viral-mediated gene delivery via different routes of injection have been tested for targeting the kidney, there have been limited reports of success.[4,11–13,30] We presented a strategy combining rAAV delivered via a retrograde renal vein injection as a practical and minimally invasive procedure for clinical application. We demonstrated by histological analysis that our technique does not induce tissue injury. Recently, Corridon et al.[31] obtained high level of transgene expression within rat kidney using an optimized method of retrograde renal vein injection. However, they used adenovirus and bacilovirus vehicles that have minimal relevance for clinical application.[32,33] We chose rAAV because this vector is safe and already used in several clinical trials.[6] Moreover, Ito et al.[34] showed that AAV-mediated kidney transduction was improved in damaged kidney compared with normal kidney, highlighting its relevance for nephropathies.

We demonstrated that systemic injection of rAAV serotypes 5, 6, 8 and 9 failed to transduce the kidney. In contrast, renal vein injection of the same rAAV serotypes at half the dose, led to successful kidney gene delivery. Therefore, renal vein injection of rAAV represents a more efficient and economical procedure. Indeed, Good Manufacturing Practice vector preparations are expensive, making the economic argument more realistic for a clinical application. Moreover, this strategy also represents a safer method by limiting the dose, which may reduce the immune responses.[35,36]

As different serotypes of AAV have different tropism, we compared rAAV5, 6, 8 and 9 for their efficiency of transducing the kidney in vivo. We showed that rAAV6, 8 and 9 injected via renal vein were efficient for transducing the kidney. However, a comprehensive study of transgene expression using two different reporter genes, GFP and luciferase, showed that rAAV6 and 8 mainly transduced the renal medulla. In contrast, rAAV9 transduced both the cortex and medulla. In addition, rAAV9 delivered via renal vein allowed the transduction of both the proximal

tubules and glomeruli in contrast to the parenchymal or the renal artery routes that led to the transduction of the tubular structures only.[11–13] This feature will be important to obtain a therapeutic gene therapy approach for many hereditary renal diseases. For instance, individuals affected with cystinosis first develop the de Toni Debré Fanconi syndrome characterized by proximal tubulopathy followed by glomerular damage that progresses to end-stage renal failure.[37] This disease is due to mutations in the gene CTNS encoding for the lysosomal transporter that allows the exit of cystine out of the lysosomes.[38–41] Our strategy could lead to a functional restoration of the transporter in the proximal tubules and glomeruli preventing both the proximal tubulopathy and kidney transplantation. Moreover, in contrast to previous studies that showed only transient expression of their transgene within the kidney, we demonstrated the long-term persistence of the transgene after a single administration of rAAV (up to 6 months which represents our last time point analyzed). For instance, Yang et al.[42] reported partial correction of the urinary concentrating defect in response to water deprivation in aquaporin-1-deficient mice by treating them with an adenoviral vector containing aquaporin-1 injected by tail vein. Aquaporin-1 expression and the resulting effects were lost over 3–5 weeks. The route of injection (renal vein vs tail vein) and the viral vector (AAV vs adenovirus) make our strategy more appropriate to reach a therapeutic level for kidney disorders.

Because rAAV8 and 9 can efficiently cross the vascular endothelial cell barrier,[43] transgene expression was detected in the kidney but also in other organs. rAAV9 uses terminal amino (N)-linked galactose as primary receptor[44] and 37/67-kDa laminin as co-receptor.[43] Shen et al.[45] showed that tissue levels of terminally galactosylated glycans are involved in the systemic tropism and the hepatotropic phenotype of rAAV9, as well as its persistence in blood circulation. Due to the risks associated with gene therapy, it is important to increase the specificity of tissue targeting. We showed that combining the strong ability of tissue transduction of rAAV9 with P1, we significantly improved the specificity and the efficiency of kidney gene delivery, although signal was also found in liver. Another advantage of combining rAAV9 with a disease-specific promoter is that we could drive transgene expression not only in the kidney but also in other potentially affected tissues. The glycogen storage disease type I is a life-threatening metabolic autosomal recessive disorder caused by the deficiency of glucose-6-phosphatase in liver and kidney.[46] The minimal human glucose-6-phosphatase promoter is fully characterized[47–49] and, in association with rAAV9 injected via renal vein, may lead to a therapeutic gene delivery of glucose-6-phosphatase in both kidney and liver. Similarly, type I tyrosinemia is caused by a deficiency of the enzyme fumarylacetoacetate hydrolase expressed in liver and kidney. Patients develop liver disease and renal failure as well as nervous system anomalies.[50] In addition, we showed that renal vein injection using a lower dose

Figure 2. Expression of luciferase after retrograde renal vein injection of rAAV5, 6, 8 or 9–CMV–luc. (a) Picture of the renal vein injection procedure. The left renal vein was isolated, clamped and 100 µl of PBS 1× containing 5×10^{10} particles of rAAV was injected using a 31G needle. (b) Representative picture of an explanted kidney after retrograde renal vein injection of trypan blue solution. (c) Representative pictures of the left (upper panel) and right (lower panel) sides of live mice at 24 weeks post injection of 5×10^{10} particles of rAAV5, 6, 8 and 9–CMV–luc in the left kidney analyzed by IVIS live imaging system. Non-injected mouse is labeled Ctrl. The luminescence was observed the area of the left kidney with rAAV6, 8 and 9 as well as in the upper body. Luciferase expression was also detected in the right side of the body with rAAV8 and 9. (d) Kinetics of luciferase expression for each serotype from 1 week to 6 months post injection, each point representing the average of the six mice per serotype, except for the control ($n = 3$). Error bars represent s.e.m. The highest transgene expression in the injected kidney was observed with rAAV9. (e) Representative image of the explanted organs scanned with IVIS, brain (B), heart (H), liver (L), spleen (S), muscle (M), left (LK) and right kidneys (RK). (f) Histogram representing the quantification of luciferase expression in the different organs as an average of the six mice per serotype and three mice for the control. Luciferase expression is improved in the injected kidney for all the serotypes compared with tail vein injection. For rAAV9, this route of injection also improved transgene expression in the heart, spleen and muscle but was decreased in the liver compared with tail vein injection. Error bars represent s.e.m. (g) Quantification of luciferase expression in the cortex and medulla of the left explanted kidneys using the luciferase assay. The relative light units (RLU) were recorded and reported to the total protein content ($n = 6$/serotype). Only rAAV9 allowed a transduction of both the cortex and the medulla of the injected kidneys. Error bars represent s.e.m.
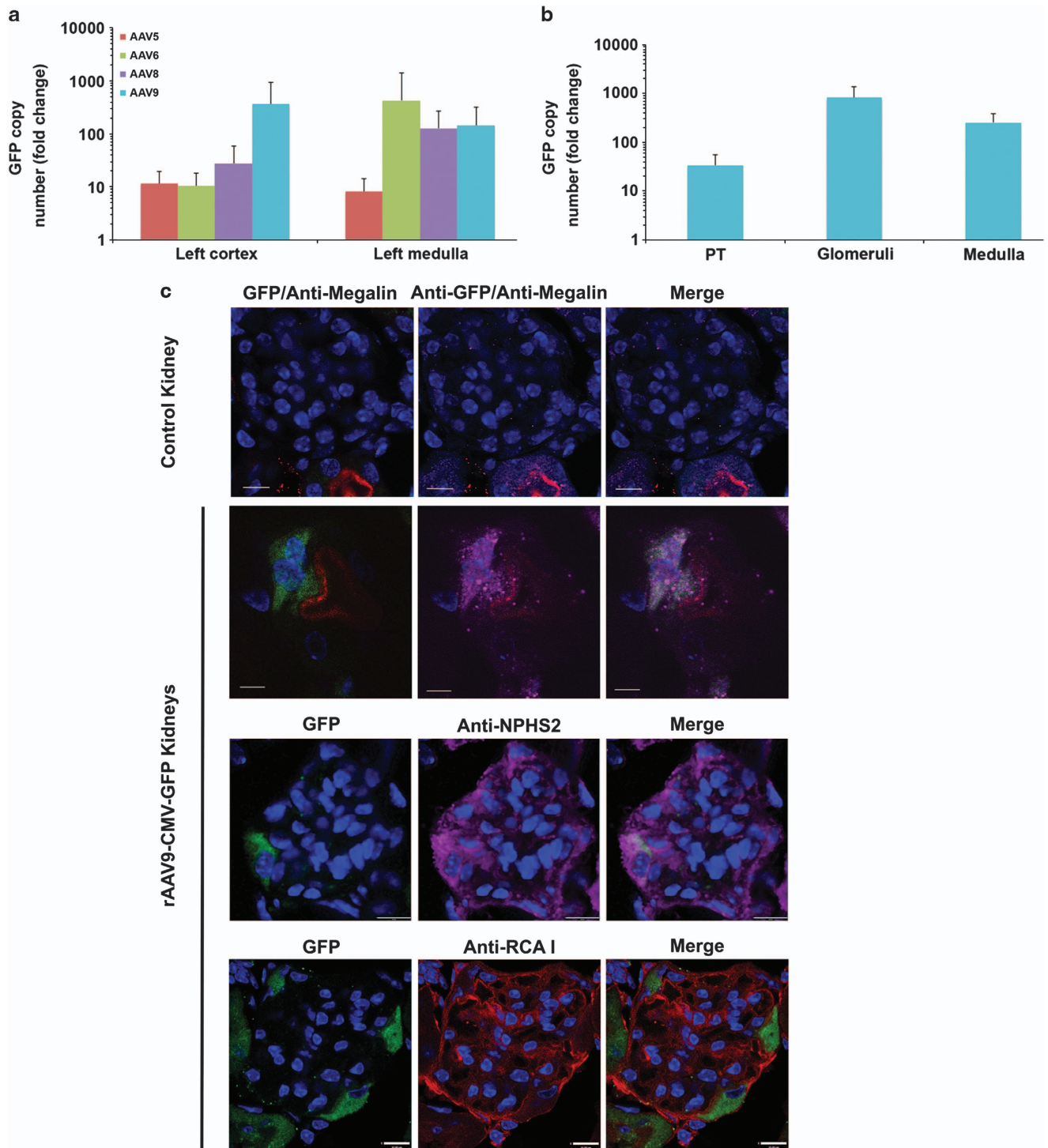
Figure 3. Kidney expression of GFP after retrograde renal vein injection of rAAV5, 6, 8 or 9–CMV–GFP. (**a**) GFP expression analyzed by reverse-transcribed quantitative PCR in the cortex and medulla of the left kidney at 12 weeks after injection of 5×10^{10} particles of AAV–CMV–GFP in the left renal vein ($n = 6$/serotype). These data confirmed that rAAV9 was the optimal serotype to transduce both the cortex and medulla. Error bars represent s.e.m. (**b**) GFP expression analyzed by reverse-transcribed quantitative PCR in the glomeruli, enriched population of proximal tubules (PT) and medulla of the left kidney at 12 weeks post injection of 5×10^{10} particles of AAV–CMV–GFP ($n = 6$/serotype). The results are expressed as fold change compared with the same cell populations isolated from non-injected kidneys. Error bars represent s.e.m. (**c**) Representative image of confocal microscopy analysis of the left kidney sections. The two top sections were stained with 4,6-diaminido-2-phenyl-indole (blue), anti-GFP (magenta), and anti-megalin (red) and GFP was observed in green. The upper panel represents a glomerulus and proximal tubules from a control kidney; no GFP expression can be seen. In the rAAV9–CMV–GFP-injected kidneys, GFP is observed in proximal tubules (second panel). The two bottom panels represent glomeruli from rAAV9–CMV–GFP-transduced kidneys. Sections were stained with anti-NPHS2 antibody (magenta) to mark the podocytes or anti-RCA I antibody (red) for the mesangial cells, 4,6-diaminido-2-phenyl-indole was observed in blue and GFP in green. Scale bars, 10 μm.
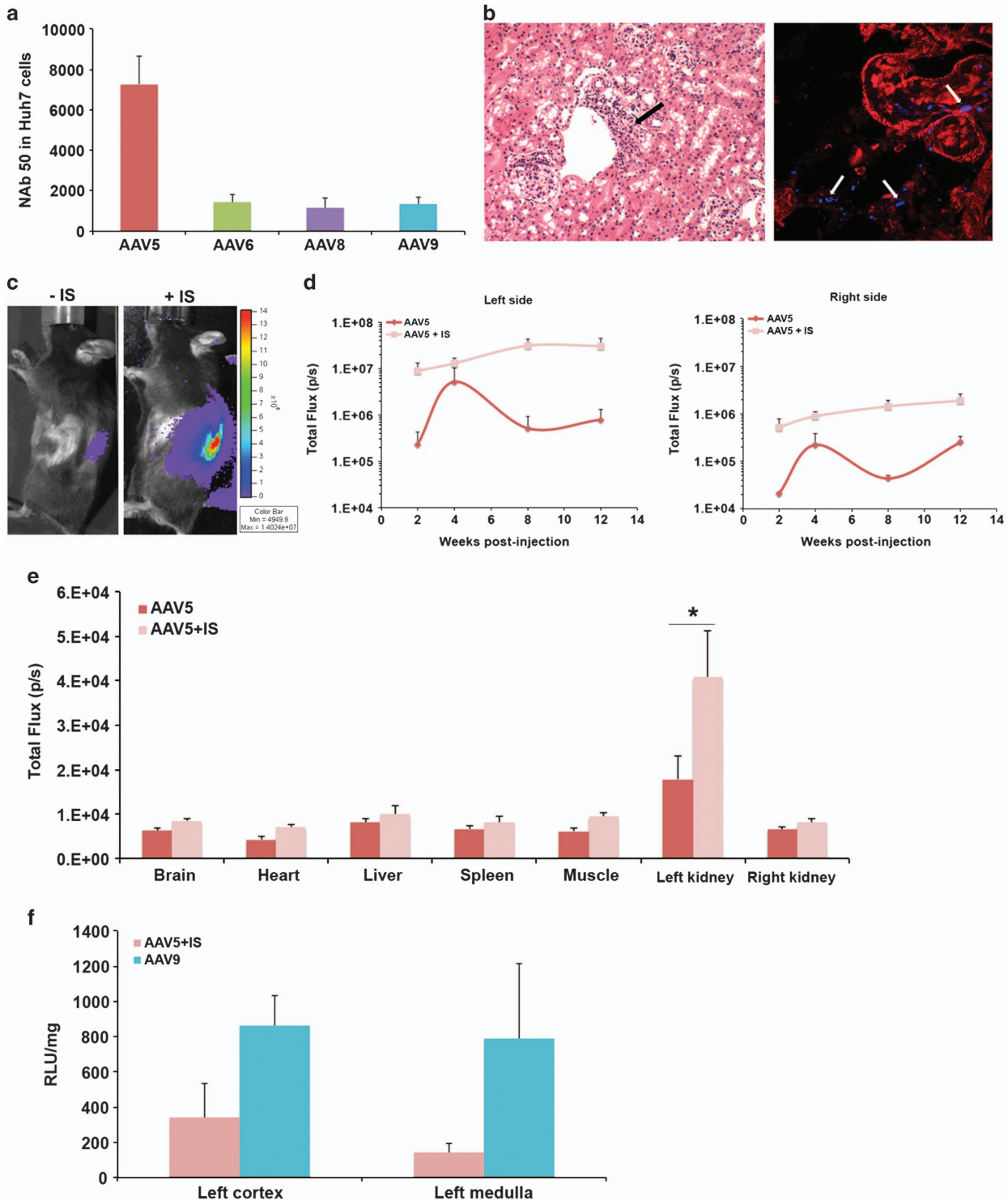
Figure 4. Immune response generated by rAAV5 and effect of transient immunosuppression. (**a**) Level of NAb directed against the rAAV capsid in sera obtained from mice injected via the left renal vein with 5×10^{10} rAAV5, 6, 8 or 9–CMV–GFP at 12 weeks post injection ($n = 6$/serotype). The highest level of NAb was seen in the animals injected with rAAV5. Error bars represent s.e.m. (**b**) Multiple mononuclear infiltrates were present within the left kidney stained with hematoxylin & eosin at 4 weeks post injection (left) and confocal analysis of the same tissue (right) stained with an anti-CD3 antibody (blue) and Phalloidin (red). (**c**) Observation of luciferase expression in mice injected with rAAV5–CMV–luc into the left renal vein in presence (+IS) or absence (− IS) of cyclosporine A and ND-CD4 at 12 weeks post injection using the IVIS imaging system. Efficient transduction of the left kidney is observed in the immunosuppressed mice. (**d**) Kinetics of luciferase expression in the right and left sides of mice injected with rAAV5–CMV–luc into the left renal vein with or without immunosuppression up to 12 weeks ($n = 6$/serotype). Error bars represent s.e.m. (**e**) Quantification of the luciferase in the explanted tissues. Luciferase is observed specifically in the left kidney. Error bars represent s.e.m. *$P < 0.05$. (**f**) Comparison of luciferase expression in mice injected with AAV9–CMV–luc and in mice transiently immunosuppressed injected with AAV5–CMV–luc via the renal vein. Luciferase assay was performed on the cortex and medulla of the left kidney of the injected mice ($n = 6$/serotype). The relative light units were recorded and reported to the protein content. The optimal serotype to transduce both the cortex and the medulla was rAAV9. Error bars represent s.e.m.
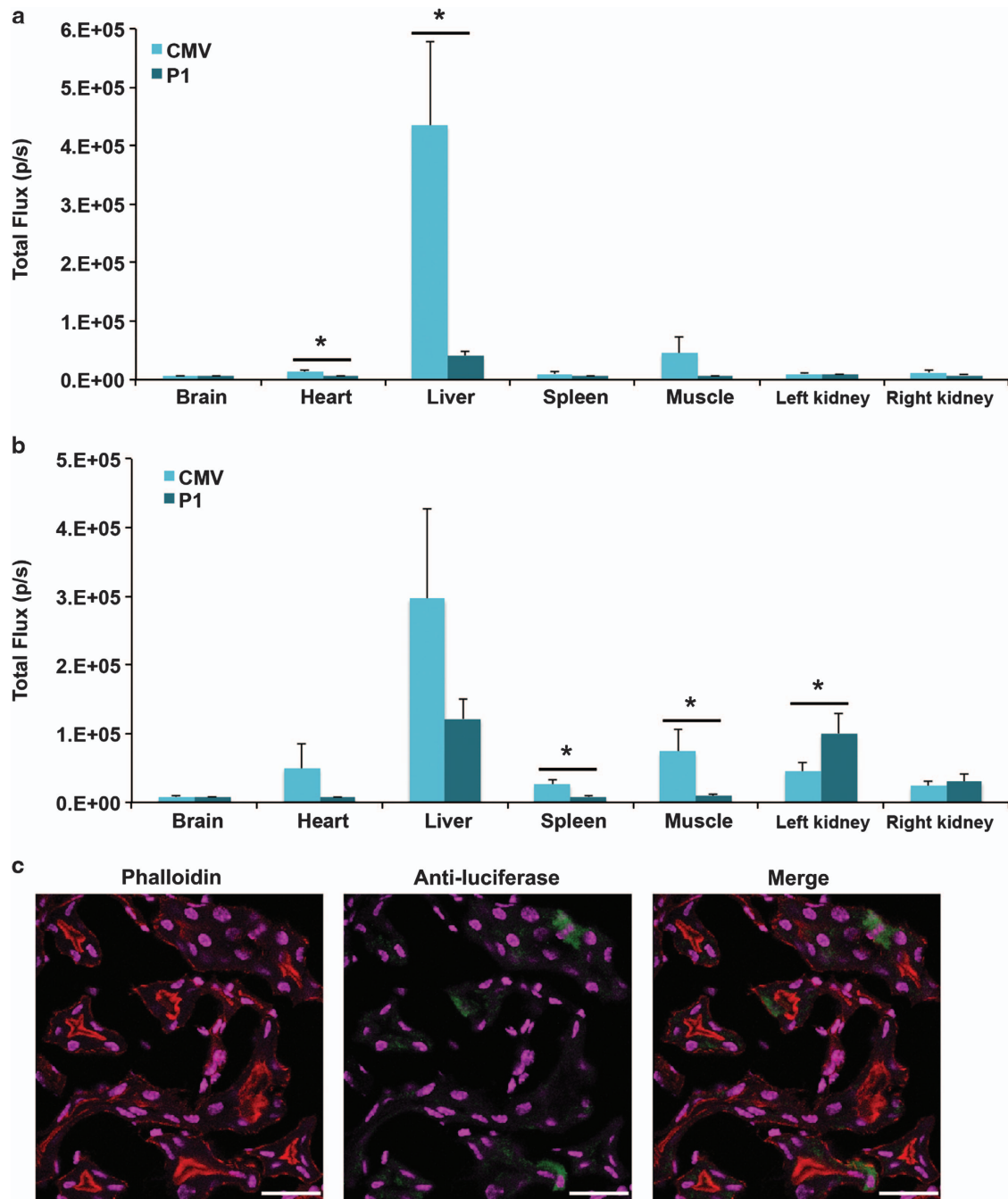
Figure 5. Comparison of luciferase expression in mice injected via renal vein with rAAV9–CMV–luc or rAAV9–P1–luc. (**a**) Level of luminescence in the explanted organs at 12 weeks post injection after tail vein injection of 10^{11} particles of AAV9–CMV–luc or AAV9–P1–luc observed using the IVIS imaging system ($n = 3$/serotype). P1 promoter led to the significant decrease of luciferase expression in all the tissues. Error bars represent s.e.m. *$P < 0.05\%$. (**b**) Level of luminescence in the explanted organs at 12 weeks post injection after renal vein injection of 5×10^{10} particles of AAV9–CMV–luc or AAV9–P1–luc ($n = 6$/serotype). Renal vein injection combined with P1 promoter improves the efficiency and the specificity of rAAV9 for kidney-targeted gene delivery. Error bars represent s.e.m. *$P < 0.05\%$. (**c**) Representative confocal image of the left kidney sections. Section was stained with anti-luciferase (green), TO-PRO (magenta) and phalloidin (red). Scale bars, 20 µm.

of rAAV could lead to a better transduction of some organs such as muscle and heart compared with tail vein injection. Thus, renal vein injection might also be considered as a new route of injection to target other tissues more efficiently.

AAV may potentially give rise to both cellular and humoral immune responses.[51] Among the serotypes we tested, only rAAV5 generated NAb in the injected mice. We showed the presence of T cells within the injected kidney, which probably explain the significant decrease of transgene expression after 3 weeks post

delivery observed in the mice injected with rAAV5–CMV–Luc. Xin et al.[52] showed that AAV serotype 5 presented with a strong tropism for dendritic cells that led to humoral and cellular responses. We overcame this issue by transiently immunosuppressing the animals with a combination of cyclosporine A that moderates T-cell function, and NDCD4ab that induces long-term antigen-specific tolerance to foreign antigens without causing depletion of CD4 lymphocytes.[53] This combination of immunosuppressors has led to promising results in the treatment of hemophilia B in

non-human primates.[54] Because the immunosuppression is only transient and allows a long-term tolerance, this strategy may allow a safe clinical application of AAV-mediated gene therapy even in patients with pre-existing NAb.

In summary, we demonstrated that retrograde renal vein injection of rAAV9 is the optimal strategy for kidney-targeted gene delivery. This clinically relevant procedure leads to an efficient, stable and economical transduction of both the renal cortex and medulla. This strategy has also the crucial advantage that combining rAAV9 with a disease-specific promoter, could not only lead to an efficient transgene expression within the kidney but also in other tissues potentially involved in the disease. We also showed that development of a T-cell response and NAb could be overcome by transient immunosuppression. This work represents the foundation for a new treatment of renal diseases.

MATERIALS AND METHODS

Mice, renal vein injection and immunosuppression

C57BL/6 mice aged 8–10 weeks were purchased from Jackson Laboratories (Sacramento, CA, USA) and bred continuously at UCSD vivarium. All mouse experiments were performed in accordance with the animal protocols approved by the Institutional Animal Care and Use Committee of UCSD. Animals received rAAV particles resuspended in 100 µl PBS 1× via tail vein injection or via retrograde renal vein injection. For renal vein injection, animals were anesthetized and the left kidney was exposed through the flank incision. The vein was clamped and rAAV particles were injected into the vein using a 31G needle. The clamp was removed after 15 min post injection and the incision sutured. To transiently immunosuppress the animals, cyclosporine A (Cell Signaling, Danvers, MA, USA) at 25 mg kg^{-1} per mouse and non-depleting CD4 YTS 177 (BioXCell, West Lebanon, NH, USA) 20 mg kg^{-1} per mouse were injected intraperitoneally on days −1, 0, +1, +3, +6 and +8 as described previously.[26]

rAAV vectors

rAAV–CMV–GFP and rAAV–CMV–luc viral particles for the serotypes 5, 6, 8 and 9 were purchased from the University of North Carolina Gene Therapy Center (Chapel Hill, NC, USA). From the CMV–luciferase plasmid, the CMV promoter was replaced by P1. The product was generated by PCR from C57BL/6 wild-type genomic DNA using the following primers P1-F: 5′-TATGGTACCGGATCCCTGGTC-3′ and P1-R: 5′-TATTCTAGACAGCTGG TGGGGG-3′. Plasmid was then amplified using the endotoxin-free maxiprep kit (Qiagen, Germantown, MD, USA) and sent to the UNC Gene Therapy Center for rAAV9–P1–luciferase particles production.

Luciferase imaging in live animals and explanted tissues

Mice injected with rAAV–luc were analyzed using the IVIS Imaging System 200 Series (Caliper Life Sciences, Hopkinton, MA, USA) 10 min after intraperitoneal injection of luciferin (Caliper, Hopkinton, MA, USA) as previously described.[55] The mice were then euthanized and the kidneys, liver, spleen, heart, muscle and brain were explanted and scanned with the IVIS (Caliper Life Sciences). Quantitative signal analysis was performed using the Living Image 2.5 software (Caliper Life Sciences). Luminescence was expressed in Total flux (photons per sec).

Luciferase assay

Explanted kidneys from mice injected with rAAV–Luciferase were dissected to separate the cortex and medulla and directly snap-frozen in liquid nitrogen. Luciferase assay was performed according to the manufacturer's instruction (Promega, Madison, WI, USA) and as previously described.[56] Briefly, frozen biopsies were individually pulverized into a fine powder and thawed. Lysis buffer was added and samples were vortexed for 15 min, frozen and thawed three times in liquid nitrogen and 37 °C water, respectively. Protein concentration of the lysates was determined using the bicinchoninic acid assay according to the manufacturer's instruction (Pierce, Rockford, IL, USA). Luminescence was measured by adding 100 µl of luciferase assay reagent to 20 µl of lysate using a luminometer with injector (TECAN, Infinite M1000 PRO, San Jose, CA, USA). Luminescence was expressed in relative light units mg^{-1} (Relative Light Units per mg of protein).

Histology

Kidneys were fixed in formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. A pathologist reviewed the slides in a blinded fashion.

Reverse transcription-quantitative PCR

Kidneys from mice injected with rAAV–GFP were explanted and RNA was isolated from the cortex and medulla biopsies using RNeasy kit according to the manufacturer's instruction (Qiagen). A total of 1 µg of RNA was reverse transcribed using iscript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative-PCR was performed using 2 µl of cDNA, the 2× Universal TAQMAN master mix (Applied Biosystems, Branchburg, NJ, USA) and the GFP-specific primer mix (F: 5′-TCCGCCCTGAGCAAAGAC-3′, R: 5′-GAACTCCAGCAGGACCATGTG-3′ and Probe: 5′-6FAMCCAACGAGAAGCG CMGBNFQ-3′) on a Bio-Rad CFX96 touch real-time PCR detection system (Bio-Rad); 18S primer mix was used as reference (Applied Biosystems, Warrington, UK). The expression level of GFP transgene, expressed as fold change, was calculated using the ΔΔCq method between the target gene and the endogenous control (18S).

Immunofluorescence analysis

Kidneys were fixed in 5% formaldehyde for 30 min, equilibrated in 20% sucrose overnight and frozen at −80 °C in tissue-Tek Optimal Cutting Temperature buffer (Sakura Finetek USA, Torrance, CA, USA). Sections of 10 µm were permeabilized using PBS–Triton 0.1% and blocked with 1% bovine serum albumin, 10% donkey serum in PBS-1×. The blocking buffer was then diluted 1:5 in PBS-1× and used to dilute antibodies. The slides were stained with 4,6-diaminido-2-phenyl-indole (1:500 dilution) or TO-PRO-3 Iodide (1:5000 dilution), BODIPY 558/568 Phalloidin (1:100 dilution), Rabbit-anti-GFP (Abcam ab290, Cambridge, MA, USA; 1:200 dilution), Goat-anti-megalin (Santa Cruz sc-16478, Santa Cruz, CA, USA; 1:200 dilution), Dylight 594 Ricinus Communis Agglutinin I (RCA I; Vector Laboratories DL-1087, Burlingame, CA, USA; 12.5 µgml^{-1}), Rabbit-anti-nephrosis 2 (NPHS2; Abcam ab50339, 1.2 µgml^{-1}), Anti-mouse CD3e PE-Cy7 (eBioscience 25-0031-81, San Diego, CA, USA; 10 µgml^{-1}) and Rabbit-anti-Firefly Luciferase (Abcam ab21176, 1:1000 dilution). As secondary antibodies, we used Donkey-anti-rabbit Cyanine 5 (Jackson ImmunoResearch, West Grove, PA, USA; 1:100 dilution), Donkey-anti-goat Alexa 546 (Invitrogen, Grand Island, NY, USA; 1:100 dilution) and Donkey-anti-Rabbit Alexa 488 (Invitrogen, 1:400 dilution). Images were acquired using the confocal Leica TCS SP5 (Leica Microsystems, Buffalo Grove, IL, USA) and analyzed with Volocity 3D image analysis software (Perkin Elmer, Santa Clara, CA, USA).

For the quantification of the transduced proximal tubules and glomeruli within rAAV9–GFP-injected kidneys, four kidney sections per mouse from four different mice were randomly analyzed with a low magnification objective (10×) after staining with anti-GFP and anti-megalin antibodies.

Glomeruli isolation

Control mice and mice injected with AAV9–CMV–GFP were anesthetized using isoflurane and perfused with 8×10^7 Dynabeads M-450 Tosylacti-vated (Life technologies, Carlsbad, CA, USA) diluted in 40 ml of HBSS (Hank's Balanced Salt Solution) through the heart. Left kidneys were removed and the cortex and medulla were dissociated. Cortex was minced into 1-mm^3 pieces, and digested in collagenase (1 mg ml^{-1} collagenase A, 100 U ml^{-1} deoxyribonuclease I in Hank's balanced salt solution) at 37 °C for 15 min. The collagenase-digested tissue was gently pressed through a 100 µm cell strainer and cell suspension was centrifuged at 200 g for 5 min. The pellet was resuspended in HBSS and the glomeruli containing Dynabeads were gathered by a magnetic particle concentrator and washed for at least three times with Hank's balanced salt solution. The supernatant containing enriched population of proximal tubules was washed and conserved for further analysis.

NAb assay

Sera from control mice and all the mice injected with rAAV–GFP were collected and sent to the Immunology Core of the University of Pennsylvania to perform a transduction inhibition-based neutralizing antibody assay as described previously.[57]

CONFLICT OF INTEREST

REFERENCES

1 Knoll G.. Trends in kidney transplantation over the past decade. Drugs 2008; 68: 3–10.

2 Davis CL, Delmonico FL. Living-donor kidney transplantation: a review of the current practices for the live donor. J Am Soc Nephrol 2005; 16: 2098–2110.

3 Cecka JM. Kidney transplantation in the United States. Clin Transpl 2008; 1–18.

4 Maruyama H, Higuchi N, Nishikawa Y, Hirahara H, Iino N, Kameda S et al. Kidney-targeted naked DNA transfer by retrograde renal vein injection in rats. Hum Gene Ther 2002; 13: 455–468.

5 Beckmann CF, Abrams HL. Renal venography: anatomy, technique, applications, analysis of 132 venograms, and a review of the literature. Cardiovas Intervent Radiol 1980; 3: 45–70.

6 Mingozzi F, High KA. Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. Nat Rev Genet 2011; 12: 341–355.

7 Daya S, Berns KI. Gene therapy using adeno-associated virus vectors. Clin Microbiol Rev 2008; 21: 583–593.

8 Arruda VR, Xiao W. It's all about the clothing: capsid domination in the adeno-associated viral vector world. J Thromb Haemost 2007; 5: 12–15.

9 Coura Rdos S, Nardi NB. The state of the art of adeno-associated virus-based vectors in gene therapy. Virol J 2007; 4: 99.

10 Choi VW, McCarty DM, Samulski RJ. AAV hybrid serotypes: improved vectors for gene delivery. Curr Gene Ther 2005; 5: 299–310.

11 Lipkowitz MS, Hanss B, Tulchin N, Wilson PD, Langer JC, Ross MD et al. Transduction of renal cells in vitro and in vivo by adeno-associated virus gene therapy vectors. J Am Soc Nephrol 1999; 10: 1908–1915.

12 Chen S, Agarwal A, Glushakova OY, Jorgensen MS, Salgar SK, Poirier A et al. Gene delivery in renal tubular epithelial cells using recombinant adeno-associated viral vectors. J Am Soc Nephrol 2003; 14: 947–958.

13 Takeda S, Takahashi M, Mizukami H, Kobayashi E, Takeuchi K, Hakamata Y et al. Successful gene transfer using adeno-associated virus vectors into the kidney: comparison among adeno-associated virus serotype 1-5 vectors in vitro and in vivo. Nephron Exp Nephrol 2004; 96: e119–e126.

14 Qi YF, Li QH, Shenoy V, Zingler M, Jun JY, Verma A et al. Comparison of the transduction efficiency of tyrosine-mutant adeno-associated virus serotype vectors in kidney. Clin Exp Pharmacol Physiol 2013; 40: 53–55.

15 Bainbridge JW, Smith AJ, Barker SS, Robbie S, Henderson R, Balaggan K et al. Effect of gene therapy on visual function in leber's congenital amaurosis. N Engl J Med 2008; 358: 2231–2239.

16 Maguire AM, Simonelli F, Pierce EA, Pugh Jr EN, Mingozzi F, Bennicelli J et al. Safety and efficacy of gene transfer for leber's congenital amaurosis. N Engl J Med 2008; 358: 2240–2248.

17 Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med 2006; 12: 342–347.

18 Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus MF et al. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. Hum Gene Ther 2010; 21: 704–712.

19 Yue Y, Ghosh A, Long C, Bostick B, Smith BF, Kornegay JN et al. A single intravenous injection of adeno-associated virus serotype-9 leads to whole body skeletal muscle transduction in dogs. Mol Ther 2008; 16: 1944–1952.

20 Katwal AB, Konkalmatt PR, Piras BA, Hazarika S, Li SS, John Lye R et al. Adeno-associated virus serotype 9 efficiently targets ischemic skeletal muscle following systemic delivery. Gene ther 2013; 20: 930–938.

21 Prasad KM, Xu Y, Yang Z, Acton ST, French BA. Robust cardiomyocyte-specific gene expression following systemic injection of AAV: in vivo gene delivery follows a Poisson distribution. Gene Ther 2011; 18: 43–52.

22 Suda T, Liu D. Hydrodynamic gene delivery: its principles and applications. Mol Ther 2007; 15: 2063–2069.

23 Wang G, Zabner J, Deering C, Launspach J, Shao J, Bodner M et al. Increasing epithelial junction permeance enhances gene transfer to airway epithelia in vivo. Am J Respir Cell Mol Biol 2000; 22: 129–138.

24 Herweijer H, Wolff JA. Gene therapy progress and prospects: hydrodynamic gene delivery. Gene Ther 2007; 14: 99–107.

25 Takemoto M, Asker N, Gerhardt H, Lundkvist A, Johansson BR, Saito Y et al. A new method for large scale isolation of kidney glomeruli from mice. Am J Pathol 2002; 161: 799–805.

26 McIntosh JH, Cochrane M, Cobbold S, Waldmann H, Nathwani SA, Davidoff AM et al. Successful attenuation of humoral immunity to viral capsid and transgenic protein following AAV-mediated gene transfer with a non-depleting CD4 antibody and cyclosporine. Gene Ther 2012; 19: 78–85.

27 McCuaig KA, Lee HS, Clarke JC, Assar H, Horsford J, White JH. Parathyroid hormone/parathyroid hormone related peptide receptor gene transcripts are expressed from tissue-specific and ubiquitous promoters. Nucleic Acids Res 1995; 23: 1948–1955.

28 Riccardi D, Lee WS, Lee K, Segre GV, Brown EM, Hebert SC. Localization of the extracellular Ca(2+)-sensing receptor and PTH/PTHrP receptor in rat kidney. Am J Physiol 1996; 271: F951–F956.

29 Amizuka N, Lee HS, Kwan MY, Arazani A, Warshawsky H, Hendy GN et al. Cell-specific expression of the parathyroid hormone (PTH)/PTH-related peptide receptor gene in kidney from kidney-specific and ubiquitous promoters. Endocrinology 1997; 138: 469–481.

30 Chetboul V, Klonjkowski B, Lefebvre HP, Desvaux D, Laroute V, Rosenberg D et al. Short-term efficiency and safety of gene delivery into canine kidneys. Nephrol Dial Transplant 2001; 16: 608–614.

31 Corridon PR, Rhodes GJ, Leonard EC, Basile DP, Gattone VH2nd, Bacallao RL et al. A method to facilitate and monitor expression of exogenous genes in the rat kidney using plasmid and viral vectors. Am J Physiol Renal Physiol 2013; 304: F1217–F1229.

32 Hu YC. Baculovirus vectors for gene therapy. Adv Virus Res 2006; 68: 287–320.

33 Schagen FH, Ossevoort M, Toes RE, Hoeben RC. Immune responses against adenoviral vectors and their transgene products: a review of strategies for evasion. Crit Rev Oncol Hematol 2004; 50: 51–70.

34 Ito K, Chen J, Khodadadian JJ, Vaughan ED Jr, Lipkowitz M, Poppas DP et al. Adeno-associated viral vector transduction of green fluorescent protein in kidney: effect of unilateral ureteric obstruction. BJU Int 2008; 101: 376–381.

35 Grimm D, Zhou S, Nakai H, Thomas CE, Storm TA, Fuess S et al. Preclinical in vivo evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. Blood 2003; 102: 2412–2419.

36 Mingozzi F, High KA. Immune responses to AAV in clinical trials. Curr Gene Ther 2007; 7: 316–324.

37 Gahl WA, Thoene JG, Schneider JA. Cystinosis. N Engl J Med 2002; 347: 111–121.

38 Cherqui S, Sevin C, Hamard G, Kalatzis V, Sich M, Pequignot MO et al. Intralysosomal cystine accumulation in mice lacking cystinosin, the protein defective in cystinosis. Mol Cell Biol 2002; 22: 7622–7632.

39 Town M, Jean G, Cherqui S, Attard M, Forestier L, Whitmore SA et al. A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis. Nat Genet 1998; 18: 319–324.

40 Kalatzis V, Cherqui S, Antignac C, Gasnier B. Cystinosin, the protein defective in cystinosis, is a H(+)-driven lysosomal cystine transporter. Embo J 2001; 20: 5940–5949.

41 Kalatzis V, Cherqui S, Jean G, Cordier B, Cochat P, Broyer M et al. Characterization of a putative founder mutation that accounts for the high incidence of cystinosis in Brittany. J Am Soc Nephrol 2001; 12: 2170–2174.

42 Yang B, Ma T, Dong JY, Verkman AS. Partial correction of the urinary concentrating defect in aquaporin-1 null mice by adenovirus-mediated gene delivery. Hum Gene Ther 2000; 11: 567–575.

43 Michelfelder S, Trepel M. Adeno-associated viral vectors and their redirection to cell-type specific receptors. Adv Genet 2009; 67: 29–60.

44 Shen S, Bryant KD, Brown SM, Randell SH, Asokan A. Terminal N-linked galactose is the primary receptor for adeno-associated virus 9. J Biol Chem 2011; 286: 13532–13540.

45 Shen S, Bryant KD, Sun J, Brown SM, Troupes A, Pulicherla N et al. Glycan binding avidity determines the systemic fate of adeno-associated virus type 9. J Virol 2012; 86: 10408–10417.

46 Bali DS, Chen YT, Goldstein JL. Glycogen storage disease type I. In: Pagon RA, Bird TD, Dolan CR, Stephens K, Adam MP (eds) Gene Reviews. Seattle (WA), 1993.

47 Lin B, Morris DW, Chou JY. The role of HNF1alpha, HNF3gamma, and cyclic AMP in glucose-6-phosphatase gene activation. Biochemistry 1997; 36: 14096–14106.

48 Schmoll D, Wasner C, Hinds CJ, Allan BB, Walther R, Burchell A. Identification of a cAMP response element within the glucose- 6-phosphatase hydrolytic subunit gene promoter which is involved in the transcriptional regulation by cAMP and glucocorticoids in H4IIE hepatoma cells. Biochem J 1999; 338: 457–463.

49 Vander Kooi BT, Streeper RS, Svitek CA, Oeser JK, Powell DR, O'Brien RM. The three insulin response sequences in the glucose-6-phosphatase catalytic subunit gene promoter are functionally distinct. J Biol Chem 2003; 278: 11782–11793.

50 Sniderman King L, Trahms C, Scott CRTyrosinemia type 1. In: Pagon RA, Adam MP, Bird TD, Dolan CR, Fong CT, Stephens K (eds) Gene Reviews. Seattle (WA), 1993.

51 Rogers GL, Martino AT, Aslanidi GV, Jayandharan GR, Srivastava A, Herzog RW. Innate immune responses to AAV vectors. Front Microbiol 2011; 2: 194.

52 Xin KQ, Mizukami H, Urabe M, Toda Y, Shinoda K, Yoshida A et al. Induction of robust immune responses against human immunodeficiency virus is supported by the inherent tropism of adeno-associated virus type 5 for dendritic cells. J Virol 2006; 80: 11899–11910.

53 Bushell A, Morris PJ, Wood KJ. Transplantation tolerance induced by antigen pretreatment and depleting anti-CD4 antibody depends on CD4+ T cell regulation during the induction phase of the response. Eu J Immunol 1995; 25: 2643–2649.

54 Mingozzi F, Chen Y, Murphy SL, Edmonson SC, Tai A, Price SD et al. Pharmacological modulation of humoral immunity in a nonhuman primate model of AAV gene transfer for hemophilia B. Mol Ther 2012; 20: 1410–1416.

55 Syres K, Harrison F, Tadlock M, Jester JV, Simpson J, Roy S et al. Successful treatment of the murine model of cystinosis using bone marrow cell transplantation. Blood 2009; 114: 2542–2552.

56 Manthorpe M, Cornefert-Jensen F, Hartikka J, Felgner J, Rundell A, Margalith M et al. Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. Hum Gene Ther 1993; 4: 419–431.

57 Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. J Infect Dis 2009; 199: 381–390.