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Safety Profile of Gutless Adenovirus Vectors Delivered into the Normal Brain Parenchyma: Implications for a Glioma Phase I Clinical Trial

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ABSTRACT

Adenoviral vectors (Ads) have been evaluated in clinical trials for glioma. However, systemic immunity against the vectors can hamper therapeutic efficacy. We demonstrated that combined immunostimulation and cytotoxic gene therapy provides long-term survival in pre-clinical glioma models. Since helper-dependent high capacity Ads (HC-Ads) elicit sustained transgene expression, in the presence of anti-adenoviral immunity, we engineered HC-Ads encoding conditional cytotoxic herpes simplex type 1 thymidine kinase and immunostimulatory cytokine fms-like tyrosine kinase ligand 3 under the control of TetOn system. Escalating doses of combined HC-Ads ($1x10^8$, $1x10^9$, $1x10^{10}$ vp) were delivered into the rat brain. We assessed neuropathology, biodistribution, transgene expression, systemic toxicity and behavioral impact at acute and chronic time points post-vector delivery. Histopathological analysis did not reveal any evidence of toxicity or long-term inflammation at the lower doses tested. Vector genomes were restricted to the injection site. Serum chemistry did not uncover adverse systemic side effects at any of the doses tested. Taken together our data indicates that doses of up to $1x10^9$ vp of each HC-Ad can be safely administered into the normal brain. This comprehensive toxicity and biodistribution study will lay the foundations for implementation of a Phase I clinical trial for GBM using HC-Ads**.**

INTRODUCTION

Despite advances in neurosurgical techniques, radiotherapy, and chemotherapy, the prognosis in Glioblastoma multiforme (GBM) remains poor, with median survival of 14.6-19.6 months.(Stupp *et al.,* 2005; Grossman *et al.,* 2010) GBM is the most common primary brain tumor in adults and its highly infiltrative nature precludes complete surgical resection; thus, the tumor almost invariably recurs. Thus, immunotherapies are attractive adjuvants for this devastating disease.

We have developed a combined, conditional cytotoxic/immune stimulatory gene therapy strategy to treat GBM.(Ali *et al.,* 2005; Candolfi *et al.,* 2009; Curtin *et al.,*, 2009; Ghulam Muhammad *et al.,* 2009) This involves treatment with two genetically engineered first generation adenoviral vectors (Ads) to deliver the conditionally cytotoxic herpes simplex type-1 thymidine kinase (TK) that kills proliferating tumor cells in the presence of pro-drug gancyclovir (GCV)(Dewey *et al.,* 1999) and human soluble fms-like tyrosine kinase ligand 3 (Flt3L), which recruits bone marrow-derived antigen presenting cells into the brain tumor microenvironment.(Ali *et al.,* 2004; Curtin *et al.,* 2006) This strategy has been shown to be highly effective in several syngeneic pre-clinical rodent GBM models, i.e., rats bearing intracranial CNS-1, 9L, and F98 tumors(Ali *et al.,* 2005; Ghulam Muhammad *et al.,* 2009) and mice bearing intracranial GL26, GL261 and B16-F10 tumors.(Candolfi *et al.,* 2009; Curtin *et al.,* 2009) Additionally, the treatment with Ad-TK+Ad-Flt3L induces GBM-specific immunological memory that is effective in treating intracranial multifocal and peripheral models of GBM.(King *et al.,* 2008; King *et al.,* 2008; Ghulam Muhammad *et al.,* 2009) Recently, this combined gene therapy strategy got approval from the FDA for a Phase I clinical trial for GBM (BB-IND 14574; NIH/OBA Protocol # 0907-990; OSU Protocol # 10089).

With the aim to improve the gene delivery platform to achieve regulatable Flt3L expression and elude anti-adenovirus immune responses which could curtail therapeutic transgene expression, we have developed helper-dependent high capacity adenovirus (HC-Ad) vectors expressing HSV1-TK under the control of a constitutive promoter and hsFlt3L under the control of the tightly regulatable mCMV-TetOn expression system.(Xiong *et al.,* 2006; Curtin *et al.,* 2008) Using these HC-Ad vectors, we recently demonstrated their therapeutic efficacy and high safety profile after delivery into a large tumor mass in a syngeneic, orthotopic rat model of GBM.(Muhammad *et al.,* 2010; Puntel *et al.,* 2010)

In the present study, as a prelude to a novel Phase I clinical trial for GBM, we conducted a dose-escalation and biodistribution study of HC-Ad-TetOn-Flt3L + HC-Ad-TK when delivered into the naïve rat brain to determine the vectors' maximum tolerated dose (MTD). Following injection of $1x10^8$, $1x10^9$, or $1x10^{10}$ vp of each HC-Ads, assessment of brain tissue integrity, immune infiltrates, vector biodistribution and transgene expression, as well as putative systemic and neurological toxicity was conducted at day 5, 1 month, 6 months or 1 year post vector delivery. Our data indicate that even at the highest dose tested, real-time quantitative PCR did not detect evidence of biodistribution of HC-Ad genomes to peripheral organs and behavioral testing did not reveal any vector mediated abnormalities. Peripheral blood cell counts and serum biochemistry were within normal ranges during all time points, at all doses tested. However, neuropathological assessment revealed evidence of neurotoxicity at the highest dose tested ($1x10^{10}$ vp of each vector), i.e., loss of brain tissue and high levels of inflammation. We did not detect any evidence of neurotoxicity or long-term inflammation at either of the two lower doses tested. HC-Ads are attractive gene delivery platforms for implementing gene therapy strategies for neurological disorders, both chronic neurodegenerative disorders and brain cancer. For

successful implementation of these experimental therapies in human patients, it will be necessary to deliver these vectors into the normal brain parenchyma. The data presented herein constitutes the first comprehensive study of the biodistribution, behavioral impact, and neuropathological implications after delivery of escalating doses of these vectors into the brain parenchyma, both at acute and chronic time points post-vector delivery. Such well controlled, pre-clinical safety and toxicity studies are required by the FDA in order to file for an pre-IND/IND and constitute a major milestone in the path towards implementation of the first Phase I clinical trial for GBM using gutless, HC-Ad vectors. These studies would also support the implementation of HC-Ad vectors to treat chronic neurological disorders.

MATERIALS AND METHODS

High-capacity adenoviral vectors

Details of the molecular characterization, rescue, amplification, and *in vitro* characterization of the HC-Ad vectors were previously published.(Candolfi *et al.,* 2006; Xiong *et al.,* 2006; Muhammad *et al.,* 2010) Briefly, HC-Ad-TK expressed herpes simplex type 1 thymidine kinase (TK) constitutively under the control of mCMV promoter and HC-Ad-TetOn-Flt3L expressed human soluble fms-like tyrosine kinase 3 ligand (Flt3L) under the control of tightly regulated mCMV-TetOn inducible expression system developed by us.(Xiong *et al.,* 2006)

Animals. Adult male Lewis rats (220-250g, Harlan, Indianapolis, IN) were used. Rats were kept in controlled conditions of light (12h light-dark cycles) and temperature (20-25^{o} C) and fed with standard lab chow and water *ad libitum*.

Intracranial injections and experiment end points

Starting 2 days before treatment, rats were fed with doxycycline mixed chow *ad libitum* (Modified LabDiet® Laboratory Rodent Diet 5001 with 2000ppm Doxycycline, PMI® Nutrition International/Purina Mills LLC, Richmond, IN). Groups of rats were injected unilaterally in the right striatum with any of the three escalating doses of HC-Ad-TK and HC-Ad-TetON-Flt3L ($1x10^8$, $1x10^9$, or $1x10^{10}$vp of each vector). The vectors were first mixed together and resuspended in a final volume of 3µl of saline. Using a 10µl Hamilton syringe fitted with 26-gauge needle, the vector mixture was administered at stereotactic coordinates: 1mm anterior and 3.2mm lateral to the bregma and the injection volume of 3µl were delivered in 3 locations (1µl each) at the depth of -5.5, -5.0, and -4.5mm from the dura. Twenty-four hours after treatment, the rats received ganciclovir (GCV, 25mg/kg, i.p.; Roche Laboratories, Nutley, NJ), twice daily for up to 10 consecutive days. The control group of rats (naïve) received 3µl of saline at the same stereotactic coordinates as the treatment groups.

Groups of rats were evaluated at 5 days, 1 month, 6 months and 1 year for biodistribution of HC-Ad vector genomes, neurotoxicity, peripheral blood cell counts and serum biochemistry, circulating levels of anti-adenovirus neutralizing antibodies and anti-TK antibodies. Additionally, the 1 month, 6 months and 1 year groups were evaluated for HC-Ad vector induced behavioral deficiencies. All animal procedures were carried out in accordance with NIH Guide for the care and use of laboratory animals and approved by the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee.

Serum biochemistry and hematology

At 5 days, 1 month, 6 months, or 1 year post treatment, blood was collected from each animal during euthanasia. The samples were sent to the laboratory of Antech Diagnostics (Irvine, CA) for analysis of routine hematological and biochemical parameters. The median, minimum, and maximum values for each parameter are shown in the results section and supplementary tables.

Circulating neutralizing anti-adenovirus antibodies

The level of adenovirus specific neutralizing antibodies was assessed as described previously.(Puntel *et al.,* 2010) Briefly, the serum samples were heat-inactivated at 56°C for 30min and serially diluted twofold in minimal essential medium (Invitrogen, Carlsbad, CA) containing 2% Fetal Bovine Serum (FBS). The range of dilutions was 1:2 to 1:4,096. Each 50µl serum dilution was incubated with 1×10^7pfu of first generation adenoviral vector expressing β-galactosidase (Ad-β-Gal) (in a 10µl volume) for 90min at 37°C. The 50µl of sample containing sera and virus was then added to the wells of a 96-well plate containing pre-seeded (1.5×10^4) HEK 293 cells per well and were incubated at 37°C for 1h. A further 50µl of medium containing 10% FBS was added to each well, and cells were incubated at 37°C for 20h before fixing with 4% paraformaldehyde in PBS (pH 7.4) and staining with 5-bromo-4-chloro-indolyl-β-d-galactoside (X-gal) (Sigma, St. Louis, MO). The neutralizing antibody titer for each animal is given as the reciprocal of the highest dilution of serum at which 50% of Ad-β-Gal-mediated transduction was inhibited. Positive and negative controls were used.

Anti-TK antibody assay

The titer of anti-TK antibodies in the sera of rats was assessed as described previously.(King *et al.,* 2011) Briefly, CNS-1 cells were infected with first generation adenoviral vector expressing TK (Ad-TK) at a multiplicity of infection of 200i.u./cell, or mock infected as a control. After 72h, the cells were harvested and freeze/thawed. Cell lysates were added to a 96-well plate (Cat # 442404, NUNC, Rochester, NY) and incubated overnight at 4°C. The serum samples were then diluted 1:4 and added to wells coated with cell lysates and incubated for 2h at room temperature. The wells were washed and incubated for 1h with rabbit anti-rat IgG/biotinylated secondary antibody (1:1000; Dako, Carpinteria, CA); wells containing positive control rabbit sera were incubated with goat anti-rabbit IgG/biotinylated secondary antibody (1:1000; Dako, Carpinteria, CA). Finally, the wells were incubated with streptavidin-HRP (R&D systems, Minneapolis, MN) and visualized with substrate solution (R&D systems, Minneapolis, MN) at 500nm wavelength. The % change of optical density was calculated for each sample incubated with TK cell lysates compared to mock lysates.

Study of vector genomes' biodistribution

Biodistribution of vector genomes was assessed at 5 days, 1 month, 6 months, or 1 year post-treatment. This was performed as described by us previously.(Muhammad *et al.,* 2010; Puntel *et al.,* 2010) Briefly, the rats were perfused without any fixative and 25mg of tissue sample was harvested from the following locations: the brain injection site, contralateral brain hemisphere, cerebellum, brain stem, spleen, liver, testes, small gut, lung, heart, cervical lymph nodes, kidney, and lumbar spinal cord. Total DNA was purified and used for the quantitation of vector genomes by Real-Time quantitative PCR using a primer and probe specific for the cosmid sequences

contained in the HC-Ad vector backbone as described by us previously.(Puntel *et al.,* 2006) The vector genomes are shown as a ratio of vector genomes/25mg of tissue (n=5 per group).

Neuropathological analysis

Neuropathological evaluation was performed at 5 days, 1 month, 6 months, or 1 year post treatment. Following perfusion with oxygenated Tyrode's solution and 4% Paraformaldehyde (PFA), brains were post fixed in 4% PFA for 3 additional days. Sixty-micrometer serial coronal sections were cut at the immediate vicinity of injection site and free-floating immunocytochemistry was performed as previously described(Candolfi *et al.,* 2007; King *et al.,* 2008; King *et al.,* 2008) with markers for oligodendrocytes and myelin sheath (mouse monoclonal anti-MBP, 1:1,000, Chemicon, Temecula, CA, USA, cat# MAB1580), dopaminergic nerve terminals (rabbit polyclonal anti-TH, 1:5,000, Calbiochem, La Jolla, CA, cat# 657012), $CD8^+$ T cells (mouse anti-CD8, 1:1,000, Serotec, Raleigh, NC, cat# MCA48G), macrophages and microglia (CD68/ED1, mouse anti-ED1, 1:1,000, Serotec, Raleigh, NC, cat# MCA341R), macrophages, microglia and immune cells (mouse anti-MHC II, 1:1,000, Serotec, Raleigh, NC, cat# MCA46G), or transgene expression, that is, TK (rabbit anti-TK, 1:10,000, custom made) or Flt3L (rabbit anti-Flt3L, 1:500, custom made). Nissl staining was performed to assess gross histopathological features of the brains. The stained sections were photographed with Carl Zeiss Optical Axioplan microscope using Axiovision Rel 4.6 and MOSAIX software (Carl Zeiss, Chester, VA).

Behavioral analysis

The neurobehavioral impact as a consequence to treatment using escalating doses of HC-Ad was assessed at 1 month, 6 months, and 1 year post treatment. Testing of amphetamine-induced rotational behavior, abnormalities in limb use asymmetry, and spontaneous motor and rearing behavior was done as described by us in detail.(King *et al.,* 2008) Briefly, amphetamine-induced rotational behavior was measured using a RotoMax apparatus and software (AccuScan Instruments, Columbus, OH) for 90min after s.c. injection of 1.5mg/kg d-amphetamine sulfate (Sigma, St. Louis, MO). To measure forelimb use asymmetry, contacts made by each forepaw with the wall of a 20.3cm wide clear cylinder were scored from videotape over a 10min period in slow motion by two independent, experimentally blinded observers. Forelimb contact with the walls of the cylinder was scored measuring only initial contact with the cylinder walls. Baseline spontaneous locomotor and rearing activity was recorded for 30min in 40.6x40.6x38.1cm closed box using photobeam breaks and optical sensors. Spontaneous locomotor and rearing activity was then monitored for 120min after s.c. injection of 1.5mg/kg d-amphetamine sulfate (Sigma, St. Louis, MO). Data was scored as the total number of beam breaks summed over the observation period.

Statistical Analysis

Statistical analyses were performed using Graphpad Prism 5 (Graphpad Software, San Diego, CA, USA). Data were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The p values of less than 0.05 were considered as significant.

RESULTS

Dose escalation of HC-Ad-TetOn-Flt3L + HC-Ad-TK delivered into the naïve rat brain followed by neuropathological analysis

To examine the effects of HC-Ad-TetOn-Flt3L and HC-Ad-TK on normal brain architecture and inflammation, we performed a comprehensive neuropathological analysis at 5 days, 1 month, 6 months, and 1 year after intrastriatal HC-Ads delivery. Naïve adult Lewis rats received a combination of either 1×10^8, 1×10^9, or 1×10^{10} vp of each HC-Ad vector or saline (**Figure 1a**). GCV was administered twice daily for up to 10 days. The rats were fed doxycycline-containing chow for up to 4 weeks because this schedule was found to be optimal for therapeutic efficacy.(Muhammad *et al.,* 2010) Neuropathological assessment revealed evidence of neurotoxicity at the highest dose tested ($1x10^{10}$ vp of each HC-Ad; total dose $2x10^{10}$ vp), i.e., loss of brain tissue and high levels of inflammation. We did not encounter any evidence of brain tissue damage at either of the lower doses tested. Nissl staining was more prominent at 5 days following delivery of the highest dose of HC-Ad when compared to saline-treated animals suggesting local inflammation: the intensity of Nissl staining was comparable among the saline treated controls and either of the lower vector doses (1×10^8 and 1×10^9 vp of each vector) (**Figure 1b**). Myelin basic protein and tyrosine hydroxylase immunoreactivity also indicated presence of tissue damage with the high total dose of $2x10^{10}$ vp. There was profuse infiltration of $CD8^+$ T cells, macrophages, and major histocompatibility complex II^+ cells were seen in the injected brain hemisphere in all groups, including saline treated controls, at the acute 5d time point. However, the observed immune cell infiltration was much more pronounced with the highest dose (1×10^{10} vp of each HC-Ad) (**Figure 1b**). Additionally, there was mild CD68/ED1 and MHC-II immunoreactivity seen in the contralateral brain hemisphere of saline and all vector

treated groups at the early time point, i.e., 5 days. The immune cell infiltration seen in all groups declined gradually over time, i.e., at 1 month, 6 months and 1 year after treatment (**Figure 1c, Figure S1**); no $CD8^+$ T cells were observed at 1 year after treatment with either of the lower doses (1×10^8 and 1×10^9 vp of each vector) or saline (**Figure 1c**). With regards to expression of the transgene proteins in the brain, immunocytochemical analysis revealed an abundance of cells expressing TK and Flt3L at day 5 following treatment (**Figure 1b**); the presence of transgene-expressing cells was also evident at 1 month and 6 months (**Figure S1**) and even at 1 year (**Figure 1c**) following HC-Ads delivery. These neuropathological data indicate that $1x10^9$ vp of each HC-Ad is the maximum tolerated dose (MTD) that can be safely administered into the brain of naïve rats.

HC-Ad-TetOn-Flt3L and HC-Ad-TK (+GCV) treated animals do not exhibit neuro-behavioral deficits

Due to the fact that the gene therapy vectors will be delivered into the surrounding brain parenchyma after tumor resection in GBM patients, it is important to assess the occurrence of any acute and/or chronic neurological deficits elicited by HC-Ads. To this end, adult naïve Lewis rats treated with escalating doses of HC-Ad-TetOn-Flt3L and HC-Ad-TK were subjected to a panel of neurobehavioral tests at 1 month, 6 months and 1 year after treatment. Analysis of amphetamine-induced total locomotor activity (**Figure 2a, S2a, S3a**), total rearing activity (**Figure 2b, S2b, S3b**), and rotational behavior (**Figure 2d, S2d, S3d**) did not reveal any abnormalities in the HC-Ad treated rats when compared with saline treated age-matched naïve controls (one-way ANOVA followed by Tukey's multiple comparison test). (**Figure 2a, 2b**). The right limb use asymmetry test also did not reveal any abnormalities except for the groups

treated with the highest dose (1×10^{10} vp of each HC-Ad) when compared with rats treated with the lowest dose (1×10^{8} vp of each HC-Ad) at 1 month (one-way ANOVA followed by Tukey's multiple comparison test; $p<0.05$).

Biodistribution of HC-Ad vector genomes is restricted to the injected brain hemisphere at all time points and HC-Ad vector's doses tested

The biodistribution of HC-Ad vector genomes was assessed in the brain, spinal cord and peripheral organs at 5 days, 1 month, 6 months, and 1 year following HC-Ad vector delivery, using quantitative PCR analysis. At all doses and time points tested, HC-Ad genomes were restricted to the injection site in the ipsilateral hemishere (**Figure 3, Figure S4**). HC-Ad vector genomes were below detectable limits in all peripheral organs, including other regions of the brain and spinal cord, even at the highest dose tested at all time points, thereby highlighting the high safety profile of this HC-Ad mediated, combined gene therapy approach.

Circulating neutralizing anti-adenovirus antibodies and anti-thymidine kinase antibodies in the serum of HC-Ad treated animals

Blood samples were collected from each animal during euthanasia to measure anti-adenovirus neutralizing antibodies and anti-thymidine kinase antibodies. Serum levels of anti-Ad neutralizing antibodies were indistinguishable form background levels at all time points with the total HC-Ad doses of $2x10^{8}$ vp and 2x109 vp. Animals injected with the highest dose of HC-Ads ($2x10^{10}$ vp) exhibited detectable, albeit low titers of circulating anti-Ad neutralizing antibodies, consistent with generation of antibodies against adenoviral proteins possibly due to some degree of leakage of the HC-Ads into the ventricular space (**Figure 4**). The highest titers were exhibited

at 1 month post vector delivery ~1:64, decreasing to titers below 1:20 at 6 months and 1 year post-HC-Ad delivery (**Figure 4**). Antibodies against thymidine kinase were also elevated in the sera of animals injected with the highest dose of HC-Ads at 1 year (**Figure 5**) (one-way ANOVA followed by Tukey's multiple comparison test; $p<0.05$).

Analysis of blood biochemistry and clinical laboratory parameters

In order to detect any possible systemic side effects following delivery of escalating doses of therapeutic HC-Ads in naïve Lewis rats' brain, serum biochemistry and blood cell counts were carried out on blood samples harvested at 5 days, 1 month, 6 months and 1 year post-treatment. Serum biochemical parameters indicated normal liver and renal functions; the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, urea, and creatinine were found to be within normal range as seen in age-matched naïve Lewis rats, at all time points and vector doses tested (**Table 1, Table S1-S3**). Red and white cell counts in the treated animals were also within normal ranges, indicating that Flt3L expression in the naive rat brain does not substantially alter the levels of circulating immune cells.

DISCUSSION

There is accumulating evidence that supports the use of immunotherapies in combination with standard anti-GBM treatment modalities, i.e., surgery, radiotherapy, and chemotherapy.(Sul and Fine 2010; Vauleon *et al.,* 2010; Castro *et al.,* 2011; Heimberger and Sampson 2011; Szabo and Carpentier 2011; Weller 2011) We have developed an effective immune-gene therapy strategy for treating GBM that comprises the combined use of conditional cytotoxicity (HSV1-TK plus systemic delivery of GCV),(Dewey *et al.,* 1999) and immune stimulation elicited by

expressing Flt3L within the tumor microenvironment.(Ali *et al.,* 2004; Curtin *et al.,* 2006) Our treatment strategy involves availability of tumor antigens and innate immune adjuvants like high-mobility group B1 protein (HMGB1) from dead/dying GBM cells initiated by TK (plus GCV) and uptake of the tumor antigens by the antigen presenting cells (the Dendritic cells, DCs) which are recruited into the tumor microenvironment by Flt3L.(Curtin *et al.,* 2006; Curtin *et al.,* 2009) Our therapeutic strategy also generates immunological memory that protects against recurrent brain tumors. We have demonstrated previously the efficacy of this treatment regime in multiple syngeneic intracranial GBM models in rats and mice,(Ali *et al.,* 2005; Candolfi *et al.,* 2009; Curtin *et al.,* 2009; Ghulam Muhammad *et al.,* 2009) including recurrent, mutifocal, and peripheral GBMs.(King *et al.,* 2008; King *et al.,* 2008; Ghulam Muhammad *et al.,* 2009; King *et al.,* 2011)

It is has been established that there is a high prevalence of systemic immunity against Ads in the human population(Chirmule *et al.,* 1999; Nwanegbo *et al.,* 2004; Sumida *et al.,* 2005), this could potentially interfere and compromise with the efficacy of gene therapies mediated by therapeutic Ad-based vectors.(Sun *et al.,* 2011) To address this problem, we developed HC-Ads as delivery platforms for the therapeutic transgenes, TK and Flt3L.(Candolfi *et al.,* 2006; Xiong *et al.,* 2006; Candolfi *et al.,* 2007; King *et al.,* 2008; Muhammad *et al.,* 2010) HC-Ads have numerous advantages, including their large cloning capacity, negligible toxicity, and long term transgene expression profile.(Parks *et al.,* 1996; Schiedner *et al.,* 1998; Morral *et al.,* 1999; Thomas *et al.,* 2001; Palmer and Ng 2005; Schillinger, Tsai *et al.,* 2005; Toietta*et al.,* 2005; Barcia *et al.,* 2007) We engineered two HC-Ad vectors, HC-Ad-TK that expresses TK constitutively(Candolfi *et al.,* 2006; King *et al.,* 2008; Muhammad *et al.,* 2010) and HC-Ad-

TetOn-Flt3L that expresses Flt3L under the tight control of regulatable mCMV-TetOn expression system.(Candolfi *et al.,* 2006; Xiong *et al.,* 2006; Muhammad *et al.,* 2010)

We used the CNS-1 GBM rat model to test the safety and efficacy of the engineered HC-Ads encoding Flt3L and TK delivered into the tumor mass.(Muhammad *et al.,* 2010) This HC-Ad medited anti-GBM gene therapy was also tested in GBM rat models that were immunized with intradermal injection of an empty Ad vector (does not express any transgene). These immunized rats displayed high levels of circulating neutralizing anti-Ad antibodies, thereby mimicking the likely scenario to be encountered during human clinical trials. We found that the GBM was eradicated in ~70% of rats within 30 days of treatment and the therapeutic strategy was effective even in the presence of systemic anti-adenoviral immune response. Further, long-term immunological memory protected the cured rats against tumor rechallenge, even at 1 year after treatment(Muhammad *et al.,* 2010) highlighting the therapeutic efficacy of this approach in countering recurrences of GBM.

Although our combination therapy with HC-Ad-TK and HC-Ad-TetOn-Flt3L was found to be highly effective and safe when injected to treat intracranial GBM, the tumor microenvironment is well known to possess unique structural features that are significantly different from those of the surrounding brain tissue.(Lorusso and Ruegg 2008; Pietras and Ostman 2010; Charles *et al.,* 2011) Hence, it was critical to conduct the present study to perform a thorough assessment of the brain tissue compatibility, biodistribution and transgene expression, as well as putative systemic and neurological toxicity of HC-Ads when delivered within the normal adult rat brain parenchyma. Accordingly, escalating doses of HC-Ads ($1x10^8$, $1x10^9$, or $1x10^{10}$ vp of each HC-Ads) were injected in the right striatum and groups of rats were euthanized at day 5, 1 month, 6 months or 1 year post treatment and tissue samples were harvested for

analysis. Rats belonging to the 1 month, 6 months and 1 year post-treatment groups were also subjected to a panel of neurobehavioral tests.

Histopathological analysis of the brain sections did not reveal any evidence of neurotoxicity or long-term inflammation at the lower doses tested (total HC-Ad dose of $2x10^8$ or $2x10^9$ vp). At the highest dose tested, i.e., total HC-Ad dose of $2x10^{10}$ vp, we observed loss of brain tissue and high levels of inflammation suggesting that this dose is unsafe to the naïve rat brain. Infiltrating immune cells were localized in the vicinity of the injection site in the ipsilateral hemisphere, comprising of CD8, CD68/ED1, and MHC-II immunopositive cells. Infiltrating immune cells decreased at 6 months and 1 year post HC-Ad vector injection; this may be in part attributed to the unique regulatable features engineered into the vector that expresses the transgene Flt3L, i.e., its expression is triggered only in the presence of Dox.

The biodistribution data revealed that HC-Ad vector genomes were confined to the brain injection site and did not leak to other areas of the brain, spinal cord, or peripheral organs. Interestingly, sustained levels of HC-Ad vector genomes were seen to persist in the brain injection site for up to 1 year after treatment suggesting that the cytotoxic effects of TK or the immune-stimulatory effects of Flt3L could be "reactivated" with the re-administration of GCV or DOX, respectively, if required.

Peripheral blood cell counts were found to be within the normal range and serum biochemical profiles indicated normal liver and renal functions in rats at all time points and doses tested. Analysis of amphetamine-induced total locomotor activity and total rearing activity also did not reveal any behavioral abnormalities when compared with saline treated age-matched naive controls. We detected low levels of neutralizing antibodies at one month post HC-Ad delivery, in the sera of animals injected with the highest dose of HC-Ads; antibodies against

thymidine kinase were also slightly elevated in the sera of animals injected with the highest dose of HC-Ads at 1 year post-injection. This could be due to the fact that although the immune-privileged status of brain can hamper immune responses against vectors delivered into the brain parenchyma, leakage into the intra-ventricular space allows systemic exposure, since the cerebrospinal fluid drains to the venous circulation thereby raising the chance of initiating an anti-vector immune response.

The present study, in conjunction with previously published work from our laboratory and others, highlights that GBM and other malignant brain tumors are attractive "target" diseases for implementing gene therapeutic strategies. In this respect, delivery of viral vectors in the brain is well established, both in animal models and also in human patients enrolled in clinical trials.(Immonen *et al.,* 2004; Markert *et al.,* 2009) Also the therapeutic efficacy of HC-Ad vectors expressing transgenes targeted to treat disease conditions such as brain cancer, colorectal cancer, hyperbilirubinemia, monogenic hypoalphalipoproteinemia, hemophilia, diabetic retinopathy, glycogen storage disease, hypertension and sensory neuronopathies, has been assessed in pre-clinical models showing high efficacy. (Schillinger *et al.,* 2005; Toietta *et al.,* 2005; Kiang *et al.,* 2006; McCormack *et al.,* 2006; Lamartina *et al.,* 2007; Oka *et al.,* 2007; Terashima *et al.,* 2009; Muhammad *et al.,* 2010; Puntel *et al.,* 2010; Gonzalez-Aparicio *et al.,* 2011) Thus, this comprehensive, pre-clinical safety and toxicity study will facilitate the implementation of Phase I clinical trials for GBM using high-capacity adenoviral vectors; it will also lay the foundations for the treatment of other devastating neurological diseases using HC-Ad-mediated gene therapy strategies.

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AUTHOR DISCLOSURE STATEMENT

We declare we have no conflict of interest.

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Table 1: Biochemical and hematological parameters at 5 days post treatment

Parameters	Naive		Saline		HC-TK+HC-Flt3L					
					$1x10^8$		$1x10^9$		$1x10^{10}$	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Total Protein (g/dL)	5.7	5.6-6.1	5.8	5.7-7.1	5.9	5-6.2	5.8	5.4-6.6	5.8	5.4-7.1
Albumin (g/dL)	2.9	2.8-3	2.9	2.5-3	2.8	2.4-3.2	2.9	2.6-3.1	2.8	2.6-3.3
Globulin (g/dL)	2.8	2.7-3.1	3	2.8-4.2	3	2.6-3.4	2.9	2.6-3.5	3	2.6-3.8
AST (IU/L)	87	63-422	110	67-593	95	70-203	108	51-342	153	74-572
ALT (IU/L)	51.5	46-89	41	30-161	37	28-68	36.5	20-72	34	14-104
Alkaline Phosphatase (IU/L)	309	242-361	199	102-241	233	129-395	227.5	129-376	108	70-283
Total Bilirubin (mg/dL)	0.1	0.1-0.1	0.2	0.1-0.6	0.2	0.1-0.3	0.2	0.1-0.6	0.2	0.1-0.6
BUN (mg/dL)	19.5	18-21	24	16-85	23	14-37	20.5	16-34	20	11-77
Creatinine (mg/dL)	0.3	0.2-0.3	0.5	0.4-1.5	0.4	0.3-0.7	0.4	0.3-0.6	0.5	0.2-1.5
Phosphorus (mg/dL)	8.1	7.2-11.2	13.9	9.6-22.1	11.3	8.2-18.6	13.2	1.7-20	13	8.4-19.8
Glucose (mg/dL)	86.5	47-105	162	57-310	189	109-473	179	68-432	155	19-230
Calcium (mg/dL)	10.4	9.9-10.9	10.6	8.9-11.5	10.4	9.6-40	10.4	9.5-12.2	9.8	8.6-10.8
Sodium (mmol/dL)	142.5	140-144	138	134-141	140	136-143	135.5	127-141	139	132-152
Potassium (mmol/dL)	6.3	5.4-7.2	10.6	8.1-13.4	8.3	5.6-13.9	9.3	6.7-16.9	9.2	5.4-15.6
Chloride (mmol/dL)	98	97-99	98	92-101	100	96-105	97	90-102	100	92-113
Cholesterol (mg/dL)	80	75-96	79	73-118	77	67-93	79.5	56-110	83	52-121
CPK (IU/L)	675.5	170-7517	493	193-23079	490	194-5747	490.5	88-17562	612	216-26054
WBC Count (x10^3/µl)	8.8	7.3-10	5.8	4.1-7.6	6.6	4.9-8.7	5.6	3.6-6.9	4.7	1.4-7.2
RBC Count (x10^6/µl)	8.2	7.9-8.6	9	8.2-9.5	9.2	8.6-9.9	8.8	7-9.5	9.5	7.1-11.6
Hemoglobin (g/dL)	13.7	13.3-14.6	15	14-15.8	15.4	14.2-16.3	14.7	11.7-16.2	15.7	11.9-18.2
MCV (fl)	51	49-52	55	52-62	57	56-60	59	56-62	57	53-60
MCH (pg)	16.8	16.5-17.5	16.6	15.9-17.1	16.7	15.8-17.1	16.8	16-17.1	16.6	15.8-17.3
Neutrophils (%)	13	10-15	18	9-32	13	9-22	11	8-21	12	7-45
Absolute Neutrophils (/µl)	1074	870-1500	928	369-2432	858	490-1496	633	400-1380	630	329-1353
Lymphocytes (%)	85	83-87	79	64-89	84	75-90	87.5	76-91	86	49-91
Absolute Lymphocytes (/µ/)	7611.5	6059-8300	4814	3649-5808	5544	4263-7396	4856	2964-5720	3956	686-6120
Monocytes (%)	1	1-2	1	1-3	2	1-4	1.5	1-3	2	1-5
Absolute Monocytes (/µl)	95.0	87-174	58	41-228	132	55-261	69	50-183	70	24-168
Eosinophils (%)	1	0-1	1	0-2	1	0-1	0	0-2	0	0-4
Absolute Eosinophils (/µl)	88.0	0-100	52	0-94	60	0-86	0	0-78	0	0-116
Basophils (%)	0	0-0	0	0-1	0	0-1	0	0-1	0	0-1
Absolute Basophils (/µl)	0	0-0	0	0-66	0	0-73	0	0-60	0	0-53
Platelet (x10^3/µl)	488.5	337-666	793	746-968	1014	917-1167	938.5	749-1496	953	709-1639

Note: Red Blood Cell Morphology appeared normal in all groups. Abbreviations used: AST, Aspartate Aminotransferase; ALT, Alanine Transaminase; BUN, Blood Urea Nitrogen; CPK, Creatine Phosphokinase; WBC, White Blood Cell; RBC, Red Blood Cell; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin.

FIGURE LEGENDS

Figure 1. Experimental design and neuropathological analysis following treatment with HC-Ads. (**a**) Diagram of experimental design utilized. Adult naïve Lewis rats were injected stereotactically in the right striatum with either saline or escalating doses of high-capacity adenoviral vectors, HC-Ad-TK+HC-Ad-TetON-Flt3L ($1x10^8$, $1x10^9$, or $1x10^{10}$vp of each vector); after 24h, they received Ganciclovir (GCV; 25mg/kg, i.p.), twice daily for up to 10 days. All rats were fed doxycycline mixed rodent chow (DOX Chow) *ad libitum* for up to 4 weeks starting 2 days before treatment. Groups of rats were evaluated at 5 days (short-term), 30 days (medium-term), 180 days or 365 days (long-term) for biodistribution of vector genomes, neuropathology, peripheral blood cell counts and serum biochemistry, circulating levels of anti-adenovirus neutralizing antibodies and anti-TK antibodies. Additionally, the 30d, 180d, and 365d post treatment, groups underwent neurobehavioral testing. The rats were euthanized at experiment end points and organs were harvested for processing. Neuropathological analysis was performed with the brains of rats at 5 days (**b**), 1 month, 6 months and 12 months (**c**). Neuropathology results at 1 month and 6 months are shown in **Figure S1**. The brains were processed for Nissl staining to show gross morphology, and immunocytochemistry using primary antibodies against myelin basic protein (MBP) to label oligodendrocytes and myelin sheaths, tyrosine hydroxylase (TH) to label striatal dopaminergic fibers, CD8 to detect $CD8^+$ T cells, CD68/ED1 for labeling macrophages and activated microglia, MHC-II to label MHC II^+ macrophages, microglia and immune cells, thymidine kinase (TK) or fms-like tyrosine kinase 3 ligand (Flt3L) to detect expression of therapeutic transgenes in the striatum. Scale bar: 1000µm for all but TK and Flt3L: 250µm.

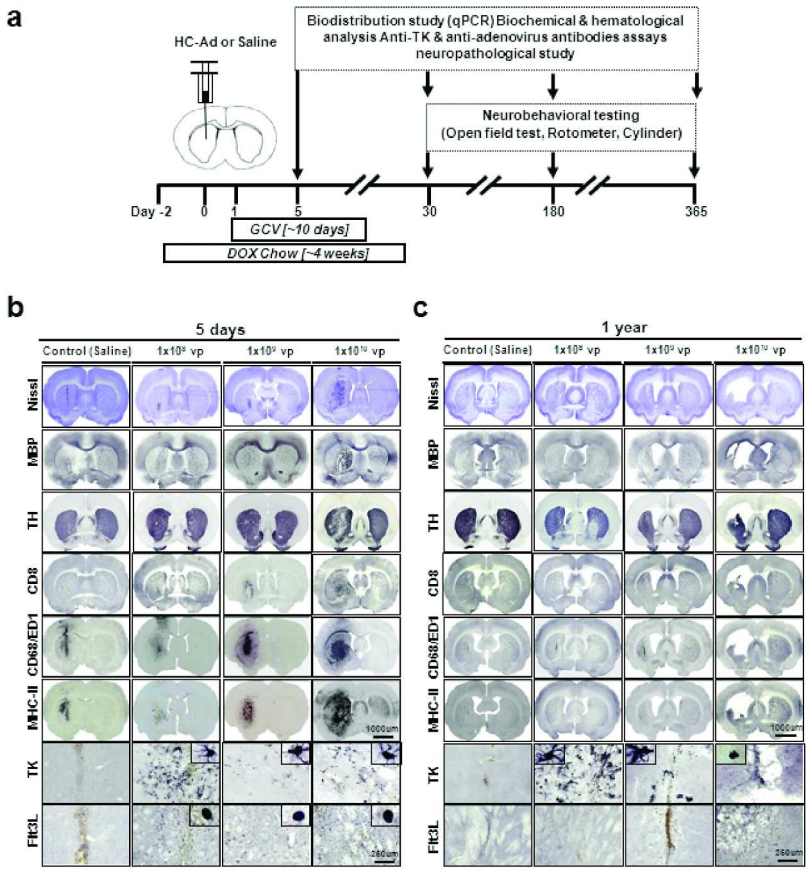
Figure 2. Behavioral assessment of rats at 1 year after treatment with high-capacity adenoviral vectors, HC-Ad-TK+HC-Ad-TetON-Flt3L. Behavioral assessment was performed before and after amphetamine treatment in the animals, at 1 month (**Figure S2**), 6 months (**Figure S3**) and 1 year (**Figure 2**) after intracranial administration of escalating doses of HC-Ad-TK+HC-Ad-TetON-Flt3L. Saline treated, age-matched rats were used as controls. (**a**) total locomotor activity; (**b**) total rearing activity; (**c**) right limb use asymmetry; (**d**) asymmetry in rotational behavior.

Figure 3. Biodistribution of HC-Ad-TK+HC-Ad-TetOn-Flt3L vector genomes following delivery into the striatum. Three escalating doses of HC-Ad-TK+HC-Ad-TetOn-Flt3L, (**a, d**) $1x10^{8}$vp, (**b, e**) $1x10^{9}$vp, and (**c, f**) $1x10^{10}$vp, were stereotactically injected into the striatum of naïve Lewis rats. Ganciclovir (25mg/kg, i.p.) was injected twice daily for up to 10 days and Dox chow was administered *ad libitum* for up to 4 weeks. Rats were euthanized at 5 days, 1 month, 6 months and 1 year end points and tissue samples (25mg) were harvested from a total of 13 sites/organs: brain injection site, brain contralateral side, brain stem, cerebellum, lumbar spinal cord, cervical lymph nodes, heart, lung, spleen, liver, kidney, small gut, and testes. Five µL of the isolated DNA from each tissue sample was used for qPCR to assess the biodistribution of the vector genomes. Vector genomes' quantification results are the average of triplicates for each DNA sample, and are shown as a ratio of vector genomes/25mg of tissue. Dotted line indicates the detection limit. Results are based on n=5 per group. The biodistribution data at 5 days (**top panel**) and 1 year (**bottom panel**) post HC-Ad delivery showed that the vector genomes' were restricted to the brain site of injection (data for 1 month and 6 months are shown in **Figure S4**).

Figure 4. Anti-adenovirus neutralizing antibodies in the serum of HC-Ad treated rats. The prevalence of anti-adenovirus neutralizing antibody in the serum of rats treated with intracranial injections of escalating doses of HC-Ad-TK+HC-Ad-TetOn-Flt3L or saline is shown. Ganciclovir (25mg/kg, i.p.) was administered twice daily for up to 10 days and Dox chow was administered *ad libitum* for up to 4 weeks. The rats were euthanized at 5 days (**a**), 1 month (**b**), 6 months (**c**), and 1 year (**d**) after treatment and sera was collected. Low levels of neutralizing antibody were detected at the highest dose $1x10^{10}$vp after 1 month of HC-Ads delivery.

Figure 5. Circulating anti-HSV1-TK antibodies in the serum of HC-Ad treated rats. Escalating doses of HC-Ad-TK+HC-Ad-TetOn-Flt3L, or saline was stereotactically injected into the striatum of Lewis rats. Ganciclovir (25mg/kg, i.p.) was administered twice daily for up to 10 days and Dox chow was administered *ad libitum* for up to 4 weeks. The rats were euthanized at 5 days (**a**), 1 month (**b**), 6 months (**c**), and 1 year (**d**) after treatment and sera was collected. Seraum from each animal was evaluated for the presence of circulating antibodies specific for thymidine kinase (TK). Circulating anti-TK antibodies were detected at the highest doses $1x10^{10}$vp after 1 year of the HC-Ads delivery into the naïve rat brain. The titer of anti-TK antibodies is represented as the % change in signal intensity from sera incubated with cell lysates from Ad-TK infected cells when compared to mock infected cells. Samples were considered positive when the % change was ≥25%; this threshold is indicated by the dashed line.

Figure 1



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antibodies against myelin basic protein (MBP) to label oligodendrocytes and myelin sheaths, tyrosine hydroxylase (TH) to label striatal dopaminergic fibers, CD8 to detect CD8+ T cells, CD68/ED1 for labeling macrophages and activated microglia, MHC-II to label MHC II+ macrophages, microglia and immune cells, thymidine kinase (TK) or fms-like tyrosine kinase 3 ligand (Flt3L) to detect expression of therapeutic transgenes in the striatum. Scale bar: 1000μm for all but TK and Flt3L: 250μm.
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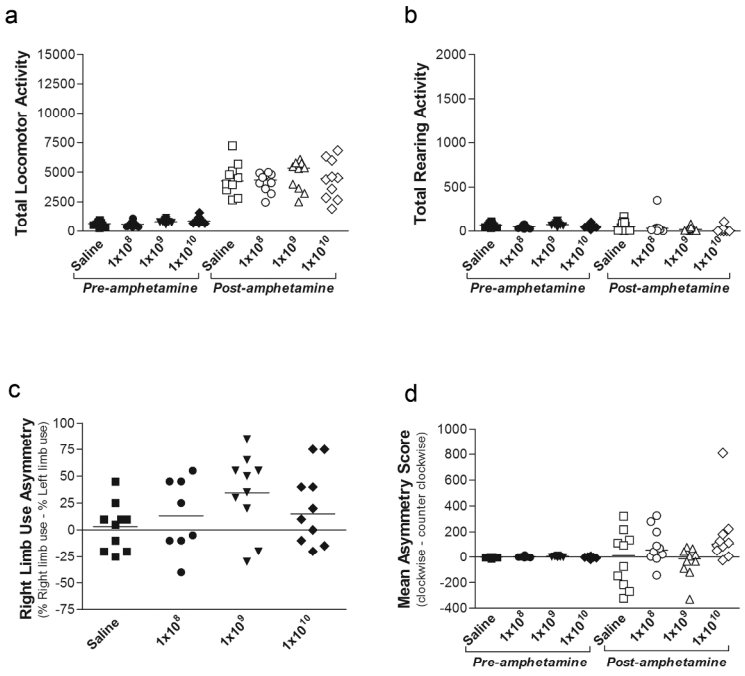
Figure 2



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215x279mm (300 x 300 DPI)

Figure 3

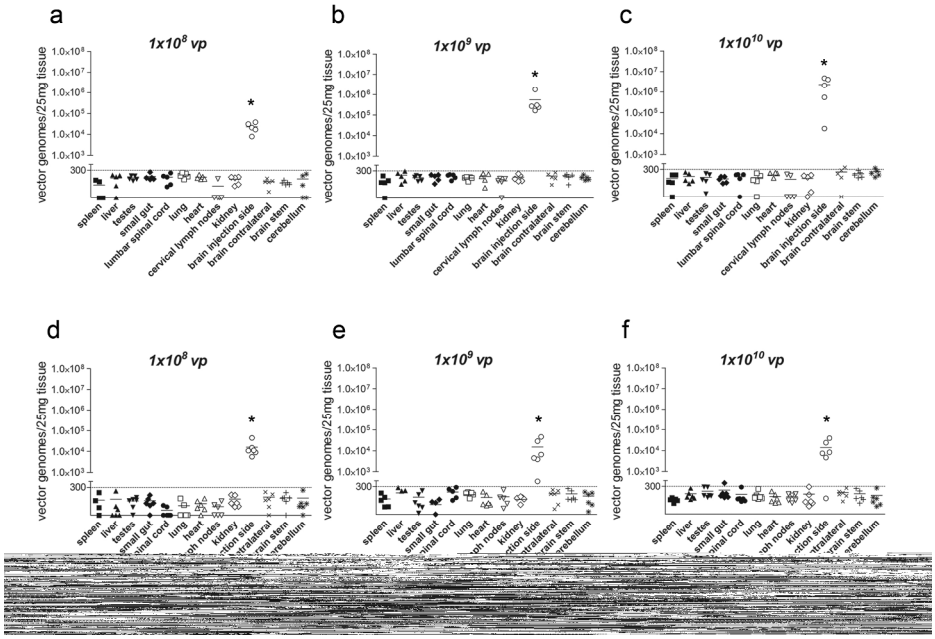


Figure 3. Biodistribution of HC-Ad-TK+HC-Ad-TetOn-Flt3L vector genomes following delivery into the striatum. Three escalating doses of HC-Ad-TK+HC-Ad-TetOn-Flt3L, (a, d) 1x108vp, (b, e) 1x109vp, and (c, f) 1x1010vp, were stereotactically injected into the striatum of naïve Lewis rats. Ganciclovir (25mg/kg, i.p.) was injected twice daily for up to 10 days and Dox chow was administered ad libitum for up to 4 weeks. Rats were euthanized at 5 days, 1 month, 6 months and 1 year end points and tissue samples (25mg) were harvested from a total of 13 sites/organs: brain injection site, brain contralateral side, brain stem, cerebellum, lumbar spinal cord, cervical lymph nodes, heart, lung, spleen, liver, kidney, small gut, and testes. Five µL of the isolated DNA from each tissue sample was used for qPCR to assess the biodistribution of the vector genomes. Vector genomes' quantification results are the average of triplicates for each DNA sample, and are shown as a ratio of vector genomes/25mg of tissue. Dotted line indicates the detection limit. Results are based on n=5 per group. The biodistribution data at 5 days (top panel) and 1 year (bottom panel) post HC-Ad delivery showed that the vector genomes' were restricted to the brain site of injection (data for 1 month and 6 months are shown in Figure S4).

215x279mm (300 x 300 DPI)

Figure 4

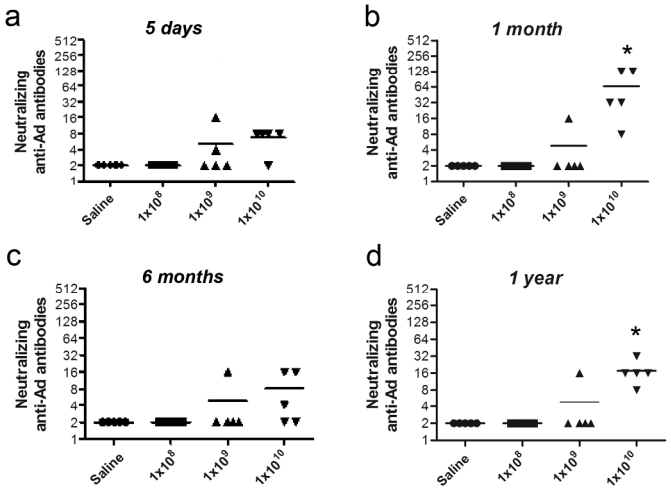


Figure 4. Anti-adenovirus neutralizing antibodies in the serum of HC-Ad treated rats. The prevalence of anti-adenovirus neutralizing antibody in the serum of rats treated with intracranial injections of escalating doses of HC-Ad-TK+HC-Ad-TetOn-Flt3L or saline is shown. Ganciclovir (25mg/kg, i.p.) was administered twice daily for up to 10 days and Dox chow was administered ad libitum for up to 4 weeks. The rats were euthanized at 5 days (a), 1 month (b), 6 months (c), and 1 year (d) after treatment and sera was collected. Low levels of neutralizing antibody were detected at the highest dose 1x1010vp after 1 month of HC-Ads delivery.
190x254mm (300 x 300 DPI)

Figure 5

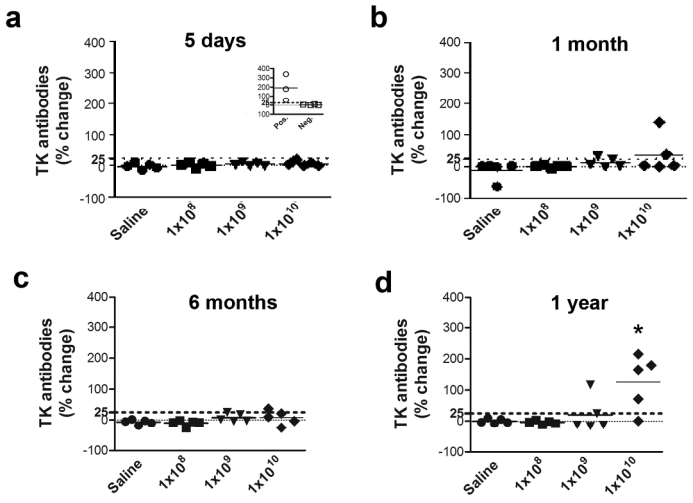


Figure 5. Circulating anti-HSV1-TK antibodies in the serum of HC-Ad treated rats. Escalating doses of HC-Ad-TK+HC-Ad-TetOn-Flt3L, or saline was stereotactically injected into the striatum of Lewis rats. Ganciclovir (25mg/kg, i.p.) was administered twice daily for up to 10 days and Dox chow was administered ad libitum for up to 4 weeks. The rats were euthanized at 5 days (a), 1 month (b), 6 months (c), and 1 year (d) after treatment and sera was collected. Seraum from each animal was evaluated for the presence of circulating antibodies specific for thymidine kinase (TK). Circulating anti-TK antibodies were detected at the highest doses 1x1010vp after 1 year of the HC-Ads delivery into the naïve rat brain. The titer of anti-TK antibodies is represented as the % change in signal intensity from sera incubated with cell lysates from Ad-TK infected cells when compared to mock infected cells. Samples were considered positive when the % change was ≥25%; this threshold is indicated by the dashed line.
190x254mm (300 x 300 DPI)

SUPPLEMENTARY MATERIALS LEGEND

Figure S1. Neuropathology at 1 month and 6 months following treatment with HC-Ads. Naïve Lewis rats received injections of saline or escalating doses of high-capacity adenoviral vectors HC-Ad-TK and HC-Ad-TetOn-Flt3L, $1x10^8$, $1x10^9$, or $1x10^{10}$vp each, in the right striatum. Ganciclovir and Doxycycline were co-administered. One month (**left panel**) or 6 months (**right panel**) later, the rats were euthanized and the brains were processed for Nissl staining to show gross morphology, and immunocytochemistry with primary antibodies against myelin basic protein (MBP) to label oligodendrocytes and myelin sheaths, tyrosine hydroxylase (TH) to label striatal dopaminergic fibers, CD8 to detect $CD8^+$ T cells, CD68/ED1 for labeling macrophages and activated microglia, MHC-II to label MHC II^+ macrophages, microglia and immune cells, thymidine kinase (TK) or fms-like tyrosine kinase 3 ligand (Flt3L) to detect expression of therapeutic transgenes in the striatum. Scale bar: 1000µm for all but TK and Flt3L: 250µm.

Figure S2. Behavioral assessment of rats at 1 month after treatment with high-capacity adenoviral vectors. Behavioral assessment was performed before and after amphetamine treatment in the animals at 1 month after intracranial administration of escalating doses of HC-Ad-TK+HC-Ad-TetON-Flt3L. Saline treated, age-matched rats were used as controls. (**a**) total locomotor activity; (**b**) total rearing activity; (**c**) right limb use asymmetry; (**d**) asymmetry in rotational behavior.

Figure S3. Behavioral assessment of rats at 6 months after treatment with high-capacity adenoviral vectors. Behavioral assessment was performed before and after amphetamine treatment in the animals at 6 months after intracranial administration of escalating doses of HC-Ad-TK+HC-Ad-TetON-Flt3L. Saline treated, age-matched rats were used as controls. (**a**) total locomotor activity; (**b**) total rearing activity; (**c**) right limb use asymmetry; (**d**) asymmetry in rotational behavior.

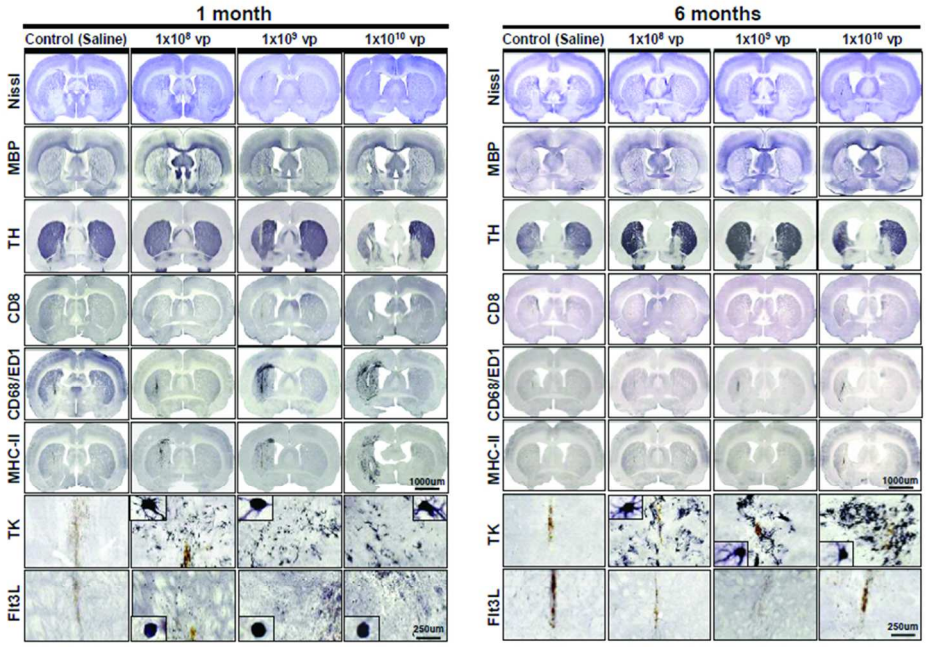
Figure S4. Biodistribution of HC-Ad-TK+HC-Ad-TetOn-Flt3L vector genomes at 1 month and 6 months post injection. Three escalating doses of HC-Ad-TK+HC-Ad-TetOn-Flt3L, (**a, d**) $1x10^{8}$vp, (**b, e**) $1x10^{9}$vp, and (**c, f**) $1x10^{10}$vp, were stereotactically injected into the striatum of naïve Lewis rats. Ganciclovir (25mg/kg, i.p.) was injected twice daily for 10 days and Dox chow was administered *ad libitum* for 4 weeks. Groups of rats were euthanized at 1 month or 6 months post vector treatment Tissue samples (25mg) were harvested from a total of 13 sites/organs: brain injection site, brain contralateral side, brain stem, cerebellum, lumbar spinal cord, cervical lymph nodes, heart, lung, spleen, liver, kidney, small gut, and testes. Five µL of the isolated DNA from each tissue sample was used for qPCR to assess the biodistribution of vector genomes. Vector genomes' quantification results are the average of triplicates for each DNA sample, and are shown as a ratio of vector genomes/25mg of tissue. Dotted line indicates detection limit. Results are based on n=5 per group. The biodistribution study at 1 month (**top panel**) and 6 months (**bottom panel**) post HC-Ad delivery showed that the vector genomes were restricted to the brain ipsilateral to the site of injection.

Table S1. Biochemical and hematological parameters at 1 month following treatment with escalating doses of HC-Ads. At 1 month post treatment, blood was collected during euthanasia and a comprehensive panel of serum chemistry and hematologic parameters was performed by Antech Diagnostics (Irvine, CA). The median, minimum, and maximum values for each parameter are shown.

Table S2. Biochemical and hematological parameters at 6 months after treatment with escalating doses of HC-Ads. At 6 months post treatment, blood was collected during euthanasia and a comprehensive panel of serum chemistry and hematologic parameters was performed by Antech Diagnostics (Irvine, CA). The median, minimum, and maximum values for each parameter are shown.

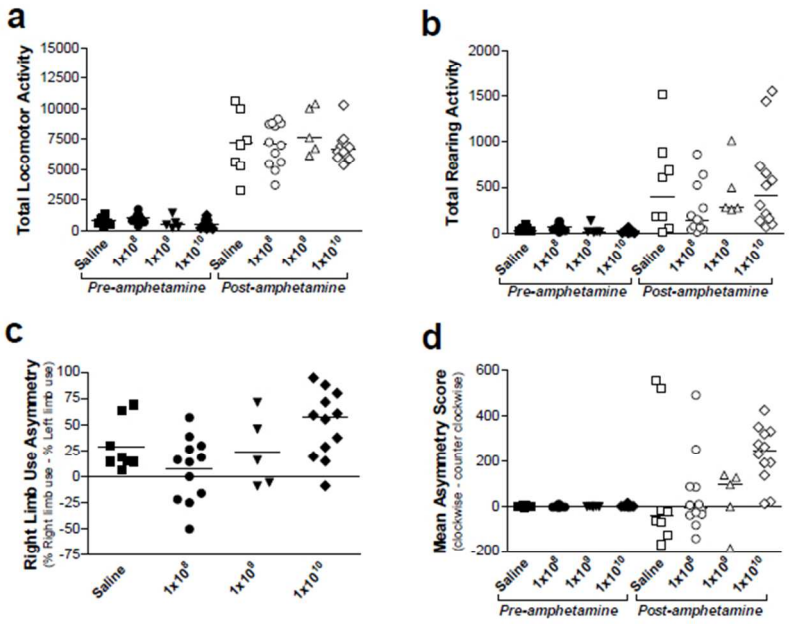
Table S3. Biochemical and hematological parameters at 1 year following treatment with escalating doses of HC-Ads. At 1 year post treatment, blood was collected during euthanasia and a comprehensive panel of serum chemistry and hematologic parameters was performed by Antech Diagnostics (Irvine, CA). The median, minimum, and maximum values for each parameter are shown.

Suppl. Figure S1. Neuropathological analysis at 1 month and 6 months following treatment with HC-Ads



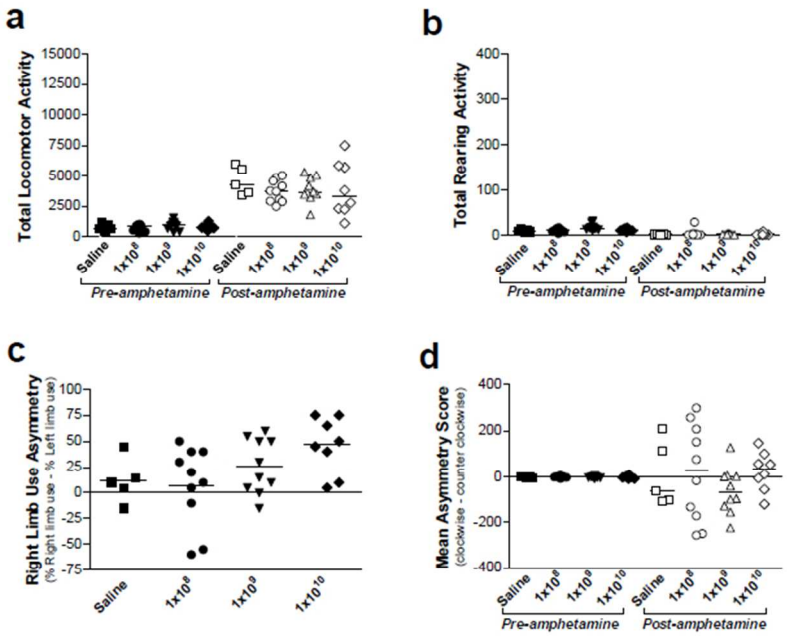
Supplementary Figure S1. Neuropathology at 1 month and 6 months following treatment with HC-Ads. Naïve Lewis rats received injections of saline or escalating doses of high-capacity adenoviral vectors HC-Ad-TK and HC-Ad-TetOn-Flt3L, 1x108, 1x109, or 1x1010vp each, in the right striatum. Ganciclovir and Doxycycline were co-administered. One month (left panel) or 6 months (right panel) later, the rats were euthanized and the brains were processed for Nissl staining to show gross morphology, and immunocytochemistry with primary antibodies against myelin basic protein (MBP) to label oligodendrocytes and myelin sheaths, tyrosine hydroxylase (TH) to label striatal dopaminergic fibers, CD8 to detect CD8+ T cells, CD68/ED1 for labeling macrophages and activated microglia, MHC-II to label MHC II+ macrophages, microglia and immune cells, thymidine kinase (TK) or fms-like tyrosine kinase 3 ligand (Flt3L) to detect expression of therapeutic transgenes in the striatum. Scale bar: 1000µm for all but TK and Flt3L: 250µm.
301x239mm (72 x 72 DPI)

Suppl. Figure S2. Behavioral assessment of rats at 1 month following treatment with high-capacity adenoviral vectors, HC-Ad-TK+HC-Ad-TetON-Flt3L



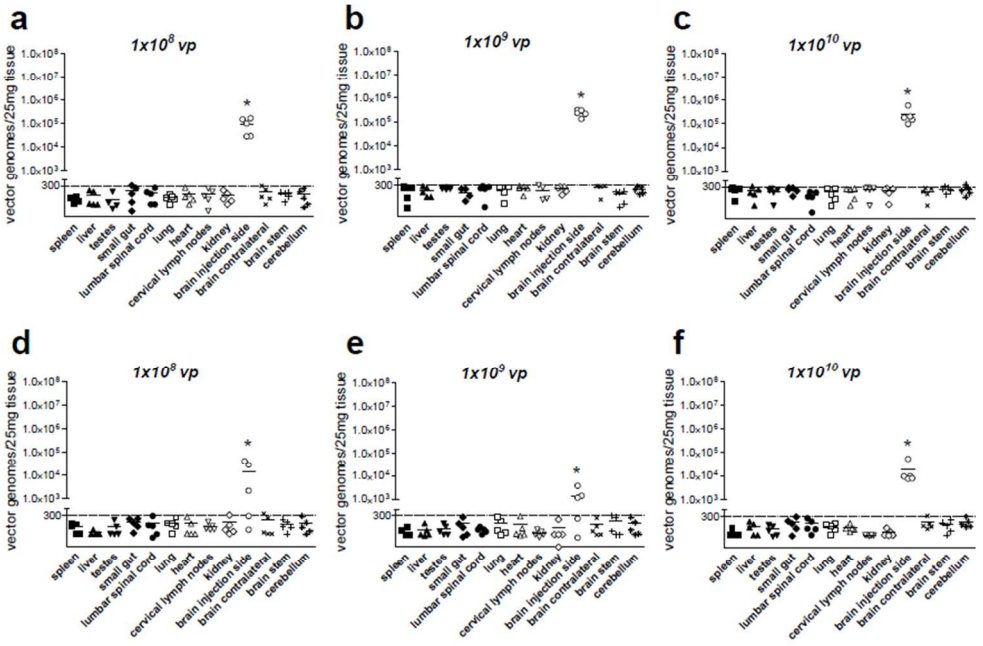
Supplementary Figure S2. Behavioral assessment of rats at 1 month after treatment with high-capacity adenoviral vectors. Behavioral assessment was performed before and after amphetamine treatment in the animals at 1 month after intracranial administration of escalating doses of HC-Ad-TK+HC-Ad-TetON-Flt3L. Saline treated, age-matched rats were used as controls. (a) total locomotor activity; (b) total rearing activity; (c) right limb use asymmetry; (d) asymmetry in rotational behavior.
271x210mm (72 x 72 DPI)

Suppl. Figure S3. Behavioral assessment of rats at 6 months following treatment with high-capacity adenoviral vectors, HC-Ad-TK+HC-Ad-TetON-Flt3L



Supplementary Figure S3. Behavioral assessment of rats at 6 months after treatment with high-capacity adenoviral vectors. Behavioral assessment was performed before and after amphetamine treatment in the animals at 6 months after intracranial administration of escalating doses of HC-Ad-TK+HC-Ad-TetON-Flt3L. Saline treated, age-matched rats were used as controls. (a) total locomotor activity; (b) total rearing activity; (c) right limb use asymmetry; (d) asymmetry in rotational behavior.
272x214mm (72 x 72 DPI)

Suppl. Figure S4. Biodistribution of HC-Ad-TK+HC-Ad-TetOn-Flt3L vector genomes following treatment at 1 month (top panel) and 6 months (bottom panel)



Supplementary Figure S4. Biodistribution of HC-Ad-TK+HC-Ad-TetOn-Flt3L vector genomes at 1 month and 6 months post injection. Three escalating doses of HC-Ad-TK+HC-Ad-TetOn-Flt3L, (a, d) 1x108vp, (b, e) 1x109vp, and (c, f) 1x1010vp, were stereotactically injected into the striatum of naïve Lewis rats. Ganciclovir (25mg/kg, i.p.) was injected twice daily for 10 days and Dox chow was administered ad libitum for 4 weeks. Groups of rats were euthanized at 1 month or 6 months post vector treatment Tissue samples (25mg) were harvested from a total of 13 sites/organs: brain injection site, brain contralateral side, brain stem, cerebellum, lumbar spinal cord, cervical lymph nodes, heart, lung, spleen, liver, kidney, small gut, and testes. Five µL of the isolated DNA from each tissue sample was used for qPCR to assess the biodistribution of vector genomes. Vector genomes' quantification results are the average of triplicates for each DNA sample, and are shown as a ratio of vector genomes/25mg of tissue. Dotted line indicates detection limit. Results are based on n=5 per group. The biodistribution study at 1 month (top panel) and 6 months (bottom panel) post HC-Ad delivery showed that the vector genomes were restricted to the brain ipsilateral to the site of injection.
316x243mm (72 x 72 DPI)

Suppl. Table S1: Biochemical and hematological parameters at 30 days post treatment

Parameters	Naive		Saline		HC-TK+HC-Flt3L					
					$1x10^8$		$1x10^9$		$1x10^{10}$	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Total Protein (g/dL)	6	5.6-6.3	6	5.6-6.6	5.7	5.4-5.9	5.9	5.5-6.4	5.8	5.5-6.6
Albumin (g/dL)	3.3	2.9-3.5	3.1	2.9-3.4	2.9	2.8-3.1	3	2.8-3.2	3	2.9-3.3
Globulin (g/dL)	2.7	2.6-2.8	2.9	2.7-3.3	2.8	2.6-3	2.8	2.6-3.4	2.8	2.6-3.4
AST (IU/L)	231	79-333	103	64-207	82	57-133	72	57-97	72	61-283
ALT (IU/L)	68.5	38-80	44	35-54	34	24-50	26.5	24-34	33	29-168
Alkaline Phosphatase (IU/L)	332.5	305-362	220.5	142-254	247.5	145-323	214	177-273	261	220-328
Total Bilirubin (mg/dL)	0.1	0-0.1	0.1	0.1-0.1	0.1	0.1-0.1	0	0-0.1	0.1	0-0.1
BUN (mg/dL)	23	20-33	22	16-30	21	16-25	21	19-26	22	17-24
Creatinine (mg/dL)	0.5	0.4-0.7	0.5	0.3-0.7	0.4	0.2-0.7	0.5	0.4-0.6	0.4	0.1-0.5
Phosphorus (mg/dL)	18.5	9.5-25	14.2	9.6-25.6	11.3	8.1-22.3	14.8	11.2-23.4	11.9	7.8-18.8
Glucose (mg/dL)	143	106-194	152.5	99-454	213	103-349	177	94-384	148	36-510
Calcium (mg/dL)	9	8.4-9.5	10.1	9.4-11.2	10.5	10-12	10.7	10.2-11.9	10.3	9.2-11.7
Sodium (mmol/dL)	135	131-142	145	139-154	141.5	138-146	144.5	141-150	144	142-146
Potassium (mmol/dL)	20.6	9-27.5	9.5	7.3-12	7.9	6.1-9.4	7.9	6.7-10.1	8.3	6.8-11.5
Chloride (mmol/dL)	101	96-102	101.5	98-103	100	96-103	100.5	97-104	102	97-105
Cholesterol (mg/dL)	110.5	76-119	83.5	76-105	80.5	71-90	84.5	76-101	83	74-97
CPK (IU/L)	8407.5	524-13263	501.5	153-2665	254	117-3886	234	142-512	203	129-6903
WBC Count (x10^3/µl)	4.8	3.8-6.6	5.6	4.6-6.6	6.1	4.5-7.3	5.7	4.1-7.4	6.1	3.9-7.6
RBC Count (x10^6/µl)	8.6	7.7-9.1	9.2	8.4-9.9	8.3	7.7-9.1	8.6	7.1-9.1	8.5	7.1-9.5
Hemoglobin (g/dL)	13.8	12.7-14.7	14.8	13.7-15.6	13.6	12.7-15.1	14	11.5-15.1	13.5	12.1-14.7
MCV (fl)	52.5	51-55	53	52-55	55	52-62	57	55-59	54	53-58
MCH (pg)	16.2	15.9-16.5	16.3	15.9-16.7	16.5	16-17.5	16.4	15.9-16.7	15.9	15.5-17.1
Neutrophils (%)	18	16-30	13.5	6-17	14	8-17	11.5	9-17	15	11-16
Absolute Neutrophils (/µl)	936	646-1500	717.5	342-1122	823	540-1095	663	396-1036	870	468-1216
Lymphocytes (%)	79	66-82	85	81-93	84	81-90	85.5	80-88	84	83-87
Absolute Lymphocytes (/µl)	3476	3002-5412	4844	3864-5655	4990.5	3690-6390	4724	3608-6216	5307	3393-6612
Monocytes (%)	1	1-1	1	1-2	1	1-4	1	1-3	1	1-2
Absolute Monocytes (/µl)	48	38-66	58.5	46-130	69.5	45-180	61	41-192	76	39-142
Eosinophils (%)	1	1-2	0	0-1	0	0-1	0	0-1	0	0-1
Absolute Eosinophils (/µl)	62	42-100	0	0-56	0	0-71	0	0-56	0	0-65
Basophils (%)	1	1-1	0	0-1	0	0-1	1	0-1	0	0
Absolute Basophils (/µl)	44	38-50	0	0-66	0	0-54	56.5	0-74	0	0
Platelet (x10^3/µl)	695	601-725	713	593-763	666	508-954	663.5	601-728	602	525-702

Note: Red Blood Cell Morphology appeared normal in all groups. Abbreviations used: AST, Aspartate Aminotransferase; ALT, Alanine Transaminase; BUN, Blood Urea Nitrogen; CPK, Creatine Phosphokinase; WBC, White Blood Cell; RBC, Red Blood Cell; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin.

Suppl. Table S2: Biochemical and hematological parameters at 6 months post treatment

Parameters	Naive		Saline		HC-TK+HC-Flt3L					
					$1x10^8$		$1x10^9$		$1x10^{10}$	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Total Protein (g/dL)	6.1	5.8-6.1	6.4	6-7	6.5	6-7	6.3	6-6.5	6.2	5.7-6.7
Albumin (g/dL)	3	2.9-3.1	3.3	3.1-3.6	3.3	3-3.4	3.1	3-3.3	3.2	3-3.3
Globulin (g/dL)	3	2.9-3.2	3.1	2.8-3.4	3.2	3-3.7	3.1	2.9-3.1	3	2.7-3.4
AST (IU/L)	79.5	67-174	81	56-133	80.5	55-133	73	63-150	64	59-310
ALT (IU/L)	42	38-50	39	32-47	33	30-39	33	28-43	38	25-72
Alkaline Phosphatase (IU/L)	214.5	183-223	182	146-232	176.5	82-236	183	90-212	176.5	147-189
Total Bilirubin (mg/dL)	0.1	0-0.1	0.1	0.1-0.1	0.1	0-0.1	0.1	0.1-0.1	0.1	0.1-0.1
BUN (mg/dL)	21.5	20-23	20	16-25	20	17-23	48.5	45-51	18.5	16-19
Creatinine (mg/dL)	0.4	0.3-0.7	0.5	0.4-0.5	0.4	0.3-0.5	0.3	0.3-0.5	0.4	0.3-0.5
Phosphorus (mg/dL)	8	6.3-10	10.4	7.8-17.1	8.7	7.1-14.5	7.8	6.4-10.8	7.7	5.7-11.8
Glucose (mg/dL)	163.5	145-293	146	110-202	133	46-148	154	131-223	152.5	116-400
Calcium (mg/dL)	10	9.7-10.3	10.2	10.1-11.7	10.4	10-10.6	10.3	9.6-10.8	10.4	9.8-11.5
Sodium (mmol/dL)	140	139-141	143	141-145	143	141-148	141	136-142	140.5	139-145
Potassium (mmol/dL)	10	8.9-11.3	8.7	6.7-10.7	7.4	6.6-10.7	9.1	7.7-11	8.7	7.6-10.7
Chloride (mmol/dL)	100	99-103	102	98-106	102.5	100-105	102	99-104	102.5	99-105
Cholesterol (mg/dL)	87.5	83-90	111	89-118	99	83-113	93.5	76-117	95.5	84-117
CPK (IU/L)	259	165-7582	413	191-890	464.5	160-1196	369	259-2550	242	144-7463
WBC Count (x10^3/µl)	4.9	4.1-6.1	3.8	2.9-4.6	4.5	3.1-6.8	4.7	4.4-6	5.2	3.9-7.7
RBC Count (x10^6/µl)	9	8.1-9.4	9.1	7.3-9.9	8.7	8.4-9.6	9.2	8.7-9.6	9	8.7-10
Hemoglobin (g/dL)	14.8	13.5-15.5	14.8	12.5-16.5	14.3	13.2-15.7	14.9	13.9-15.7	14.8	13.7-15.9
MCV (fl)	51	50-53	54	49-57	53	51-54	53	51-55	52	51-55
MCH (pg)	16.6	16.3-16.7	16.6	15.6-17	16.1	6.9-16.7	16.2	16-16.9	16.2	6-16.8
Neutrophils (%)	17	12-19	22	16-29	23	17-29	22	19-30	18	15-22
Absolute Neutrophils (/µl)	807.5	656-1159	839	638-1242	918	585-1392	1056	748-1350	940	594-1206
Lymphocytes (%)	80.5	79-86	76	68-81	73	68-80	73	65-77	79	4-82
Absolute Lymphocytes (/µ/)	3904	3321-4819	2808	808-3432	3374	2387-5372	3512	2925-4620	4129	3003-6237
Monocytes (%)	1	1-2	2	1-4	2	2-3	3	2-4	3	2-3
Absolute Monocytes (/µl)	52.5	47-82	76	29-184	90	62-204	157.5	88-225	142	100-231
Eosinophils (%)	0	0-1	1	0-1	1	0-1	1	1-1	1	0-1
Absolute Eosinophils (/µl)	0	0-49	36	0-46	44.5	0-68	46.5	44-102	45.5	0-77
Basophils (%)	1	1-1	0	0-0	0	0-1	0	0-0	0	0-0
Absolute Basophils (/µl)	48.5	41-61	0	0-0	0	0-45	0	0-0	0	0-0
Platelet (x10^3/µl)	594.5	515-618	596	491-672	621	517-788	614	494-683	576	418-699

Note: Red Blood Cell Morphology appeared normal in all groups. Abbreviations used: AST, Aspartate Aminotransferase; ALT, Alanine Transaminase; BUN, Blood Urea Nitrogen; CPK, Creatine Phosphokinase; WBC, White Blood Cell; RBC, Red Blood Cell; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin.

Suppl. Table S3: Biochemical and hematological parameters at 1 year post treatment

Parameters	Naive		Saline		HC-TK+HC-Flt3L 1x10^8		1x10^9		1x10^{10}	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Total Protein (g/dL)	6.2	5.9-6.3	6.4	6-6.7	6.2	6-6.5	6.6	6.3-7.2	6.3	6-6.7
Albumin (g/dL)	3.1	2.9-3.2	3.3	3.1-3.5	3.2	3-3.3	3.4	3.3-3.6	3.3	3.2-3.5
Globulin (g/dL)	3.1	2.9-3.2	3.1	2.8-3.2	3	2.8-3.2	3.3	2.8-3.6	3	2.8-3.2
AST (IU/L)	67	62-77	82.5	59-106	68	64-98	83.5	61-245	71.5	57-107
ALT (IU/L)	40	37-52	40	36-47	34	29-37	37	28-67	36	30-49
Alkaline Phosphatase (IU/L)	213	191-268	169	146-207	142.5	113-168	177	144-261	163.5	129-217
Total Bilirubin (mg/dL)	0.1	0.1-0.1	0.1	0.1-0.1	0.1	0.1-0.1	0.1	0.1-0.6	0.1	0.1-0.5
BUN (mg/dL)	20.5	18-23	19.5	16-22	16	13-18	19	12-22	16	15-19
Creatinine (mg/dL)	0.3	0.3-0.4	0.4	0.4-0.5	0.4	0.3-0.5	0.4	0.3-0.5	0.4	0.3-0.4
Phosphorus (mg/dL)	6.8	6-8	8.6	7.8-12.7	7.6	6.2-9.6	9.3	4.7-17.8	7.8	5.8-11.2
Glucose (mg/dL)	187.5	143-206	161.5	140-202	177.5	145-260	131.5	80-228	130.5	85-188
Calcium (mg/dL)	10.2	9.8-10.2	10.2	10.1-10.2	9.9	9.7-10.7	9.2	8.2-11.3	9.8	8.6-10.3
Sodium (mmol/dL)	140.5	140-143	144	141-145	143	142-145	146	144-151	146.5	143-153
Potassium (mmol/dL)	8.9	7.7-9.4	9.8	7.6-10	9.5	7.8-10.6	8.5	7.8-10.3	8	7-10.3
Chloride (mmol/dL)	103	100-103	103	101-106	105	103-107	103.5	99-108	104.5	101-109
Cholesterol (mg/dL)	100.5	93-104	112.5	107-118	116	105-127	126.5	110-139	110.5	98-143
CPK (IU/L)	175	149-858	345	191-547	244.5	157-623	196	106-2025	144	92-528
WBC Count (x10^3/µl)	3.8	2.7-4.5	3.5	2.9-3.9	3.5	2.2-5.2	3.7	2.7-5	3.4	2.6-4.2
RBC Count (x10^6/µl)	8.7	7.8-8.9	8.9	8.8-9.9	8.9	8.2-9.2	8.8	7.7-9.5	8.7	7.6-9.5
Hemoglobin (g/dL)	14.1	13.3-14.4	14.9	14.7-16.5	14.8	13.8-15.6	14.6	12.8-15.5	14.6	12.8-15.8
MCV (fl)	51.5	51-54	55	52-55	54	53-56	51.5	49-55	51	51-54
MCH (pg)	16.3	15.8-17.1	16.7	16.5-16.8	16.8	16.6-17	16.5	16.1-16.9	16.6	16.2-17.2
Neutrophils (%)	16.5	14-22	23	20-29	26.5	21-29	31.5	10-42	30	20-48
Absolute Neutrophils (/µl)	629	378-880	838	638-1014	971.5	462-1352	1129.5	270-2016	1044.5	520-1632
Lymphocytes (%)	81.5	75-83	74.5	69-78	71.5	68-76	64	53-87	66	44-79
Absolute Lymphocytes (/µl)	3035.5	2241-3690	2660	2204-2850	2442	1672-3692	2347	2112-2900	2175	1496-2829
Monocytes (%)	1.5	1-2	2	1-2	2	1-3	3	2-6	3	1-5
Absolute Monocytes (/µl)	64	38-106	70	29-78	62	44-156	105	54-234	99	29-170
Eosinophils (%)	1	1-1	0	0-1	1	0-1	1	0-3	1	0-2
Absolute Eosinophils (/µl)	37.5	27-45	0	0-38	26	0-37	33.5	0-123	35.5	0-70
Basophils (%)	0	0-0	0	0-0	0	0-0	0	0-1	0.5	0-1
Absolute Basophils (/µl)	0	0-0	0	0-0	0	0-0	0	0-41	15	0-42
Platelet (x10^3/µl)	525.5	516-582	597.5	548-669	572.5	501-617	517	392-774	581	361-740

Note: Red Blood Cell Morphology appeared normal in all groups. Abbreviations used: AST, Aspartate Aminotransferase; ALT, Alanine Transaminase; BUN, Blood Urea Nitrogen; CPK, Creatine Phosphokinase; WBC, White Blood Cell; RBC, Red Blood Cell; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin.