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Regulated and Liver-Specific Tamarin Alpha Interferon Gene Delivery by a Helper-Dependent Adenoviral Vector

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Gene therapy approaches based on liver-restricted and regulated alpha interferon (IFN-α) expression, recently shown to be effective in different murine hepatitis models, appear promising alternatives to inhibit hepatitis C virus (HCV) replication in patients and minimize side effects. Tamarins (*Saguinus* species) infected by GB virus B (GBV-B) are considered a valid surrogate model for hepatitis C to study the biology of HCV infection and the development of new antiviral drugs. To test the efficacy of local delivery and expression of IFN-α in this model, we have developed HD-TET-tIFN, a helper-dependent adenovirus vector expressing tamarin IFN-α (tIFN) under the control of the tetracycline-inducible transactivator rtTA2^s-S2. Expression of tIFN was successfully induced both in vitro and in vivo in rodents by doxycycline administration with consequent activation of IFN-responsive genes. More importantly, tIFN efficiently inhibited GBV-B replicon in a Huh-7 hepatoma cell line at low HD-TET-tIFN doses. A certain degree of transcriptional control of tIFN was achieved in tamarins injected with HD-TET-tIFN, but under the conditions used in this study, infection and replication of GBV-B were only delayed and not totally abrogated upon virus challenge. Hepatic delivery and regulated expression of IFN-α appear to be a possible approach for the cure of hepatitis, but this approach requires more studies to increase its efficacy. To our knowledge, this is the first report showing a regulated gene expression in a nonhuman primate hepatitis model.

For the treatment of hepatitis C virus (HCV), systemic injection of recombinant alpha interferon (IFN-α) is efficient in only 40% of patients and is associated with severe side effects, causing the withdrawal of 20% of patients from therapy (9, 25). In fact, high IFN doses are required since pharmacokinetics studies indicate that IFN-α exhibits a short half-life in the bloodstream after parenteral protein administration (15, 37), suggesting that the unsatisfactory results of IFNs in hepatitis treatment may be caused, at least in part, by insufficient or lack of sustained delivery of this protein to the liver.

Gene therapy approaches could overcome, at least in part, these limitations. The ideal therapeutic vector should have a natural tropism to the liver and should express only the therapeutic transgene, and expression should be strictly tissue specific. At the moment, suitable vectors meeting these requirements are the fully deleted helper-dependent adenoviruses (HD) (19, 29). These vectors are completely devoid of any viral coding sequences, are maintained extrachromosomally, and result in prolonged transgene expression in immunocompetent mice upon systemic delivery (2, 4, 21, 22, 32). We examined the effects of IFN-α2 gene delivery mediated by HD vectors to the mouse liver (2, 4) in obtaining long-term and hepatospecific expression. The combination of an HD vector and a liver-specific promoter resulted in intrahepatic IFN-α expression, which protected liver parenchyma in acute hepatitis models. Recently, the same approach was applied to woodchuck hepatitis virus-infected woodchucks, showing a reduction of virus replication in the liver after transduction with an HD vector expressing woodchuck IFN-α (13).

To exert control over the timing and level of transgene expression, an inducible gene expression system would be the most appropriate. Ideal inducible systems should be regulated by an exogenous, nontoxic, orally active compound which does not affect endogenous cellular genes. In addition, expression of the transgene should be silent in the absence of inducer as well as reversible upon its withdrawal. The tetracycline-responsive promoter system, based on the Tn*10*-specified tetracycline resistance operon of *Escherichia coli*, seems to fulfill these criteria (14). In the Tet-on version, the addition of tetracycline (Tet) or one of its derivatives, such as doxycycline (Dox), activates transcription via the chimeric transcription factor reverse Tet-controlled transactivator (rtTA) (5). Dox is currently used for antibiotic therapy and has an excellent safety profile with minimal side effects (18). Recently, the rtTA transactivator series was improved through generating variants called rtTA2^s-S2 and rtTA2^s-M2, which show better properties such as lower leakiness and higher inducibility (36). These components have been inserted in the context of HD vectors (2, 28). In particular, the association of the HD vectors with the Tet-on system allowed tight control and liver-specific mouse IFN (mIFN)-α2 gene expression (2). The amount of expressed cytokine could be easily regulated both by dosage of the HD vector and by the amount of doxycycline administered, and this correlated with the activation of IFN-induced antiviral genes. Expression of mIFN-α2 induced by doxycycline resulted in prolonged survival and reduced liver damage in HD-TET-IFN-injected mice challenged with a lethal dose of a coronavirus (2).

In the past, the development of therapeutic agents against

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hepatitis C (17, 24, 26) has been hampered by the lack of a suitable small-primate model, since HCV can infect only humans and chimpanzees. Monkeys of the genus *Saguinus*, commonly called tamarins, undergo hepatitis upon infection with the flavivirus GB virus B (GBV-B) (23, 33). The significant similarity between HCV and GBV-B at the genomic and biochemical levels led to the proposal of the GBV-B/tamarin system as a good surrogate model for hepatitis C as an alternative to chimpanzees (7). Both viruses are responsible for a similar liver pathology in primates, and corresponding regions of their genomes play similar roles during the infection (31, 38). GBV-B infection in tamarins has been achieved by intrahepatic injection of RNA in vitro transcribed from molecular constructs (8, 30). This allows the study of the effects of new pharmacological agents or improvement of those already in use. In addition, as a complementary tool to verify the pharmacological effects in GBV-B before testing them in animals, a cell-based system able to sustain GBV-B RNA replication has been recently established (10). In this study, we tested a gene therapy approach in the GBV-B/tamarin system by means of liver-regulated expression of IFN-α.

MATERIALS AND METHODS

Cell lines. 293Cre4.15 cells were grown in modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. L-929 (mouse fibroblasts), A549 (human lung), B76.1/Huh-7 (cell line derived from human hepatoma Huh7, bearing the Neo-RepB GBV-B replicon), and Hep3B (human hepatoma) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. A total of 0.800 mg/ml of G418 was added to replicon cell line medium.

Mouse and rat strains. Mice used in this study were immunocompetent, 6- to 8-week-old (at the time of injection) C57B/6 females purchased from Charles River. Female Sprague-Dawley rats were obtained from Charles River. Groups of animals received injections in the tail vein of HD-TET-tIFN adenovirus vectors diluted in physiologic solution in volumes of 200 μl. Doxycycline (D-9891, 200 μg/ml in 5% sucrose, pH 6.0; Sigma) was added in the drinking water at the indicated time points. Blood was obtained by retroorbital bleeding, and serum was stored at −80°C. At the indicated time, mice were euthanized and organs were rapidly frozen in liquid nitrogen and stored at −80°C.

Tamarins. The GBV-B study was carried out in cotton-top tamarins (*Saguinus oedipus*) housed at the Biomedical Primate Research Centre (Rijswijk, The Netherlands). They were mature animals in good physical health, and their weights were about 500 g. Moreover, they were free of GBV-B virus and of adenovirus and anti-adenovirus neutralizing antibodies and had not been experimentally infected previously with related agents or subjected to treatments affecting the immune system responsiveness. During the experiment, the animals were housed separately, with each animal in one cage. They were provided a daily diet of rice, potatoes, eggs, fresh fruit, and vegetables. Drinking water was available ad libitum via a water bottle. The study was performed under biocontainment conditions at environmental temperatures.

Construction of Tet-inducible tIFN-α expression cassette. The rtTA2^S-S2 transactivator gene was recovered from the plasmid pHUD52.1 (kindly provided by H. Bujard, ZMBH, Im Neunheimer Feld, Heidelberg, Germany) as an EcoRI/BamHI fragment and subcloned into the vector pTTR-bGH (4), containing the liver-specific transthyretin (TTR) gene minimal enhancer and promoter and the bovine growth hormone (bGH) poly(A) site. To improve the level of expression, an artificial intron was amplified by PCR from pCAT3basic (Invitrogen) and subcloned HindIII/EcoRI between the TTR promoter/enhancer and the mIFN cDNA, generating pTTR-intr-rTA2-bGH. The intron A from the vector pVIJnsA as a SacII/EcoRI fragment was subcloned into the vector pHUD10.3 between the Tet-responsive element (TRE) and the simian virus 40 (SV40) polyadenylation site. A PacI site and a NotI site were inserted upstream and downstream, respectively, of the SV40 poly(A) by PCR. At this point the TRE-intron A-SV40 fragment was excised as an XhoI/KpnI fragment and inserted into pTTR-intr-rTA2-bGH, thus generating pTet−/−. The tamarin IFN-α (tIFN-α) gene was amplified by PCR adding a BamHI site 5′ and a PacI site 3′ and subcloned into pTet−/−, generating pTet-tIFN-α.

Adenoviral vectors. To construct pC4-Tet-tIFN, the tIFN-α expression cassette was excised with NotI from pTet-tIFN-α and subcloned into the NotI site of pC4-HSU (29).

To rescue the HD-TET-tIFN vector, the pC4-Tet-tIFN plasmid was cleaved by PmeI and transfected into 293Cre4.15 cells (29). Subsequently, the cells were infected with the helper adenovirus H14 (29). The titer of the HD vector during the amplification passages on 293Cre4.15 cells was followed by infecting Hep3B cells with lysates from each passage and determining the amount of tIFN-α in cell supernatants with and without Dox at 48 h postinfection (p.i.) by enzyme-linked immunosorbent assay (ELISA). Multiple viral passages were performed to reach a high titer, and vector was purified by double CsCl gradient. Physical particles (pp) were measured by optical density of DNA. HD-GFP is a helper-dependent adenovirus carrying green fluorescent protein (GFP) under the cytomegalovirus promoter. Genomic structure of the HD vectors in comparison to starting plasmids was determined by restriction analysis and radioactive labeling of the extracted DNA. No rearrangements were detected. To determine helper virus contamination, purified vectors were analyzed by TaqMan quantitative PCR using both helper-dependent virus-specific (forward, CCACCACTACAT AGCCCACAGT; reverse, ACAAAGAATGGCTGAGCAAGC; probe, F-TGC CCCAGCCACAGCATCCTT-T) and helper virus-specific (forward, TCTGAG TTGGCACCCCTATTC; reverse, GTTGCTGTGGTCGTTCTGGTA; probe, F-TTCAGGGATGCCACATCCGTTGA-T) primers and probe set. For both probes, F is 6-carboxyfluorescein fluorophore and T is 6-carboxytetramethylrhodamine quencher. Internal standard control curves were used to determine the viral genomes. In the preparations used for this study, the helper virus contamination was measured as 0.03% for HD-TET-tIFN and 0.02% for HD-GFP.

tIFN measurement. The secreted tIFN in cell supernatant or in serum was measured by a human IFN-α ELISA kit (PBL) according to the manufacturer's directions. This kit does not cross-recognize mouse and rat IFN-α. The viral cytopathic inhibition assay using vesicular stomatitis virus (VSV) has been described elsewhere previously (1). tIFN-α activity is expressed in units/ml. The tIFN-α activity on mouse L-929 cells was previously determined as 0.23 pg/unit (3).

Northern blot analysis. Frozen tissues were mechanically pulverized and RNA was isolated from tissues using Ultraspec RNA reagent (Biotecx Laboratories) according to the manufacturer's instructions. Total RNA (20 μg) was used in Northern blot analysis. The intensity of bands was quantified by PhosphorImager analysis.

Inhibition of GBV-B replication. B76.1/Huh7 cells carrying the GBV-B replicon were used to test the effect of HD-TET-tIFN on replication. A total of 1×10^5 cells were plated into each well of a series of wells of 6-multiwell plates (Falcon) in medium without G418. After 16 h, cells were infected with HD-TET-tIFN or HD-GFP at the indicated doses for 30 min at 37°C and medium with or without Dox at 1 μg/ml. For tIFN exocrine function assay, 1×10^5 cells were plated into cell culture removable membrane inserts (catalog no. 353090, PET track-etched 0.4-μm pore size; Falcon), and the day after, they were infected with HD vectors at the indicated doses. After being washed twice with phosphate-buffered saline, inserts were placed into 6-multiwell plates containing B76.1/Huh7 cells and 2 ml medium with or without Dox. Cells were grown for 3 days and finally lysed with TRIzol reagent (Life Technologies). Total RNA was purified as described in the manufacturer's protocol and quantified by using a spectrophotometer. GBV-B RNA extracted from mock-treated cells was used as a calibrator. tIFN-α protein was produced by transfection of HeLa cells and quantified by ELISA and Coomassie blue gel staining.

TaqMan quantification of GBV-B RNA. GBV-B RNA was quantified by a real-time, 5′ exonuclease PCR (TaqMan) assay using a primer/probe set that recognized a portion of the GBV-B 5′ untranslated region. The primers (GBV-B-F3 [GTAGGCGGCGGGACTCAT] and GBV-B-R3 [TCAGGGCCATCCA AGTCAA]) and probe (GBV-B-P3 [6-carboxyfluorescein-TCGCGTGATGAC AAGCGCCAAG-*N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine]) were used at 10 pmol/50-μl reaction, and the probe was used at 5 pmol/50-μl reaction. The reactions were performed using a TaqMan Gold reverse transcription (RT)-PCR kit (PE Applied Biosystems) and included a 30-min reverse transcription step at 48°C followed by 10 min at 95°C and 40 cycles of amplification using the universal TaqMan standardized conditions (15-s, 95°C denaturation step followed by a 1-min, 60°C annealing/extension step). RNA transcribed from a plasmid containing the first 2,000 nucleotides of the GBV-B genome was used as a standard to establish genome equivalents. Standard RNA was transcribed using a T7 Megascript kit (Ambion) and was purified by DNase treatment, phenol-chloroform extraction, Sephadex-G50 filtration, and ethanol precipitation. RNA was quantified and normalized by absorbance at 260 nm and stored at −80°C. All reactions were run in duplicate by using the ABI Prism 7900 sequence detection system (PE Applied Biosystems).

TaqMan quantification of HD-TET-tIFN genomes. Low-molecular-weight DNA was extracted from infected B76.1/HuH7 cells according to the HIRT procedure (16) with slight modifications. Briefly, the cell pellet was resuspended in 1 ml lysis buffer (10 mM Tris-Cl, 1 mM EDTA, 1% sodium dodecyl sulfate) with 200 μg/ml proteinase K and incubated for 2 h at 42°C. Next, 250 μl of 5 M NaCl was added dropwise, and samples were incubated overnight at 4°C. After centrifuging for 30 min at 4°C, supernatant was extracted with phenol-chloroform, and DNA was precipitated by adding an equal volume of isopropanol. DNA was washed with 70% ethyl alcohol, resuspended in Tris-EDTA buffer plus RNase A, and then quantified by optical density at 260 nm. HD genomes were measured by TaqMan using the same set of primers/probe described above for determination of HD genomes in viral preparations.

Tamarin treatment and GBV-B challenge. Animals 95-14 and 96-15 were inoculated with 2×10^{11} pp of HD-TET-tIFN. Monkeys 96-20 and 97-01 were inoculated with 1×10^{11} pp of HD-TET-tIFN. Consequently, the amount of injected virus was about 4×10^{11} pp/kg and 2×10^{11} pp/kg, respectively. Stock viruses were diluted in sterile phosphate-buffered saline in a total volume of 1 ml and administered by injection into the saphenous vein. Dox was administered orally by using a syringe as a ready-to-use solution (Vibravet) or was mixed in the food. Each monkey received about 10 mg/kg Dox per administration.

For GBV-B challenge, animals were inoculated intravenously with an aliquot of serum (10^{-4} dilution in saline to a final volume of 250 μl) of tamarin B242 (26). The serum had been in vivo titrated, and it was calculated that this inoculum contained 30 50% tamarin infectious doses (TID_{50}) (30).

For blood sampling, the animals were sedated with ketamine. Care was taken that the total amount of blood per month did not exceed 1% of the body weight. Blood samples were collected aseptically from the femoral or saphenous vein using Vacutainer blood collection tubes (Becton Dickinson) and allowed to clot for at least 30 min before they were centrifuged.

For hematological analysis, the following exams were routinely performed: hemochrome, electrolytes, creatininemia, enzymes measurement (alkaline phosphatases, gamma glutamyl transpeptidase, alanine aminotransferase [ALT], and aspartate aminotransferase), cholesterolemia, glycemia, and protidogram.

GBV-B viremia in the serum was determined by PCR as described elsewhere previously (30).

Statistical analysis. Paired and unpaired Student's *t* tests were used for statistical analyses (34). A *P* value of <0.05 was considered significant. Results were expressed as mean values and standard deviations.

RESULTS

Description of HD-TET-tIFN vector. To generate a liver-directed and pharmacologically regulated expression of tIFN, an inducible cassette based on the Tet system was constructed (Fig. 1A). The tetracycline-sensitive transactivator rtTA2^s-S2 was cloned under the liver-specific TTR promoter and enhancer followed by the SV40 polyadenylation signal. In the opposite orientation, the tIFN gene was inserted downstream of the P_{Tet-1} promoter (13), followed by a bGH polyadenylation site. This expression cassette was then inserted into the HD plasmid pC4HSU, thus generating pHD-TET-tIFN. To verify the liver-specific Dox-inducible tIFN expression, Hep3B hepatoma cells and A549 (human lung) cells were infected with different doses of HD-TET-tIFN vector and transgene expression maintained as a function of Dox addition (Fig. 1B). In the absence of Dox, tIFN expression as dosed in the cell culture supernatant was very low but not completely absent at all the HD doses. This was probably due to the leakiness of the P_{Tet-1} promoter. On the contrary, when Dox was added, the tIFN level rose sharply, and at the lowest dose used (30 pp/cell), a 1,462-fold induction was measured in Hep3B cells. No significant tIFN expression was measured in A549 cell supernatants in the presence of Dox (not shown). These results indicate that HD-TET-tIFN provides potent, cell-specific, and regulated expression of tIFN in vitro.

Regulated and prolonged expression of tIFN in mice and rats injected with HD-TET-tIFN. To test the efficacy of secretion and gene regulation of tIFN in vivo, C57B/6 mice were injected intravenously with 1×10^{11} pp/kg of HD-TET-tIFN vector. Five days after the injection, Dox was added to the drinking water, and the released tIFN was measured in the serum after 3 days of treatment (Fig. 2A). An average of 23,670 (±4,200) pg/ml of tIFN was determined in mice sera, corresponding to 100-fold induction of expression. After Dox withdrawal, the level of tIFN dropped to baseline within 48 h (not shown). However, at this vector dose, tIFN was always detectable even without induction (not shown). To follow reinducibility of tIFN expression over time, C57B/6 mice were injected intravenously with a 200-fold-lower vector dose (5×10^8 pp/kg) (Fig. 2B). Similar levels of expression were obtained upon Dox administration during the entire course of the experiment (2 months). As a tissue specificity control, HD-TET-tIFN injection at a high dose (1×10^{11} pp/kg) in the muscle and induction with Dox did not lead to detectable tIFN levels in the bloodstream (data not shown). These results show that HD-TET-tIFN allows in vivo liver-specific and long-lasting gene expression.

To determine the functionality and stability of the vector in a larger animal model, rats were injected intravenously (i.v.) with 6×10^9 pp/kg of HD-TET-tIFN vector (Fig. 3). Fifty-four

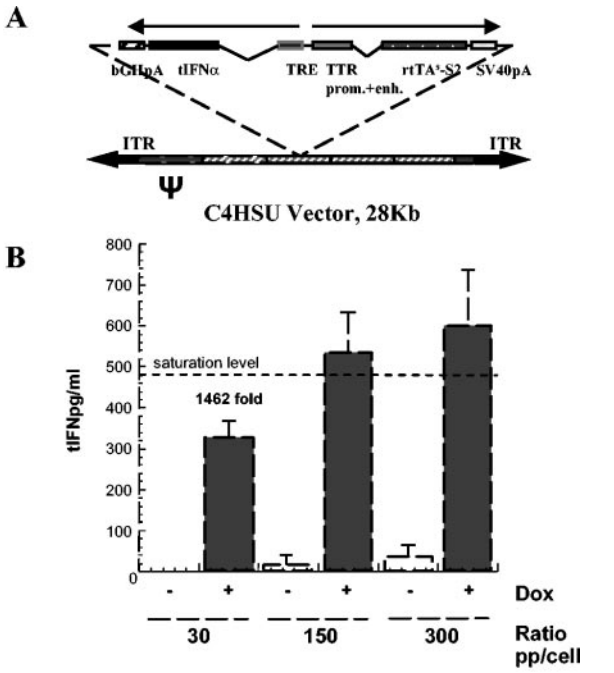

FIG. 1. (A) Structure of HD-TET-tIFN vector. The Dox-inducible transactivator rtTA2^s-S2 was inserted under the control of the liver-specific TTR promoter/enhancer (prom.+enh.). In the opposite orientation, the P_{Tet-1} promoter (TRE) controls the expression of tIFN. To stabilize the messengers and increase the level of expression, two introns were inserted downstream of the promoters. The expression cassette was inserted in the helper-dependent backbone C4HSU. ITR, inverted terminal repeat. (B) Expression and inducibility of tIFN by HD-TET-tIFN. Human hepatoma Hep3B cells were infected at the indicated doses of HD-TET-tIFN, and 48 h later, secreted tIFN was measured in supernatants by ELISA. Dox was added at 1 μg/ml. From left to right, tIFN levels were 0.23 pg/ml, 327.5 ± 8.2 pg/ml, 19.3 ± 8.4 pg/ml, 536.0 ± 9.3 pg/ml, 36.4 ± 5.6 pg/ml, and 600.1 ± 35.9 pg/ml. ELISA saturation level is indicated by the line.

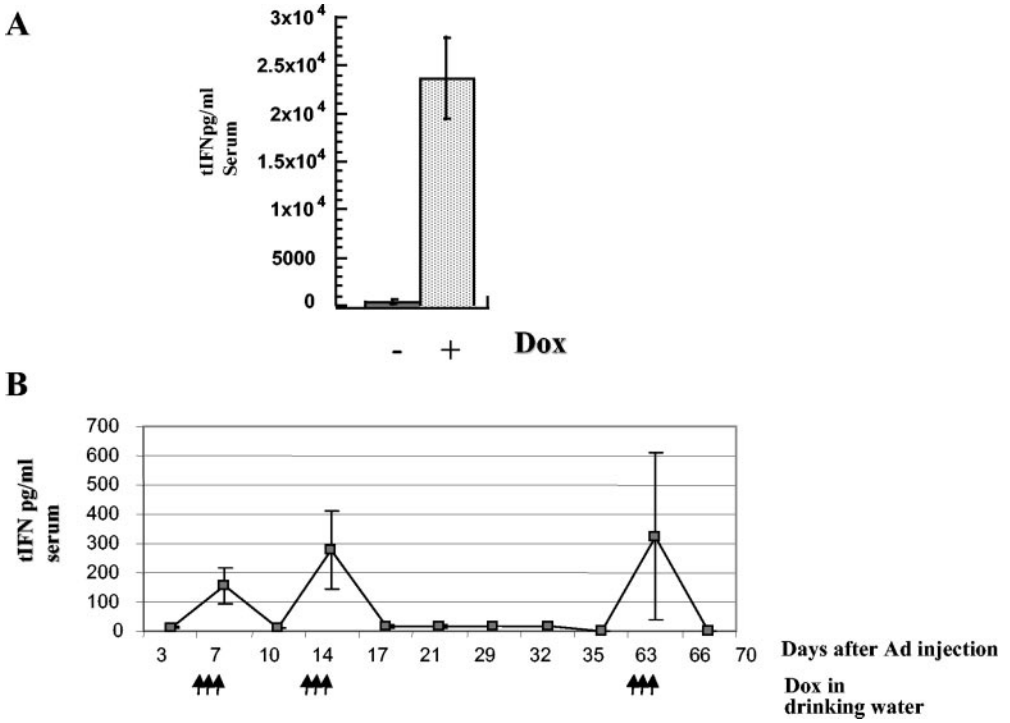

FIG. 2. (A) Induction of tIFN expression. C57B/6 mice were injected in the tail vein with 1×10^{11} pp/kg of HD-TET-tIFN vector. At day 5 p.i., Dox was added to the drinking water. At day 8, mice were bled and tIFN was measured in serum by ELISA. tIFN levels were 235 ± 50 pg/ml before induction and 23,670 ± 200 pg/ml after Dox administration ($P < 0.05$). (B) Repetitive induction of tIFN over time. C57B/6 mice were injected i.v. at the dose of 5×10^8 pp/kg of HD-TET-tIFN. Dox was administered at the indicated time points. Groups were made up of five to six mice. Ad, adenovirus.

days after the injection, Dox was added to the drinking water, and tIFN secretion in the bloodstream was measured 3 days later. Again, high levels of tIFN were measured in treated rat sera with respect to bleeds obtained before Dox treatment, showing 237.9-fold induction over the basal level, thus confirming the efficient and long-term inducibility in another rodent and indicating similar levels of transduction and expression between these two species.

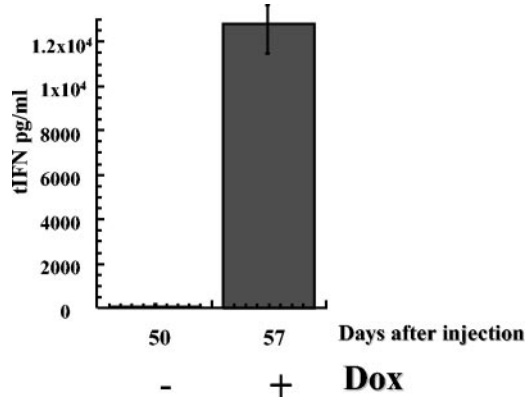

FIG. 3. Expression of tIFN in rats. Three rats were injected i.v. at a dose of 6×10^9 pp/kg. At day 50, rats were bled to determine the basal tIFN level (53.6 ± 0 pg/ml). At day 54, Dox was then added to the beverage, and at day 57, circulating tIFN was measured by ELISA. The level of circulating tIFN rose to 12,750 ± 100 pg/ml ($P < 0.05$).

IFN-mediated gene expression by transcriptional activation of tIFN in mice liver. We previously reported the biological activity of tIFN protein on mouse cells in culture and in vivo upon muscle electroporation gene transfer of a DNA plasmid expressing this gene (3). To test activation of the IFN pathway in HD-transduced tissues, C57B/6 mice were injected i.v. with 8×10^8 pp of HD-TET-tIFN and treated or not treated with Dox at day 5 p.i. Two days later, liver and muscle RNA was extracted, and the induction of an IFN-activated downstream gene was verified by Northern blot. As a consequence of tIFN expression, 2′5′-oligoadenylate synthetase transcript was elevated in liver upon Dox induction (Fig. 4). No difference could be observed in muscle, indicating a liver-localized effect of gene expression. These experiments therefore show that HD-TET-tIFN can induce and regulate the IFN signal transduction genes necessary to elicit its antiviral activity.

Inhibition of GBV-B in the replicon cell line. To analyze the effects of HD-TET-tIFN as an antiviral agent on GBV-B replication, we used the GBV-B replicon system (10, 11). B76.1/Huh7 cells were infected with different doses of HD-TET-tIFN, and tIFN expression was induced or not induced by the presence of Dox in the medium. tIFN protein was used as a positive control for inhibition of replication, and HD-GFP, an HD vector expressing green fluorescent protein, was used as a negative control. Seventy-two hours later, RNA was extracted and GBV-B replication was quantified by TaqMan quantitative RT-PCR assay (Fig. 5A). In the presence of Dox, a vector dose-dependent inhibition of GBV-B replication was observed.

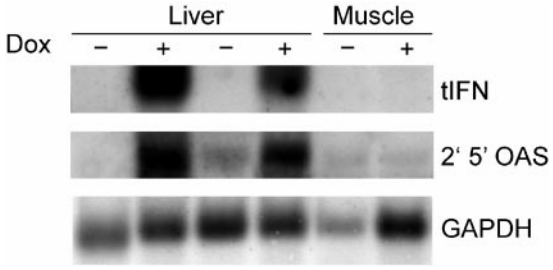

FIG. 4. Biological activity of tIFN. C57B/6 mice were injected i.v. with 8×10^8 pp. Five days later, expression of tIFN was induced with Dox or not induced for 3 days. Liver and muscle RNA (20 μg) were analyzed by Northern blot for the expression of tIFN and 2′5′-oligoadenylate synthetase (2′5′OAS). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping-normalization messenger.

At 10 pp/cell, the inhibition was 95% and the level of tIFN measured in the supernatant was 63 pg/ml (Fig. 5A). At the lower vector dose (1 pp/cell), the GBV-B inhibition level was 73%, comparable to the addition of 10 pg/ml of tIFN as protein. Slight inhibition of GBV-B replication was also measured in absence of the antibiotic but was statistically different from the Dox-induced samples ($P = 0.028$). The partial inhibition was probably due to the low level of tIFN expression in the absence of inducer, which was not measurable by ELISA (Fig. 5A). At 10-fold-higher doses (100 pp/cell), HD-GFP vector had no significant effect on GBV-B replication nor did it result in measurable endogenous IFN secretion. Differently, at this high dose, HD-TET-tIFN infection resulted in a nearly complete inhibition of GBV-B replication in the absence of Dox also (data not shown).

To determine the percentage of transduced cells expressing tIFN, B76.1/HuH7 cells were infected with HD-TET-tIFN, and cell DNA was extracted 48 h later. HD-TET-tIFN genomes were quantitated by TaqMan PCR assay (see Materials and Methods), and results are shown in Table 1. Interestingly, at the doses of 10 and 1 pp/cell, only a low percentage of cells were effectively transduced by HD-TET-tIFN, thus suggesting a bystander effect mediated by tIFN exocrine activity on GBV-B replication. To further support this observation, we sought to determine the effect of HD-TET-tIFN in a cell culture system in which vector-transduced and IFN-responsive nontransduced replicon-containing cells were separated by a membrane permeable only to proteins. The inhibition of replicon mediated by secreted tIFN deriving from infected B76.1/HuH7 cells was measured by TaqMan quantitative RT-PCR assay (Fig. 5B); this experiment basically reproduced the results obtained by the direct infection of cells, thus confirming that tIFN acts through an exocrine mechanism on bystander cells.

TABLE 1. Results of quantitation of HD-TET-tIFN genomes

No. of pp/cell	No. of HD-TET-tIFN genomes/1,000 cells	Estimated upper limit of transduction efficiency (%)
1,000	54,729 ± 29,310	100
100	12,143.7 ± 4,216.2	100
10	44.1 ± 19.2	4.41 ± 1.92
1	6.4 ± 4.6	0.64 ± 0.46

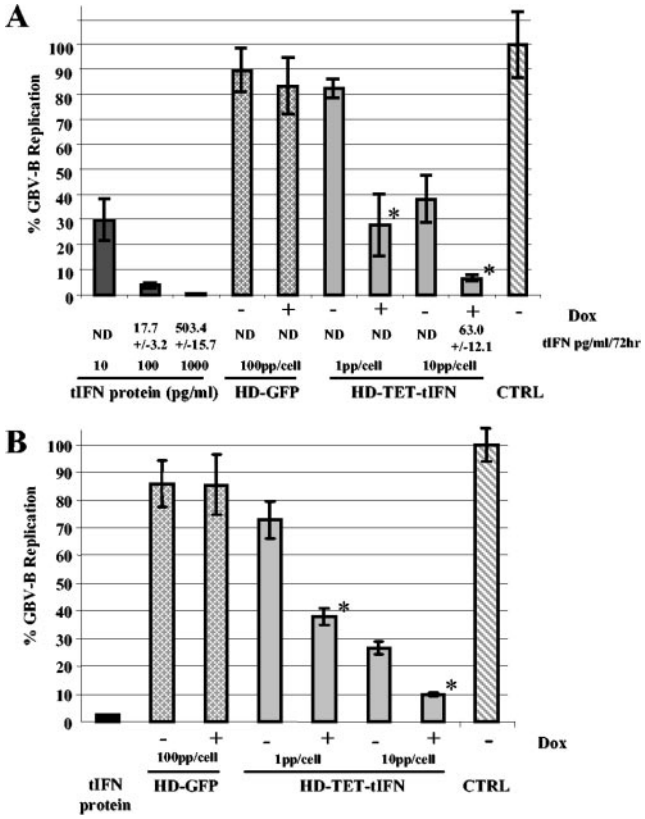

FIG. 5. Inhibition of GBV-B in replicon cell line. (A) B76.1/Huh7 cells were infected with either HD-TET-tIFN or HD-GFP at the indicated doses. Different quantities of tIFN protein were added to cells as a positive control. Three days later, RNA was extracted, and the GBV-B replicon was quantified by TaqMan quantitative PCR assay. Results are expressed as the percent inhibition of the ratio of GBV-B to RNA optical density at 260 nm related to noninfected cells (100%). tIFN protein secreted in the supernatant 72 h after infection of cells or left after exogenous tIFN addition is reported (ND, not detectable; CTRL, control, noninfected cells). Values represent the means ± the standard deviations of triplicate experiments. Asterisks indicate statistically significant values ($P = 0.044$ and $P = 0.028$ for 10 and 1 pp/cell, respectively) compared to Dox-untreated samples. The experiment was repeated twice with comparable results. (B) B76.1/Huh7 cells were plated onto the membrane of cell culture inserts permeable to proteins but not to cells and infected with HD vectors or left untreated. Inserts were then placed into wells containing untreated B76.1/Huh7, and 3 days later, their GBV-B replicon level was measured by TaqMan quantitative RT-PCR assay. tIFN (1,000 pg/ml) was used as a positive control. Values represent the means ± the standard deviations of sextuplicate experiments. Asterisks indicate statistically significant values ($P = 0.00085$ and $P = 0.00064$ for 10 and 1 pp/cell, respectively) compared to Dox-untreated samples.

These results demonstrate that HD-TET-tIFN can efficiently inhibit GBV-B replication in a cell-based replicon system, and this effect is also observed when few cells are effectively transduced.

tIFN expression and GBV-B challenge in tamarins. Recently, tamarins (*Saguinus* species) infected with GBV-B have been proposed as a surrogate model for HCV infection (6–8, 30, 31, 38). In order to test the transcriptional regulation and therapeutic effect of IFN hepatic expression in tamarins, HD-TET-tIFN was injected intravenously into four monkeys. Two tamarins (95-14 and 96-15) were injected at a dose of 4×10^{11}

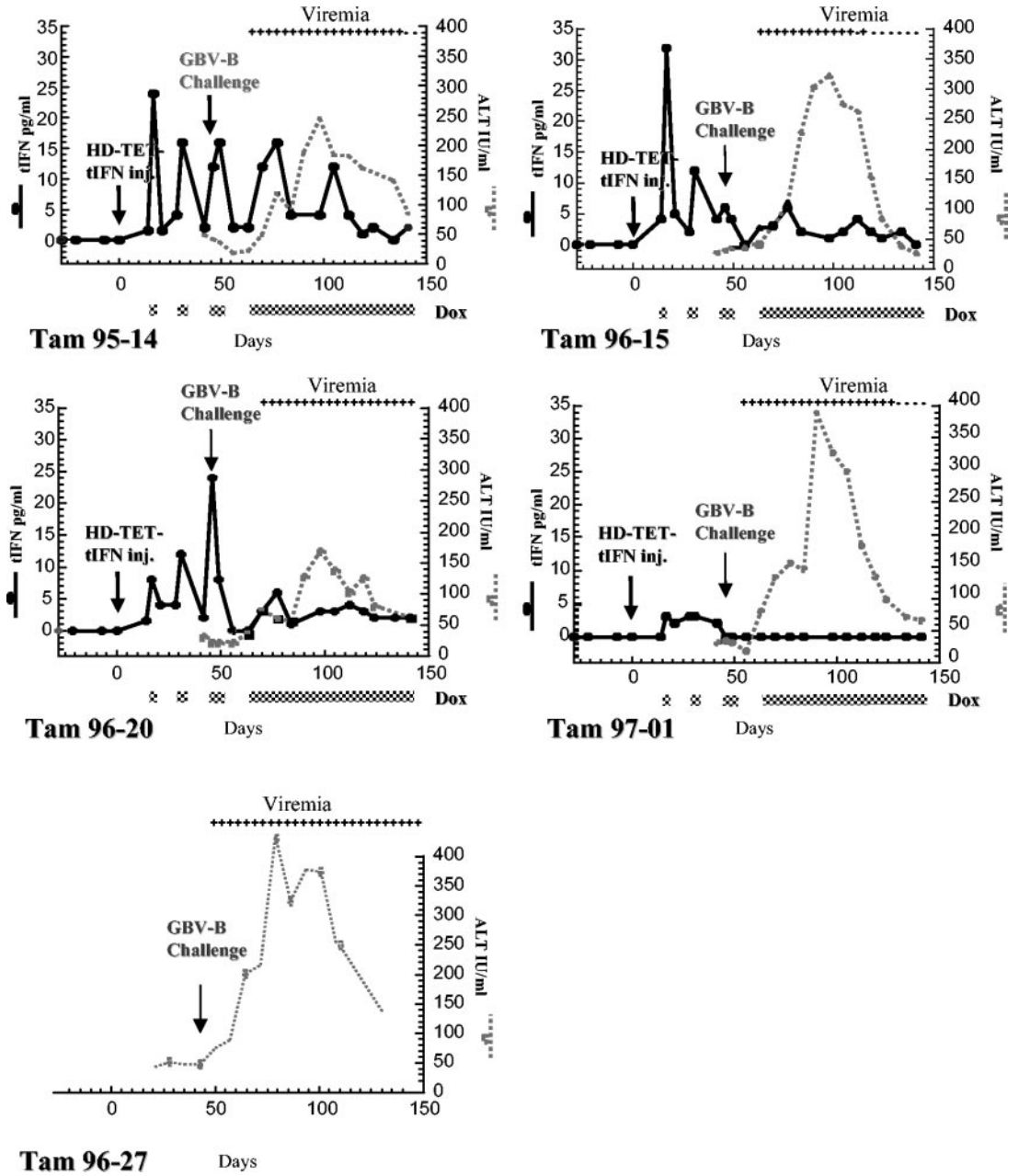

FIG. 6. tIFN-α regulation of expression and GBV-B challenge in HD-TET-tIFN-injected tamarins. Tamarins (Tam) were injected (inj.) in the saphenous vein either with 4×10^{11} pp/kg (95-14 and 96-15) or with 2×10^{11} pp/kg (96-20 and 97-01) of HD-TET-tIFN. tIFN-α expression was induced at days 14, 28, and 42 for a duration of 3 days by Dox administration in beverage or food. Measurement of circulating tIFN-α was performed by VSV cytopathic inhibition assay. All tamarins were challenged intravenously with 30 TID_{50} of GBV-B inoculum at day 42 p.i., and Dox was administered daily until the end of the study. Tamarin 96-27 was used as the control for the infection. Elevations of transaminases (ALT, black solid line) and the serum tIFN-α level (hatched gray line) were followed over time. Viremia was determined by PCR from blood (+ symbol indicates PCR amplification).

pp/kg, and the other two (96-20 and 97-01) were injected at a dose of 2×10^{11} pp/kg. Dox was given to the monkeys by oral administration at day 14 to day 17 after the injection, and levels of serum tIFN were analyzed by inhibition of VSV cytopathic effect (Fig. 6). In monkeys 95-14 and 96-15, the measured tIFN circulating levels were 24 and 32 pg/ml, respectively, obtaining an induction of expression of at least 16- and 8-fold on the baseline, respectively, while for monkeys 96-20 and 97-01, the levels were lower (3 and 8 pg/ml, respectively). At day 28 p.i., a second administration of doxycycline was given for 3 days, and similar increased levels of expression were obtained in all monkeys. These data demonstrate that HD-TET-tIFN vector works in tamarins and that tIFN expression is reinducible.

To verify if our vector could have a therapeutic efficacy against the hepatitis induced by GBV-B, at day 42 p.i., we both induced the expression of tIFN with doxycycline for 3 days and administered i.v. a GBV-B inoculum corresponding to 30 TID_{50} to the tamarins (Fig. 6). From day 69 up to the end of

TABLE 2. GBV-B challenge in tamarins

Monkey	Peak tIFN level (pg/ml)	Viremia onset day	Peak ALT level (IU/liter)
95-14	24[a]	21	245
96-15	32[a]	17	323
96-20	24[b]	17	169
97-01	3[a]	10	390
96-27 (control)	<1	8	440

[a] Day 17 postinjection.
[b] Day 46 postinjection.

the study (day 150), Dox was given daily to the monkeys to obtain augmented local liver expression of tIFN. In the successive bleedings, besides tIFN levels, we measured the transaminases (ALT) as an index of liver inflammation and GBV-B viremia in the blood by PCR (Fig. 6). Importantly, blood tests did not reveal any evident parameter alteration, indicating that this treatment was well tolerated by the animals (data not shown and see Materials and Methods). We observed that all the tamarins became viremic 2 to 3 weeks after GBV-B challenge and that transaminases gradually rose to high levels (shown in Fig. 6 and summarized in Table 2). However, while control animal 96-27 became viremic at day 8 postchallenge, as was also observed for other tamarins infected with the same inoculum in a previous study (30), the higher-dose HD-TET-tIFN-treated monkeys 95-14 and 96-15 showed detectable viremia only at day 21 and day 17, respectively. Animals 96-20 and 97-01 showed circulating GBV-B virus at days 17 and 10 postchallenge, respectively (Table 2). Interestingly, we found that the higher the peak of circulating tIFN level before challenge, the later the hepatitis onset (Fig. 6 and Table 2). In fact, the earliest increase in both viremia and transaminase levels occurred in tamarin 97-01, which showed a low tIFN expression throughout the entire experiment. On the other hand, in tamarins 95-14 and 96-20, which both had 24 pg/ml circulating tIFN before challenge, a milder hepatitis occurred, as suggested by ALT level. As commonly observed in GBV-B infection of tamarins, the final outcome was an acute self-limiting disease. Notably, continuous administration of Dox resulted in a low but constant secretion of tIFN above the background in the injected monkeys, with the exception of animal 97-01, which did not show measurable levels of tIFN and developed a severe hepatitis comparable to that of the control monkey.

Thus, treatment of tamarins with HD-TET-tIFN vector allows regulated hepatic expression of tIFN and results in delayed hepatitis onset upon GBV-B virus challenge as a function of cytokine secretion.

DISCUSSION

In this study, we explored the application of a gene therapy approach for the treatment of hepatitis in the GBV-B/tamarin model. In a previous report (2), we described the application and efficacy of regulated liver-specific IFN-α expression in the mouse.

Here, we addressed the issue of suitability of HD vectors to this kind of application. For this reason, we generated HD-TET-tIFN; this HD adenovirus vector efficiently regulated the expression of tIFN through Dox administration both in vitro and in vivo. Secretion of the cytokine was repeatedly induced at high levels in mice and rats for a long period. As previously observed, liver-specific expression of tIFN activated the IFN signal transduction pathway essential for the induction of antiviral genes (3) and, more importantly, did so only in the liver upon Dox treatment. Once the regulation properties of HD-TET-tIFN had been characterized, we verified its efficacy as an antiviral agent. For this purpose, we used the B76.1/Huh-7 cell line bearing the GBV-B neo-RepB replicon. These cells actively support replication of GBV-B RNA and are suitable to test the effect of IFN and other antiviral molecules (10). Infection by HD-TET-tIFN and the addition of Dox (Fig. 5A) efficiently inhibited GBV-B replication both when all cells were transduced (100 pp/cell) and even at very low doses (1pp/cell); since at this dose, only a small percentage of cells was effectively transduced (Table 1), this suggests that tIFN paracrine and exocrine action was sufficient to block viral replication in most cells. As further confirmation of this hypothesis, the effect of HD-TET-tIFN on GBV-B replication was observed also when infected cells were separated by a membrane (Fig. 5B). These results showed that HD-TET-tIFN is extremely powerful in this in vitro system. Similar effects mediated by an HD vector constitutively expressing woodchuck IFN-α under the control of TTR liver-specific promoter were described by our group in the woodchuck hepatitis model also. Primary hepatocytes from chronically woodchuck hepatitis virus-infected animals showed consistent reduction of viral proteins upon treatment with an HD-woodchuck IFN vector in vitro (27). Accordingly, chronically infected woodchucks showed a reduction of viral replication after treatment with the same vector in vivo (13).

The HD-TET-tIFN vector was also tested in the GBV-B/tamarin system. Four monkeys were injected with HD-TET-tIFN, and repeated induction by Dox administration showed expression of tIFN in the bloodstream for several days. Vector-injected tamarins were challenged with GBV-B, and Dox was administered again. At day 8 postchallenge, the control monkey became viremic and developed hepatitis, as measured by ALT determination, while the HD-TET-tIFN-injected Dox-treated monkeys showed no circulating viral particles. Although somewhat delayed, viremia and hepatitis symptoms were also detected in the treated monkeys. In fact, GBV-B viremia was detected at days 10 to 21 postchallenge in HD-TET-tIFN-injected monkeys, and ALT levels reached their peak at days 42 to 49 after challenge, going gradually back to the basal level after more than 3 months. Even if the number of animals of this study was limited, levels of tIFN expression and hepatitis onset and symptoms suggested a correlation. However, we cannot exclude the possibility that the observed partial suppression of GBV-B may in part be due to the bystander effect of the innate or adaptive immune response against the vector per se, in addition to the effect of tIFN-α expressed by the vector. Nevertheless, vector-injected but low-tIFN-expressing monkey 97-01 developed a disease similar to that of the control monkey. In addition, GBV-B challenge was performed at day 42 p.i.; at this point in time, there should be no further effect of a possible acute inflammation induced by adenovirus particles.

The lack of complete protection from viral hepatitis by HD-TET-tIFN can be due to several reasons. First, we cannot

exclude a low density of coxsackie adenovirus receptor on tamarin hepatocytes and consequent poor transduction. This could explain the low tIFN levels measured in tamarins in comparison with those observed after delivery of the same vector in mice and rats (Fig. 2 and 3). In fact, expression of the transgene obtained in monkeys and rodents differed by at least 3 orders of magnitude using similar vector dosages. Second, despite their similarity, HCV and GBV-B could exhibit some differences in the development of hepatitis. For example, the average viral titer measured in HCV patients is approximately 10^5 to 10^6 viral particles/ml; in experimentally infected tamarins, the reported circulating GBV-B titer is about 10^8 to 10^9 viral particles/ml (6, 8, 30). This means that GBV-B could propagate in its host 1,000-fold more efficiently than HCV, and replication could be much more difficult to inhibit. Third, we cannot exclude that extrahepatic replication of GBV-B in other organs could continuously infect the liver and overcome the hepatic tIFN-mediated block. Fourth, effective Dox concentration in the liver might not have been sufficient to fully activate the transactivator rtTA2^s-S2. To this end, utilizing the new variant rtTA2^s-M2 could increase the sensitivity to Dox by at least 10-fold (reference 36 and L. Aurisicchio et al., unpublished results). In conclusion, the strong antiviral efficacy of HD-TET-tIFN in vitro does not fully correlate with the situation in vivo, where many more factors contribute to the outcome of the infection.

Interestingly, tIFN expression was controllable even in an inflamed tissue like liver undergoing hepatitis. In fact, in tamarins 95-14, 96-15, and 96-20, we observed tIFN levels of expression over the background also during the rise of transaminases (Fig. 6). Despite this, we observed a gradual reduction of tIFN expression over time in all four tamarins to baseline. One explanation is that hepatitis could partially and aspecifically inactivate HD-TET-tIFN transduced hepatocytes. This would diminish the active tIFN producer cells. Additionally, an immune response against the rtTA2^s-S2 transactivator could also contribute to the decrease of tIFN expression. The effect of this response could have been enhanced in the liver microenvironment, where active secretion of IFN may have promoted a Th1 response (35). This could specifically eliminate rtTA2^s-S2-expressing cells. In fact, in recent works, rtTA transactivators were shown to be immunogenic in monkeys if delivered intramuscularly by AAV (12) or DNA or adenovirus (12, 20).

To our knowledge, this is the first IFN-mediated gene therapy study in a nonhuman primate hepatitis model. Although HD-TET-tIFN was able to efficiently inhibit GBV-B replication in vitro, we were not able to prevent tamarins from GBV-B infection and spreading, since tIFN expression was capable of only delaying the onset of hepatitis. Nonetheless, regulation of tIFN expression was achieved in this system, and more studies are now required to increase both transduction of tamarin hepatocytes and optimization of tIFN expression.

It is important that this study exemplifies the issues involved in scaling up therapeutic protocols from rodents to monkeys and subsequently to humans, which are not always straightforward, and gene therapy trials should take this into account.

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