THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Treatment of Complement Regulatory Protein Deficiency by Retroviral In Vivo Gene Therapy[1]

Dirk Spitzer,[2] Xiaobo Wu,* Xiucui Ma,† Lingfei Xu,† Katherine P. Ponder,† and John P. Atkinson[2]**

Gene therapy is an attractive means to replace a deficient or defective protein. Using a murine retroviral vector, we provide an example of reconstituting a C regulator by neonatal in vivo gene transfer. The fusion gene containing the mouse C receptor 1-related gene/protein y (Crry) and a single chain Ab fragment with specificity for mouse glycophorin A was placed under transcriptional control of a liver-specific promoter. Shortly after birth, Crry KO mice were injected with the retroviral vectors. Protein expression progressively increased over the next 6–8 wk after which an equilibrium was established. Coating levels on RBCs were obtained that inhibited C activation similar to wild-type cells and remained constant for >1 year. Thus, gene therapy with targeted regulators represents a treatment option to provide a long-term and sustained protein supply for the site-specific blockade of undesirable complement activation. *The Journal of Immunology,* **2006, 177: 4953–4956.**

The C system is a powerful barrier against invading pathogens. However, tight regulation of innate immunity is required to prevent undesirable damage to host cells. A group of plasma membrane regulatory proteins evolved to protect self from C activation. Deficiencies or genetic mutations that lead to reduced activity profiles of these inhibitors can be linked to diseases such as atypical hemolytic uremic syndrome (aHUS)[3] (1), age-related macular degeneration (AMD) (2), glomerulonephritis (3), and paroxysmal nocturnal hemoglobinuria (PNH) (4).

Two principle strategies can be envisioned to modulate the undesirable effects of this effector system. One approach is to block a component that is required in the activation process. For example, patients with PNH have been successfully treated by inhibiting C5 and thereby lysis of their regulator-deficient cells (5). Another promising concept has been described in mouse models of autoimmune disease and of ischemia/reperfusion injury, which can be favorably influenced by providing additional regulation (6). In both cases, the systemic C blockade has a downside due to reduced ability of being efficiently activated in the setting of an infection. Another strategy is to provide the host with the regulatory elements at the site(s) where damage is occurring. This approach centers on the generation of chimeras between a C regulator and either Abs or Ab fragments that bind a specific target where more inhibition is needed (7, 8) or between a regulator and protein domains that recognize an inflammatory milieu (9).

To provide patients with long-lasting, protective levels of targeted C regulators, retrovirus-mediated gene therapy is an attractive treatment option. Because of the limited size of retroviral vector genomes (10), we have constructed C regulator-scFv fusion proteins. These are comprised of single polypeptides and fit into commonly used retroviral vectors. The approach that we have developed involves the direct attachment of C regulators such as the decay-accelerating factor (DAF) (11) and C receptor 1-related gene/protein y (Crry) (12) to the RBC membrane to modulate C activation at this specific site.

This proof-of-concept study was designed to determine, in a mouse model of Crry deficiency, whether in vivo retroviral gene transfer with a vector encoding a membrane-targeted regulator could reconstitute the deficient RBCs with the missing protein.

Materials and Methods

Generation of retroviral constructs

The cDNAs of the RBC-targeted Crry forms (12) were excised with *Eco*RI/*Xba*I (Crry4-Ter, 1664 bp) and *Eco*RI/*Asp*718 (Crry5-Ter, 1930 bp). This was followed by blunt end modification of the restriction site overhangs. hAAT-WPRE-767 (13) was linearized with *Not*I (6001 bp), blunt ended, and ligated with the above-described fragments to generate RV-4 and RV-5, respectively.

Animals

C57BL/6 wild-type (WT) mice (2- to 4-mo old) were used as recipients for RBC transfusions and as a serum source. Mice deficient in both Crry and C3 (referred to herein as Crry knockout (KO) mice) have been previously described (14) and are on a mixed C57BL/6 (B6) and sv129 background (B6.129-Crry^{tm1Hmo}C3^{tm1Hrc}). They were injected via the temporal vein 2 to 3 days

Department of Internal Medicine, *Division of Rheumatology and †Division of Hematology, Washington University School of Medicine, St. Louis, MO 63110

Received for publication June 16, 2006. Accepted for publication August 9, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants 5RO1AI37618 and RO1AI41592 (to J.P.A.).

[2] Address correspondence and reprint requests to Dr. John P. Atkinson, Department of Medicine, Division of Rheumatology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8045, St. Louis, MO 63110; E-mail address: jatkinso@im.wustl.edu or Dr. Dirk Spitzer, Department of Medicine, Division of Rheumatology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8045, St. Louis, MO 63110; E-mail address: dspitzer@im.wustl.edu

[3] Abbreviations used in this paper: aHUS, atypical hemolytic uremic syndrome; AMD, age-related macular degeneration; CCP, C control protein repeat; Crry, complement receptor 1-related gene/protein y; hAAT, human α1 anti-trypsin; PNH, paroxysmal nocturnal hemoglobinuria; scFv, single chain Ab fragment; SEC, sinusoidal endothelial cell; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; WT, wild type.

0022-1767/06/$02.00

after birth. RBC coating levels were determined by FACS and normalized to that of native Crry on RBCs of WT control mice. Blood samples were collected as described (12). Experiments were performed in accordance with the institutional regulations of Animal Welfare.

Cells, transfections, virus production, and assessment

The 293GPG packaging cell line was provided by Daniel Ory (Washington University School of Medicine). These cells were transiently transfected with the expression plasmids using LipofectAMINE (Invitrogen Life Technologies). The virus-containing supernatants were concentrated by ultracentifugation (15). The titer was determined by real-time PCR of genomic DNA (Qiagen) of infected 3521 mouse cells using WPRE-specific primers (13). Also, construct-specific proviral integration events were detected by PCR (from infected 3521 cells (Fig. 1*C*) or mouse organs (Fig. 4*A*)). Primer sequences (Integrated DNA Technologies) are available upon request.

In vivo RBC survival

To assess the clearance of mouse RBCs in vivo, cells from WT, nontreated Crry KO, and the experimental animals were labeled ex vivo with the membrane dye PKH-26 (Sigma-Aldrich) (12). They were introduced into WT mice via the tail vein in a 300 μl cell suspension. For FACS analysis, blood samples were collected by tail clipping. Donor RBC survival was calculated as reported (16).

Flow cytometry

RBCs were washed and then incubated with the rat anti-Crry mAb 1F2 followed by incubation with FITC-conjugated secondary goat anti-rat IgG pAb (BD Pharmingen). Cells were next washed and analyzed by flow cytometry (FACScan, BD Biosciences) as described (12). C activation was monitored by direct immunostaining with FITC-conjugated rabbit anti-mouse C3 polyclonal Ab ($F(ab')_2$, ICN Pharmaceuticals). To assess for free fusion protein in plasma, naive Crry KO RBCs (2×10^7 cells/ml) were incubated with 1–10% sera from mice R3, L2, and L4, washed and then submitted for FACS analysis (12).

In vitro C deposition assays

Ab-induced C3 deposition on mouse RBCs was performed as previously described (12). Briefly, RBCs from WT, Crry KO and the experimental mice were sensitized with an anti-mouse CD24 mAb J11d (rat IgM; Research Diagnostics) before exposure to 5% WT mouse serum.

Immunofluorescence analysis of liver sections

Following fixation of 5-μm liver cryosections with acetone, the sections were blocked with 5% normal goat serum. The sections were then double-stained for Crry (rat anti-Crry mAb followed by FITC-conjugated secondary Ab) and for CD16/CD32 (FcRII/III) with PE-conjugated rat mAb 2.4G2 (BD Pharmingen) (17).

Results and Discussion

The cDNAs encoding two C-terminally targeted forms of mouse Crry (Ref. 12; Fig. 1) were cloned into the retroviral vector hAAT-WPRE (13), resulting in RV-4 and RV-5, respectively. 293GPG-derived virus (15) was transiently produced and assessed by PCR because of the lack of a marker gene on the retroviral vector. Following infection of 3521 mouse fibroblasts, genomic DNA was analyzed by PCR for the presence of proviral integrates. A primer combination specific for CCP 4 of Crry and the artificial linker sequence of the C-terminal scFv (Fig. 1, diamond-flanked lines) exclusively amplified the transgenes (data not shown), indicative of successful generation of infectious retrovirus. This strategy also enabled us to discriminate the two Crry forms on the DNA level, with the CCP 5-containing RV-5 being 190 bp larger than RV-4, which lacks this CCP (as shown in Fig. 4*A*). Titer determination was performed by real-time PCR with WPRE-specific primers (13) and resulted in $\sim 3 \times 10^7$ IU/ml for both virus preparations.

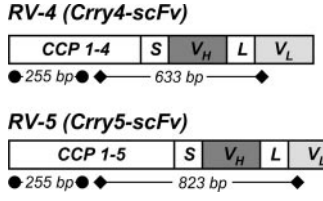

FIGURE 1. Structural organization of RBC-targeted Crry. Protein structures of the mature, secreted Crry-scFv forms are shown (12). Primer locations and calculated size of PCR products are indicated below the constructs. S, Spacer; L, linker.

The 2- to 3-day-old Crry KO mice were injected via the temporal vein with 100 μl of the retrovirus preparations (3×10^6 IU/animal). Two mice received RV-4, two mice RV-5, and three mice a mixture of both viruses (to mimic a treatment with two different C regulators). The initial Crry transgene expression was detected by immunostaining of freshly isolated RBCs as early as 8 days postinfection and ranged from 4 to 60% relative to the native protein on WT RBCs with a homogeneous coating pattern (Fig. 2*A*). The levels increased for all mice during the initial 6 to 8 wk after which an equilibrium was established. Subsequently, the levels remained stable for >12 mo (Fig. 2*B*). The average coating level for each mouse ranged from 34 to 465% relative to the copy number of native Crry on wild-type cells, i.e., all but mouse L5 achieved levels on their RBCs greater than the endogenous level. Western blot analysis (12) confirmed the expected molecular weights of the host-derived C regulators (not shown). Of note, development of Abs against the expressed fusion protein using a FACS-based assay (12) were not detected (not shown). This likely reflects their immature immune system at the time of vector injection and has been reported for mice (18) and dogs (19). Furthermore, the inhibitors were not detectable in serum (data not shown). Thus, the downside of a globally impaired C system is avoided.

The constitutive production and coating of peripheral RBCs with the recombinant fusion proteins with copy numbers nearly

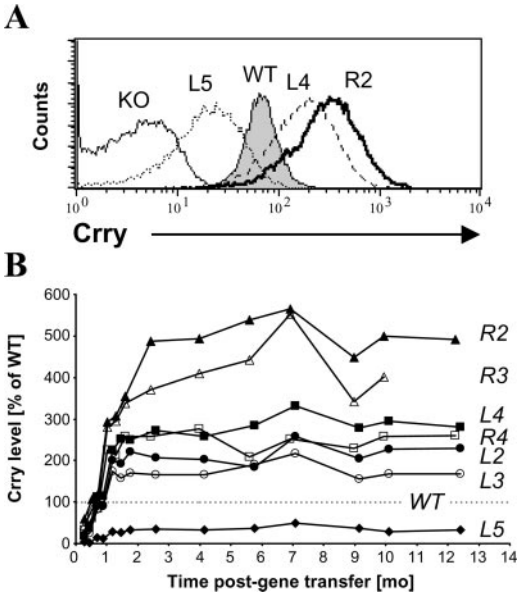

FIGURE 2. Long-term, stable coating of RBCs with targeted Crry following neonatal in vivo gene transfer. Two mice received RV-4 (L2 and L5), two mice received RV-5 (L3 and L4), and the others received a 1:1 mixture of both viruses (R2, R3, and R4). *A*, Crry expression profiles from RBCs of selected mice used to generate the graph in *B* obtained 10 mo postgene therapy. *B,* Crry expression follow-up by FACS analysis. The data are normalized to the level of Crry on WT RBCs (dotted line).

6 times the native Crry (Fig. 2*A*, mouse R2) did not affect hematologic parameters such as RBC count, hemoglobin, hematocrit, or reticulocyte count obtained 10 mo postgene transfer. The animals gained weight and matured normally. They have not developed signs of disease >1 year posttreatment.

To demonstrate C regulation, experiments were performed (12) using the RBCs coated in vivo with the host-derived inhibitors. In a FACS-based C activation assay, deposition of C3 fragments on IgM-sensitized RBCs was highest in the complete absence of Crry (Fig. 3*A*, KO). However, for those mice in which 1.5-fold or higher levels of the regulator were attached, C deposition was reduced to or below that of wild-type RBCs. As little as 25% of the normal Crry copy number (L5) reduced the amount of deposited C3 by >50%. These results are in agreement with those obtained from earlier studies in which the Crry-scFv fusion proteins were generated by standard transfection procedures (12).

Protection from C-mediated clearance in vivo was next examined by infusing RBCs obtained from the treated mice into wild-type recipients. Crry-deficient RBCs were rapidly cleared from the circulation, in accord with previously published results, and wild-type cells survived normally (Fig. 3*B*) (16, 20). In comparison, Crry KO RBCs, reconstituted with host-derived RV-4 and RV-5 proteins, remained in the circulation with a half-life of up to ~2 days (Fig. 3*B*, R2). These results are consistent with what has been shown for RBCs coated ex vivo with the same proteins. The reduced survival rate of Crry-coated KO RBCs relative to WT cells reflects the progressive transfer of regulators from the treated RBCs to the host's RBC pool (not shown, but see Ref. 12).

We next examined the preference of the virus for selected target organs. Genomic DNA from 4 organs of mice R3, L2, and L4 was analyzed by PCR using a primer combination that would specifically amplify the integrated Crry/scFv cDNA (Fig. 1, diamond-flanked primers). As a loading control, the same DNA was amplified with primers (Fig. 1, circle-flanked lines) specific for CCPs 1 and 2 (Ref. 21) and Fig. 4*A*, *lower panel*). No amplification product was observed from DNA of a nontreated Crry KO mouse (Fig. 4*A*, *lanes 1*, *5*, *9*, and *13*). However, signals of 633 (RV-4) and 823 bp (RV-5) were generated primarily from the livers of the treated mice R3, L2, and L4. It also verifies the existence of two types of integrated proviral DNA, because mouse R3 was infected with a mixture of RV-4 and RV-5. The other two mice received either RV-4 (L2) or RV-5 (L4). The faint PCR signals from lung, kidney, and spleen of the treated mice indicates that these organs are not the

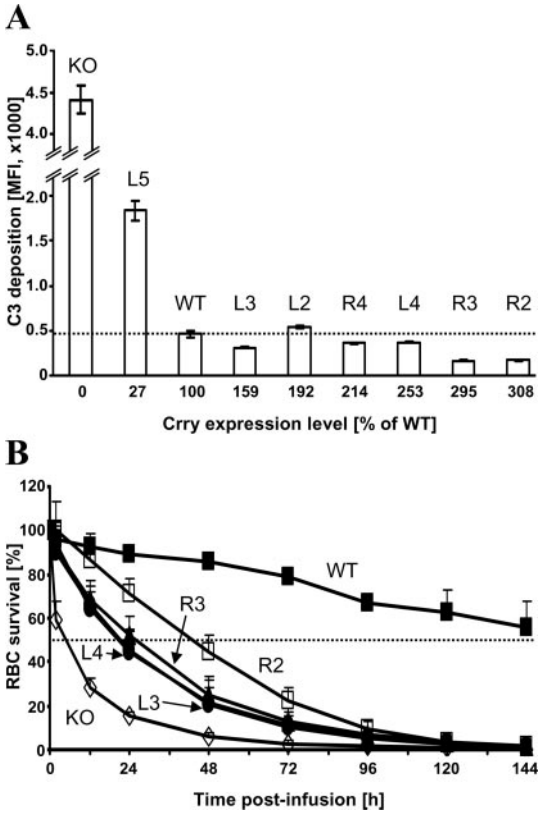

FIGURE 3. Targeted Crry on RBCs of mice receiving neonatal gene therapy protects against C attack in vitro (*A*) and in vivo (*B*). *A*, At 1.5 mo postgene transfer, the Crry levels of peripheral RBCs were assessed by FACS (as shown in Fig. 2*A*). At the same time, IgM-sensitized RBCs from the experimental group, WT and the KO mice were challenged with 5% WT mouse serum. C activation was detected with FITC-conjugated anti-mouse C3 polyclonal Ab. The Crry expression level is plotted as percentage of the native level of WT RBCs. C deposition is expressed as mean fluorescence intensity (MFI, mean of duplicates with error bars representing ±SD). *B*, Nontreated WT recipient mice received via the tail vein equivalent numbers of donor RBCs and the time needed to reduce their starting concentration by 50% was used to determine their half-life (dotted line). Error bars, ±SD. The data are normalized to the recovery of each RBC type at 5 min postinjection ($n = 4$).

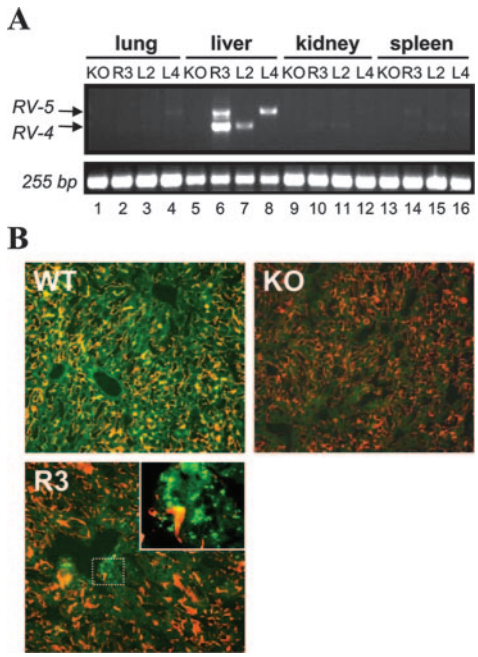

FIGURE 4. RBC-targeted Crry/scFv is primarily produced by the liver. *A,* Genomic DNA from lung, liver, kidney, and spleen of four mice (KO, R3, L2, and L4) was subjected to PCR analysis. The construct-specific primers (Fig. 1, diamond-flanked lines) resulted in two fragments of the expected size of 823 bp (RV-5) and 633 bp (RV-4) primarily in the liver of the treated mice (*upper panel*). An amplification product with a primer combination specific for CCPs 1 and 2 served as a loading control (*lower panel*) (21). *B,* Immunofluorescence analysis of the expression pattern of Crry (green) and CD16/CD32 (FcRII/III, red) on liver cryosections of the indicated mice. Shown are representative areas (original magnification, ×20). The inset for the treated mouse R3 highlights an area of hepatocytes rich in vesicular Crry expression.

primary target of the injected vectors. With such low PCR signals and the retroviral vector containing a liver-specific promoter, we do not expect a significant contribution of these organs to protein production.

To further demonstrate protein expression of targeted Crry from the primary target organ, immunofluorescence analysis of liver sections was performed. Liver sections from WT mice revealed a positive staining pattern, characteristic of a ubiquitously expressed plasma membrane protein (Fig. 4*B*, WT). As expected, mice lacking Crry were negative for the C regulator (Fig. 4*B*, KO). In the case of retrovirally transduced liver cells, a more scattered and/or clustered signal pattern was anticipated. Indeed, most of the cells were negative for Crry and the few positive signals exhibited a punctuate pattern (Fig. 4*B*, R3). This would be anticipated for vesicle localization of a secreted protein. In light of the hepatocyte-specific promoter used (hAAT) (22), this liver cell type would be the major source of the secreted fusion proteins. To verify this assumption, liver sections were also stained with an anti-CD16/CD32 mAb that recognizes Fcγ receptors II and III (FcRII/III). They are primarily expressed by sinusoidal endothelial cells (SECs) and liver macrophages but not by hepatocytes and vascular endothelial cells (17). Using anti-Crry-FITC and anti-FcRII/III-PE Abs, we identified the hepatocyte as the major cell type of secreted RV-4/-5 (Fig. 4*B*, R3, *inset*).

To examine for adverse effects that might have occurred secondary to the infection process of neonatal liver cells, e.g., tumor formation via insertional mutagenesis (23), histological examination (H&E staining) did not reveal liver pathology (not shown). This may be explained by the relatively low amount of virus used, being 16-fold lower compared with a previous study in which a similar type of virus was neonatally injected (24).

In summary, these data establish that targeting of a C regulator to a specific site via fusion with a single chain Ab fragment results in production of host-derived proteins sufficient to protect from a C-mediated insult. Thus, liver-directed synthesis of scFv-targeted C regulators represents a treatment strategy for a long-term protein supply to block undesirable C activation at specific sites. It has the potential to deliver a broad range of other therapeutic effector molecules to their site of action.

Acknowledgments

We thank Daniel Ory (Division of Cardiology, Washington University School of Medicine) for providing us with the 293GPG packaging cell line and his laboratory members for helpful advice in handling these cells. We thank the Division of Comparative Medicine and the Digestive Disease Resource Core Center (Grant P30 DK52574; Washington University School of Medicine) for performing blood analyses and animal tissue preparation, respectively. We thank Madonna Bogacki and Lorraine Whiteley for their assistance in manuscript preparation.

Disclosures

The authors have no financial conflict of interest.

References

1. Goodship, T. H. J., M. K. Liszewski, E. J. Kemp, A. Richards, and J. P. Atkinson. 2004. Mutations in CD46, a complement regulatory protein, predispose to atypical HUS. *TRENDS Mol. Med.* 10: 226–231.
2. Bok, D. 2005. Evidence for an inflammatory process in age-related macular degeneration gains new support. *Proc. Natl. Acad. Sci. USA* 102: 7053–7054.
3. Thurman, J. M., and V. M. Holers. 2006. The central role of the alternative complement pathway in human disease. *J. Immunol.* 176: 1305–1310.
4. Takeda, J., T. Miyata, K. Kawagoe, Y. Iida, Y. Endo, T. Fujita, M. Takahashi, T. Kitani, and T. Kinoshita. 1993. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal haemoglobinuria. *Cell* 73: 703–711.
5. Hillmen, P., C. Hall, J. C. W. Marsh, M. Elebute, M. Bombara, B. E. Petro, M. J. Cullen, S. J. Richards, S. A. Rollins, C. F. Mojcik, and R. P. Rother. 2004. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.* 350: 552–559.
6. Thurman, J. M., P. A. Royer, D. Ljubanovic, B. Dursun, A. M. Lenderink, C. L. Edelstein, and V. M. Holers. 2006. Treatment with an inhibitory monoclonal antibody to mouse factor B protects mice from induction of apoptosis and renal ischemia/reperfusion injury. *J. Am. Soc. Nephrol.* 17: 707–715.
7. Zhang, H., S. Lu, S. L. Morrison, and S. Tomlinson. 2001. Targeting of functional antibody-decay-accelerating factor fusion proteins to a cell surface. *J. Biol. Chem.* 276: 27290–27295.
8. He, C., M. Imai, H. Song, R. J. Quigg, and S. Tomlinson. 2005. Complement inhibitors targeted to the proximal tubule prevent injury in experimental nephrotic syndrome and demonstrate a key role for C5b-9[1]. *J. Immunol.* 174: 5750–5757.
9. Atkinson, C., H. Song, B. Lu, F. Qiao, T. A. Burns, V. M. Holers, G. C. Tsokos, and S. Tomlinson. 2005. Targeted complement inhibition by C3d recognition ameliorates tissue injury without apparent increase in susceptibility to infection. *J. Clin. Invest.* 115: 2444–2453.
10. Verma, I. M., and N. Somia. 1997. Gene therapy—promises, problems and prospects. *Nature* 389: 239–242.
11. Spitzer, D., J. Unsinger, M. Bessler, and J. P. Atkinson. 2004. ScFv-mediated in vivo targeting of DAF to erythrocytes inhibits lysis by complement. *Mol. Immunol.* 40: 911–919.
12. Spitzer, D., J. Unsinger, D. Mao, X. Wu, H. Molina, and J. P. Atkinson. 2005. In vivo correction of complement regulatory protein deficiency with an inhibitor targeting the RBC membrane. *J. Immunol.* 175: 7763–7770.
13. Xu, L., M. E. Haskins, J. R. Melniczek, C. Gao, M. A. Weil, T. M. O'Malley, P. A. O'Donnell, H. Mazrier, N. M. Ellinwood, J. Zweigle, et al. 2002. Transduction of hepatocytes after neonatal delivery of a Moloney murine leukemia virus based retroviral vector results in long-term expression of β-glucuronidase in mucopolysaccharidosis VII dogs. *Mol. Ther.* 5: 141–153.
14. Xu, C., D. Mao, V. M. Holers, B. Palanca, A. M. Cheng, and H. Molina. 2000. A critical role for the murine complement regulator Crry in fetomaternal tolerance. *Science* 287: 498–501.
15. Ory, D. S., B. A. Neugeboren, and R. C. Mulligan. 1996. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc. Natl. Acad. Sci. USA* 93: 11400–11406.
16. Miwa, T., L. Zhou, B. Hilliard, H. Molina, and W. C. Song. 2002. Crry, but not CD59 and DAF, is indispensable for murine erythrocyte protection in vivo from spontaneous complement attack. *Blood* 99: 3707–3716.
17. Ahmed, S. S., H. Muro, M. Nishimura, I. Kosugi, Y. Tsutsi, and H. Shirasawa. 1995. Fc receptors in liver sinusoidal endothelial cells in NZB/W F_1 lupus mice: a histological analysis using soluble immunoglobulin G-immune complexes and a monoclonal antibody (2.4G2). *Hepatology* 22: 316–324.
18. Daly, T. M., T. Okuyama, C. Vogler, M. E. Haskins, N. Muzyczka, and M. S. Sands. 1999. Neonatal intramuscular injection with recombinant adeno-associated virus results in prolonged β-glucuronidase expression in situ and correction of liver pathology in mucopolysaccharidosis type VII mice. *Hum. Gene Ther.* 10: 85–94.
19. Ponder, K. P., J. R. Melniczek, L. Xu, M. A. Weil, T. M. O'Malley, P. A. O'Donnell, V. W. Knox, G. D. Aguirre, H. Mazrier, N. M. Ellinwood, et al. 2002. Therapeutic neonatal hepatic gene therapy in mucopolysaccharidosis VII dogs. *Proc. Natl. Acad. Sci. USA* 99: 13102–13107.
20. Molina, H., T. Miwa, L. Zhou, B. Hilliard, D. Mastellos, M. A. Maldonado, J. D. Lambris, and W. C. Song. 2002. Complement-mediated clearance of erythrocytes: mechanism and delineation of the regulatory roles of Crry and DAF. *Blood* 100: 4544–4549.
21. Paul, M. S., M. Aegerter, K. Cepek, M. D. Miller, and J. H. Weis. 1990. The murine complement receptor gene family. III. The genomic and transcriptional complexity of the Crry and Crry-ps genes. *J. Immunol.* 144: 1988–1996.
22. Kugler, W., U. Wagner, and G. U. Ryffel. 1988. Tissue-specificity of liver gene expression: a common liver-specific promoter element. *Nucleic Acids Res.* 16: 3165–3174.
23. Neil, J. C., and D. Forrest. 1987. Mechanisms of retrovirus-induced leukaemia: selected aspects. *Biochim. Biophys. Acta.* 907: 71–91.
24. Themis, M., S. N. Waddington, M. Schmidt, C. von Kalle, Y. Wang, F. Al-Allaf, L. G. Gregory, M. Nivsarkar, M. Themis, M. V. Holder, et al. 2005. Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice. *Mol. Ther.* 12: 763–771.