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Journal

Gene Therapy, 22(2)

ISSN

0969-7128

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Publication Date

2015-02-01

DOI

10.1038/gt.2014.96

Peer reviewed

Published in final edited form as:

Gene Ther. 2015 February ; 22(2): 202–208. doi:10.1038/gt.2014.96.

High cerebrospinal fluid levels of interleukin-10 attained by AAV in dogs

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Abstract

Intrathecal (IT) gene transfer using adeno-associated virus (AAV) may be clinically promising as a treatment for chronic pain if it can produce sufficiently high levels of a transgene product in the cerebrospinal fluid (CSF). While this strategy was developed in rodents, no studies investigating CSF levels of an analgesic or anti-allodynic protein delivered by IT AAV have been performed in large animals. Interleukin-10 (IL-10) is an anti-allodynic cytokine, for which target therapeutic levels have been established in rats. The present study tested IT AAV8 encoding either human IL-10 (hIL-10) or enhanced green fluorescent protein (EGFP) in a dog model of IT drug delivery. AAV8/hIL-10 at a dose of 3.5×10^{12} genome copies induced high hIL-10 levels in the CSF, exceeding the target concentration previously found to be anti-allodynic in rodents by >1000-fold. AAV8/EGFP targeted the primary sensory and motor neurons and the meninges. hIL-10, a xenogeneic protein in dogs, induced anti-hIL-10 antibodies detectable in the dogs' CSF and serum. The high hIL-10 levels demonstrate the efficacy of AAV for delivery of secreted transgenes into the IT space of large animals suggesting a strong case for further development towards clinical testing.

Keywords

Pain; interleukin-10; dogs; adeno-associated virus; intrathecal; cerebrospinal fluid

INTRODUCTION

Chronic pain is among the major health problems affecting more than 116 million Americans and costing more than $560 billion per year in the US alone[1]. Currently available treatments fail in a substantial number of patients, which has prompted the search for novel analgesic drugs. One of the promising agents is interleukin-10 (IL-10) – an anti-

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Supplementary information is available at Gene Therapy's website.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

inflammatory cytokine with established anti-allodynic and anti-hyperalgesic properties. IL-10 counteracts the effect of pro-inflammatory cytokines, such as IL-1β and TNF-α. Upregulation of these cytokines results in glial activation, which has been identified as a critical process in the pathogenesis of chronic neuropathic pain[2]. When tested in a rodent pain model, intrathecal (IT) administration of recombinant IL-10 has been shown to inhibit glial activation and increase the threshold of mechanical allodynia[3,4].

Direct delivery of recombinant IL-10, however, has been found to be impractical because IL-10 does not cross the blood brain barrier[5] and, when given IT, its half-life of elimination in the CSF is 2 hours[6]. Potentially therapeutic IL-10 concentration could therefore only be achieved by repeated IT delivery of the recombinant protein. The need for invasive administration (either by repeated bolus or infusion), together with generally high manufacturing costs of recombinant proteins, makes simple use of the recombinant IL-10 to treat chronic pain not feasible in the clinical setting. As a result, IT administration of IL-10 has never been tested in humans.

To overcome the limitations associated with IT administration of the recombinant IL-10, gene therapy has been suggested as an alternative strategy. Delivery of the IL-10 gene by several independent vector systems resulted in an anti-allodynic response in rodent models. In addition, the studies directly measuring the IL-10 levels in the CSF found that the presence of IL-10 was closely linked to the duration of the therapeutic effect. Plasmid DNA or adenovirus mediated gene transfer led to initially robust but eventually transient expression of IL-10[6–9]. In contrast, use of adeno-associated virus (AAV)[10] or herpes simplex virus (HSV)[11,12] led to stable IL-10 production over the entire survival period, which extended up to 3 months in the case of AAV. Despite considerable variation in the achieved CSF levels of IL-10, these studies established a concentration range needed for therapeutic response and suggested that even concentrations as much as 30-fold higher might lead to no overt adverse reaction to the treatment in the rat model[9].

It has not been determined, however, whether any of these approaches can lead to a similarly high IL10 level in a large animal model – the critical step towards clinical translation. The need for large animal experiments has been highlighted by several recent studies that investigated AAV gene transfer to the central nervous system (CNS)[13–16]. These studies reported wider distribution and more prominent immune response against the vector and xenogeneic transgene products in large animals compared to rodents. Up to date, no gene therapy vector has been shown to achieve a potentially anti-allodynic CSF concentration of IL-10 in any large animal species. However, a recent report by Haurigot et al[17] showed high levels sulfamidase, a secreted transgene product, in the CSF of dogs receiving IT AAV, suggesting that achieving therapeutic-range levels of IL-10 in the CSF might a feasible translational goal.

The present study uses IT delivery of AAV8 in dogs to examine the distribution of the vector given by the IT route and explores whether potentially therapeutic levels of human IL-10 (hIL-10) can be achieved in the CSF of a large animal model.

RESULTS AND DISCUSSION

Tropism of AAV8 in the dog IT space

Administration of AAV8 expressing EGFP (AAV8/EGFP) into the lumbar cistern of the thecal sac resulted in transduction of the primary sensory neurons in the DRG (Fig. 1A). The transduction rates were observed to be proportionate to the vector dose administered and to the proximity to the anatomical site of vector delivery to the IT space, the lumbar subarachnoid cistern. The animal receiving the highest dose tested (1.2×10^{13} genome copies, GC) showed DRG transduction rates of 11.3% in the lumbosacral-, 9.3% in the thoracic-, and 6.1% in the cervical segment. The dogs that were given the medium (7×10^{12} GC) or low (3.5×10^{12} GC) dose demonstrated markedly lower transduction of the lumbosacral DRG, 7% and 3.9%, respectively, with only occasional transduced cells found in the more rostral segments. Examination of the sensory tracts in the spinal cord showed transduction of the axons in the dorsal roots, dorsal root entry zones, and gracile fascicles. Rostro-caudal extent of transduction in the spinal cord matched that of the DRG.

Analysis of the motor system in dogs revealed that AAV8 targeted the primary motor neurons (Fig. 1B). The animal receiving the high vector dose showed strong EGFP expression in the anterior horn neurons, ventral roots, and ventral root entry zones of the lumbar and sacral spinal segments. The animals receiving either the medium or the low dose demonstrated only occasional motor neuron transduction, with only weak EGFP fluorescence found in the nerve roots and their respective entry zones. The transduction of motor neurons in dogs contrasts with previous finding in rodents, where AAV8 selectively targeted the sensory system[10,18,19]. Differences between rodents and larger models have been previously described in other AAV applications[15,20]. While the underlying mechanisms have not been fully elucidated, these findings are essential to outline the range of potential outcomes that could be encountered in a human trial.

Examination of spinal meninges showed transduction of the pia mater and the arachnoid at all spinal levels (Fig. 1C). Transduction in the intracranial region was limited to the meninges and no evidence of gene expression was found in the brain parenchyma or in the choroid plexus. Although no previous report examined the targeting of meninges by AAV8, meningeal transduction was found after IT delivery of AAV2 in rats[21]. In addition, the transduction of the meninges found here in dogs was attained by adenovirus based gene transfer in rodents, which also led to high levels of a secreted transgene product in the CSF but where the meninges were the only tissue targeted[22].

The observed transduction pattern suggests that AAV8 targets only tissues with which it comes into direct contact after IT delivery to the CSF and does not penetrate the neural parenchyma. This mechanism explains the transduction of the primary sensory and motor neurons, whose axons are exposed to the virus suspended in the CSF, as well as of the meninges. The lack of tissue penetration by AAV8 contrasts with other serotypes, such as AAV9, which is reported to result in ubiquitous neuronal and glial transduction upon IT delivery[16,20]. The properties of AAV8 observed here may be regarded as a desirable vector feature if the goal of gene therapy is to attain a therapeutic transgene level in the CSF without widespread transduction of the neural tissue.

Supra-physiological hIL-10 concentration in the CSF achieved by IT administration of AAV8

AAV8 expressing human hIL-10 (AAV8/hIL-10) was given IT at a total dose of 3.5×10^{12} GC (equivalent to the low dose of AAV8/EGFP). CSF was repeatedly sampled from both the lumbar cistern and the *cisterna magna*. All animals showed a robust transgene expression (Fig. 2). The IL10 concentration reached its peak within 1 week after vector administration. The maximum hIL-10 levels measured 6 days after vector administration exceeded 45 ng/ml in all animals and reached mean concentration of 79.5 ng/ml in the cisternal and 187.9 ng/ml in the lumbar CSF. The difference between the cisternal and lumbar hIL-10 concentrations, however, might have been confounded by the presence of the indwelling IT catheter, which might provide surface affecting the distribution of the vector or of the transgene product within the CSF. The early peak found at 6 days after vector administration was followed by a decline of hIL10 levels, evident at 18 days. By the time of sacrifice at either 28 or 39 days after vector administration, the concentration of hIL-10 leveled off to a lower plateau (124 pg/ml in the lumbar CSF) or fell below the detection limit of the assay (1.76 pg/ml).

In addition to the CSF, serum samples were obtained at the same time points to assess hIL-10 levels in the systemic circulation. The analysis showed presence of hIL-10 in all dogs receiving AAV8/hIL-10 vector. The maximum systemic transgene levels were approximately 2.5% of the lumbar CSF levels (4.4 ng/ml vs. 187.9 ng/ml, respectively). The systemic hIL-10 levels could be attributed to extrathecal escape of the hIL-10 by means of venous and potentially lymphatic drainage of the CSF, as described previously for other cytokines administered IT[23,24]. Alternatively, extrathecal distribution of the vector could result in peripheral production of hIL-10 – an explanation supported by the presence of vector genomes in the liver (as detailed in the Vector distribution section below).

In healthy humans low levels of IL-10 are detectable in the CSF and the upper limit of normal was reported to be 2.2 pg/ml[25]. Up to 10-fold elevation above the normal concentration of IL-10 can occurs in various clinical scenarios, such as long-term IT catheterization or aseptic encephalitis[25,26]. While potential toxicities associated with higher levels hIL-10 in the CSF have not been directly studied, toxicity profile of up to 100 μg/kg recombinant hIL-10 administered systemically has been investigated in humans[27,28], revealing acute flu-like symptoms, followed by neutrophilia, monocytosis, lymphopenia, and down-regulation of pro-inflammatory cytokines upon repeated administration.

Rodent experiments performed by us[10] and by others[6–9] showed that a median CSF concentrations of 85 pg/ml led to therapeutic responses (reversal of allodynia) in models of neuropathic pain. The hIL-10 concentrations demonstrated here in dogs exceeded the rodent anti-allodynic concentration 1,000-fold while being attained by relatively moderate doses of AAV compared to other studies investigating IT delivery of AAV in large animals[13,14,16].

Dog immune response against xenogeneic transgene products

In order to investigate the decline of hIL-10 levels over time, immune response to both transgenes used was investigated by measuring anti-hIL-10 and anti-EGFP antibodies in the CSF and serum (Fig. 3). In all animals, administration of AAV8 resulted in the emergence of

transgene-specific antibodies by the time of sacrifice. The antibody levels were higher in the serum compared to the CSF, and the humoral immune response against hIL-10 appeared to be more pronounced than that against EGFP, which may be related to the fact that hIL10 is a secreted transgene product, while EGFP is confined to the transduced cells. Repeated measurements of hIL-10 and anti-hIL-10 antibody levels in the CSF and sera showed that the emergence of antibodies paralleled the decline of hIL-10 antigen levels. Low levels of antibodies were detectable at day 6 after vector administration. Antibody levels then rose until the time of sacrifice.

The decline of hIL10 levels is therefore likely related to an immune response against the human transgene product. hIL-10 is a secreted protein and, as a result, the humoral immunity is a critical component of the immune response. The human transgene was selected for the present study in an attempt to test the exact vector that would be used clinically. However, recent studies of either AAV-based gene transfer or direct delivery of xenogeneic proteins to the CNS in large animals demonstrated – in contrast to rodent species – consistent immune response against xenogeneic transgenes, which in some cases also included systemic seroconversion[17,29–32]. At the same time, recent studies of IT AAV showed that peak levels of a xenogeneic transgene might predict the long-term levels achievable with a syngeneic transgene[17], suggesting that the hIL-10 levels achieved in dogs in the short-term, i.e. before the immunity developed, might provide a meaningful estimate of what could be expected from the same vector in the clinical setting as no immune shutdown of hIL-10 expression would be expected humans.

The hIL-10 decline attributable to the transgene-specific immune response and presence of EGFP specific antibodies suggest that EGFP expression visualized *post mortem* by microscopy might underestimate the extent of AAV transduction before the immune response had emerged. EGFP is also a protein xenogeneic to the dog and immune response against EGFP has been reported in multiple species[29,33–35]. Presence of anti-EGFP antibodies therefore serves as a marker of the EGFP specific immune response, likely consisting of both the cellular and humoral component and warrants caution that the AAV transduction pattern observed may be more widespread early after vector administration than could be observed at the time of sacrifice.

Vector biodistribution

Detection of vector DNA by quantitative polymerase chain reaction revealed rostro-caudal IT distribution and extrathecal escape of both AAV8/EGFP and AAV8/hIL-10 (Fig. 4). AAV genomes were detected within the DRG and spinal cord at all spinal levels and, unlike for the DRG transduction rates determined by EGFP expression, no trend towards higher numbers at the lumbar segment or in animals receiving higher doses of the vector was observed. Vector distribution in the brain was markedly lower than in the DRG and spinal cord. Analysis of the peripheral tissues showed highest numbers of the vector genome copies in the liver and spleen of the dog receiving the high dose (33.4 and 32.2 GC per diploid host genome, respectively). While the peripheral distribution of the vector was notably lower in the remaining animals, the liver preference could also be appreciated, with median values of

1.8 GC per host diploid genome for the dogs receiving the medium dose and 0.2 GC per host diploid genomes for the dogs receiving the low dose.

AAV8 is known to be hepatotropic upon systemic administratin[36] and the observation of liver transduction therefore suggests that vector may have leaked from the IT space into the periphery. While implantation of the IT catheter was performed 3 days prior to vector administration to allow for wound healing, the catheter insertion path through the atlanto-occipital membrane represents a potential site of vector escape from the IT space. Another explanation would be that AAV8 crossed the blood-brain-barrier in the reverse direction, though this possibility would not be suggested by the literature available about this serotype[37,38]. For these reasons, future studies may need to address the issue of AAV escape from the IT space by alternate procedure of IT vector delivery such as by interventional radiology assisted lumbar puncture. Such experiments may be a valuable addition to the use of the long-standing, “gold standard” dog model that was used in the present study[30,39–43].

CONCLUSION

The present study provides the only report available to date of a high concentration of IL-10 in the CSF of a large animal species achieved by gene therapy. The IL-10 levels achieved in dogs exceeded levels previously found to be anti-allodynic in rats. While the target level that a clinical trial of IT IL-10 would aim to achieve are likely lower, the present study demonstrates that vector doses needed to establish a dose-response relationship and determine the maximum tolerated dose of hIL-10 – critical prerequisites for potential clinical translation – are within the dose range of precedent AAV preclinical- and clinical studies.

In summary, the present study demonstrates that AAV delivery to the IT space can produce pharmacologically desired levels of transgene product in the CSF in large animals. Our findings provide a rationale for further development of this approach in therapeutic applications where a long-term infusion of a therapeutic protein into the CSF is desired and cannot be achieved by chronic IT catheterization for delivery of a recombinant protein.

MATERIALS AND METHODS

Animals

These studies were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego, in AAALAC-accredited facilities. Male beagle dogs, Marshall BioResources, North Rose, NY, USA), weighing 9.4 – 12.8 kg at the time of injection, were used. For the experiments with the EGFP reporter, one dog was injected with 1.2×10^{13} GC and two dogs were injected with 7×10^{12} GC of AAV8/EGFP. All animals were sacrificed 4 weeks after vector administration. For the hIL-10 studies, two dogs were given 3.5×10^{12} GC of AAV8/IL10 and 3.5×10^{10} GC of AAV8/EGFP, one allowed a survival period of 4 weeks and one of 6 weeks (Table 1). The vector was suspended in 3 mL of phosphate-buffered saline and delivered as a slow injection in all animals. The animals were monitored daily for overall appearance, appetite, stool and

urine output, arousal, muscle tone, and coordination. Body temperature was measured every other day and a comprehensive neurological examination was performed weekly.

Vector preparation

Self-complementary AAV expressing either EGFP reporter or human hIL-10 coding DNA sequence was used. The expression cassette further consisted of CMV promoter/enhancer and either rBG (EGFP) or bGH (hIL-10) polyA sequence. Viral particles were packaged in the AAV8 capsid. The vector was produced by the Penn Vector Core (University of Pennsylvania, Philadelphia, PA, USA).

IT catheterization in dogs

The lumbar IT catheter was surgically placed in anesthetized dogs by passing the catheter from the cisterna magna approximately 40 cm to the lumbar enlargement as described previously[30,44]. For anesthesia, the dogs received a pre-anesthetic intramuscular (IM) dose of atropine (0.04 mg/kg), which was given 10 minutes prior to IM injection of xylazine (1.5 mg/kg) and followed by 3.5% isoflurane in 40% 0_2 / 60% N_20 via mask. Animals were then intubated and anesthesia was maintained with 1–2% isoflurane in 40% 0_2/60% N_20. On day 6 after vector administration, the animals were anesthetized and the indwelling IT catheter removed. Cisternal CSF was collected at the time of catheter placement (3 days before vector administration) and, in addition, a lumbar sample was obtained at that time through the implanted IT catheter. The cisternal and lumbar CSF samples were taken in the identical fashion at the time of IT catheter removal (6 days after vector administration) and at the time of sacrifice (28 or 39 days after vector administration). The dogs were also anesthetized 18 days postoperatively and a cisternal CSF sample was obtained by percutaneous puncture. In addition to the CSF samples, blood was collected from the cephalic vein at the same timepoints. All procedures were carried out under rigid aseptic precautions.

Euthanasia and tissue harvest

All animals were deeply anesthetized with intravenous injection of pentobarbital (Nembutal, Akorn Inc., Lakeforest, IL, USA) and exsanguinated with ice-cold PBS followed by perfusion with 10% formalin. The DRGs, spinal cord, and the brain were harvested, post-fixed in 4% paraformaldehyde for 24 hours, and cryoprotected in 30% sucrose overnight. A panel of peripheral tissue was placed on dry ice and stored at −70° Celsius until further analysis.

EGFP detection and quantification of transduction

The samples were embedded in OCT compound (Sakura Finetek, Torrance, CA, USA) and sectioned at 16 μm slide thickness (cryostat CM3050 S, Leica Biosystems, Nussloch, Germany). The sections were mounted on slides using mounting media containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) and viewed on a laser scanning microscope (LSM 780, Carl Zeiss Microscopy, Jena, Germany). EGFP expression was visualized as reported[10]. Briefly, native EGFP fluorescence was detected in λ-stack mode and the specific EGFP signal distinguished from a non-specific tissue autofluorescence by linear unmixing.

To determine the DRG transduction rates, serial sections of each DRG (160 μm apart) were obtained and three sections per DRG were selected for quantification, which was performed by a blinded experimenter exactly as described previously[45].

Measurement of human hIL-10 concentration

The human IL10 concentration in the CSF was measured by human IL-10 enzyme-linked immunosorbent assay (ELISA) kit (Pierce Biotechnology, Rockford, IL, USA). The samples were assayed in duplicates exactly according to the manufacturer's instructions. The CSF of animals injected with AAV8/EGFP served as negative controls and the measurements were used to determine the detection limit of the assay (the upper 95% confidence interval of the mean). Canine IL-10 concentrations of up 600 pg/mL (the upper limit of the hIL-10 standard curve range) were tested and no cross-reactivity was observed.

Detection of antibodies against human hIL-10

The presence of human hIL-10 specific antibodies in CSF and serum was detected by ELISA (Supplemental Fig. S1). First, serial dilutions of the experimental samples in a coating solution (Kirkegaard & Perry Laboratories, Gaithersburg, MA, USA) were adsorbed to a polystyrene 96-well plate in duplicates (Corning, New York, USA) blocked with 1% bovine serum albumin. Next, either recombinant human hIL-10 (1 μg/ml; Shenandoah Biotechnology, Warwick, PA, USA) or recombinant EGFP (0.125 μg/ml; BioVision, Milpitas, CA, USA) was added to the plate, followed by respective biotinylated detection antibodies (anti-hIL-10: 0.3125 μg/ml; Pierce Biotechnology, Rockford, IL, USA; or anti-EGFP: 0.005 μg/ml; Abcam, Cambridge, MA, USA) and streptavidin – horseradish peroxidase conjugate (1:400; Pierce Biotechnology, Rockford, IL, USA). The assay was developed by adding 3,3′,5,5′-tetramethylbenzidine substrate and the stop solution (Kirkegaard & Perry Laboratories, Gaithersburg, MA, USA). The plate was washed with 0.002 M imidazole buffered saline with 0.02% Tween 20 three times between all steps. As a standard, known concentration of either anti-human hIL-10 IgG (Abcam, Cambridge, MA, USA) or anti-EGFP IgG (Life Technologies, Grand Island, NY, USA) were adsorbed to the plate instead of the experimental CSF or sera, with a dynamic range of 12.5 – 0.125 μg/ml or 4 – 0.125 μg/ml, respectively. The human hIL-10 or EGFP capturing capacity of the experimental samples (consisting of antibodies of all isotypes) was thereby quantified as an equivalent of a capturing capacity of the respective IgG at the given concentration.

Biodistribution of the vector

DNA from the peripheral tissue samples was extracted by DNAeasy Blood and Tissue Kit (69506, Qiagen Sciences, Germantown, MA, USA). Quantitative PCR based on the SYBR Green detection system (0470756001, Roche Diagnostics, Mannheim, Germany) was used and the copies of vector genomes were normalized to the copies of host diploid genomes. Either EGFP (forward, 5′-GAGCAAAGACCCCAACGAGA-3′; reverse, 5′-GTCCATGCCGAGAGTGATCC-3′) or human hIL-10 (forward, 5′-CTACGGCGCTGTCATCGAT-3′; reverse, 5′-TGGAGCTTATTAAAGGCATTCTTCA-3′) specific primers were used for a detection of the vector target sequence. Host GAPDH primers (forward, 5′-GAGGTTCCTTGCTGCTGTTC-3′; reverse, 5′-ATTCAAGGTCCAATTTTGCG-3′) were used as a reference for the relative quantification.

The amplification was performed using LightCycler 480 (Roche Diagnostics, Mannheim, Germany).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The study was supported by NIH grant R01NS063022 and funds from the Schulze Family Foundation. The authors would like to very much thank the anonymous reviewer for pointing towards the literature on cytokine exit from the CSF to the general circulation and references 23 and 24 included in the revised version of the manuscript.

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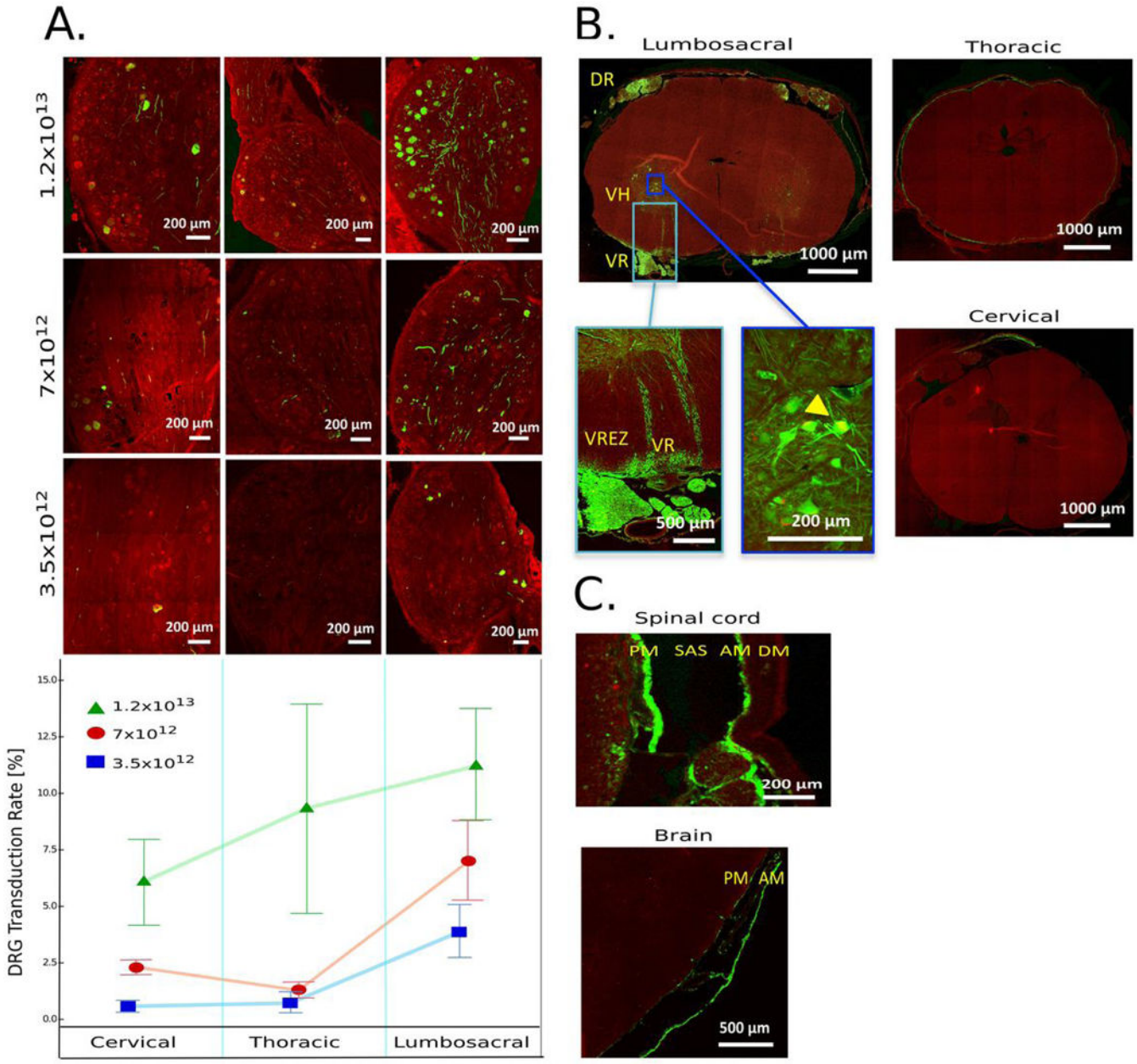

Figure 1. Transduction of the peripheral nervous system (PNS) and the meninges by IT AAV8/EGFP

AAV8 displayed tropism for primary sensory neurons, primary motor neurons, and meninges. The vector was administered into the dog lumbar cistern at three different doses (high, 1.2×10^{13} genome copies, GC; medium, 7×10^{12} GC; and low, 3.5×10^{12} GC). Animals were sacrificed after 28 days and the EGFP expression was analyzed by laser scanning microcopy.

A. DRG. AAV8 targeting of the DRG was evident in all animals. EGFP positive cells demonstrated the anatomical characteristics of the primary sensory neurons with large cell bodies and occasionally visualized pseudounipolar axons. 11.3% of DRG cells were transduced at the lumbar and sacral spinal levels in the dog receiving the high vector dose,

with 9.3% transduced at the thoracic- and 6.1% and the cervical level. The dogs receiving the medium and low doses showed transduction rates of 7% and 3.9% within the lumbosacral DRG, respectively, and only occasional EGFP positive cells were observed on the thoracic and cervical spinal levels.

B. Spinal cord. EGFP expression was found in both motor and sensory systems on the lumbosacral spinal cord here demonstrated for the dog receiving the high AAV8/EGFP dose. The animal showed a robust transduction of the primary motor neurons *(arrowhead)* in the ventral horns *(VH)*. In addition, EGFP expression was present in both dorsal *(DR)* and ventral roots *(VR)*, as well as in the respective root entry zones *(ventral root entry zone, VREZ)*. No transduction was noted in the thoracic or cervical spinal segments. The dogs receiving the medium or low vector dose demonstrated only occasional motor neurons transduced and moderate transduction of the nerve roots and entry zones on the lumbosacral spinal level (not shown).

C. Meninges. Transduction of the leptomeninges *(PM, pia mater; SAS, subarachnoid space; AM, arachnoid mater)* encompassed the entire neuraxis and extended to the cerebral meninges. No EGFP expression was found in the dura mater *(DM)* and the brain parenchyma.

Magnification: 200×; Error bars: standard error of the mean (SEM).

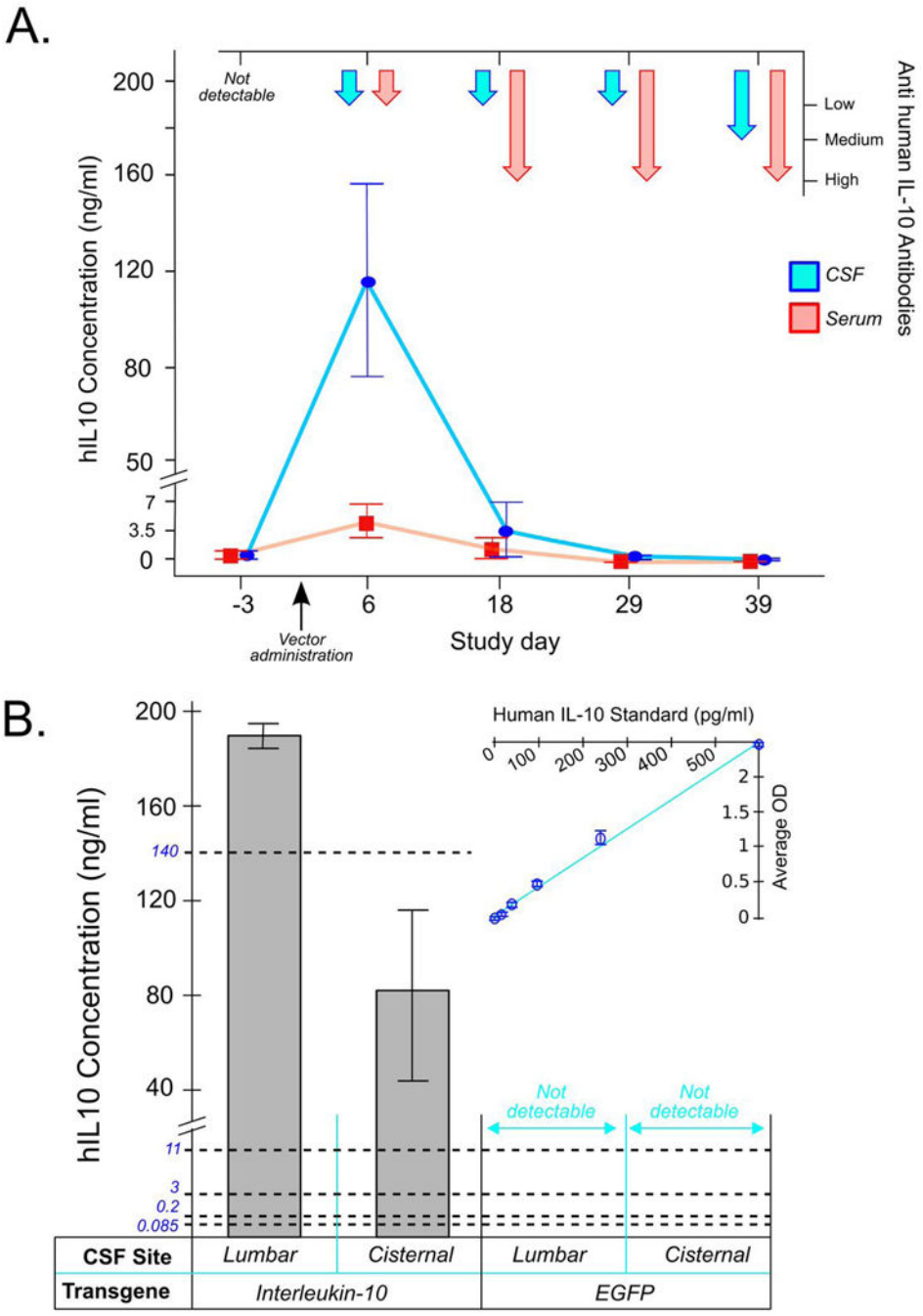

Figure 2. Levels of human interleukin-10 (hIL-10) in the cerebrospinal fluid (CSF) observed after IT AAV8/hIL-10

The hIL-10 levels in the dog CSF reached or exceeded the pharmacological target range in all animals. The vector expressing human hIL-10 was administered IT at a dose of 3.5×10^{12}. Levels of hIL-10 in the CSF were quantified by enzyme-linked immunosorbent assay (ELISA) at the time points indicated.

A. Time-course of hIL-10 in the CSF and humoral immune response against the transgene product. hIL-10 expression peaked within 1 week after the vector administration (> 50 ng/ml), which was followed by a marked decline detectable 18 days after vector administration.

No hIL-10 could be measured 39 days after vector administration, the latest time point tested. hIL10 could also be detected in the serum with a time-course corresponding to that in the CSF but with hIL-10 concentration not exceeding 7 ng/mL at any time point. The decline in the hIL-10 levels was associated with emergence of anti-hIL-10 antibodies detected in CSF and in serum. The antibodies first appeared on a day 6 both in the CSF and serum, and the concentration gradually increased until the animals were sacrificed. These results suggest that the immune response against the xenogeneic transgene may be responsible for the loss of hIL-10 transgene product. Antibody quantification: low, sample hIL-10 binding capacity corresponds to that of the IgG standard at a concentration ≤ 1000 μg/ml; medium, 1000 – 100,000 μg/ml; high, ≥ 100,000 μg/ml.

B. The levels of hIL10 in context to precedent efficacy studies. The hIL-10 concentrations were measured in the CSF sampled from both the *cisterna magna* and the lumbar cistern. The results were compared to a reference range derived from published rodent studies investigating the anti-allodynic efficacy of IL-10. The levels measured in the lumbar CSF exceeded those found in the cisternal CSF by approximately 2-fold. The IL10 CSF level previously found to induce therapeutic response are marked by dashed lines: 85 pg/ml[10]; 200 pg/ml[7]; 3 ng/ml[9]; 11 ng/ml[6]; 140 ng/ml[8]. ELISA characteristics showed a wide linear range covering the measured concentration at the appropriate dilution of CSF in the reaction buffer. No hIL-10 was detected in the animals receiving AAV8/EGFP, confirming the specificity of the assay.

Error bars: SEM in all panels.

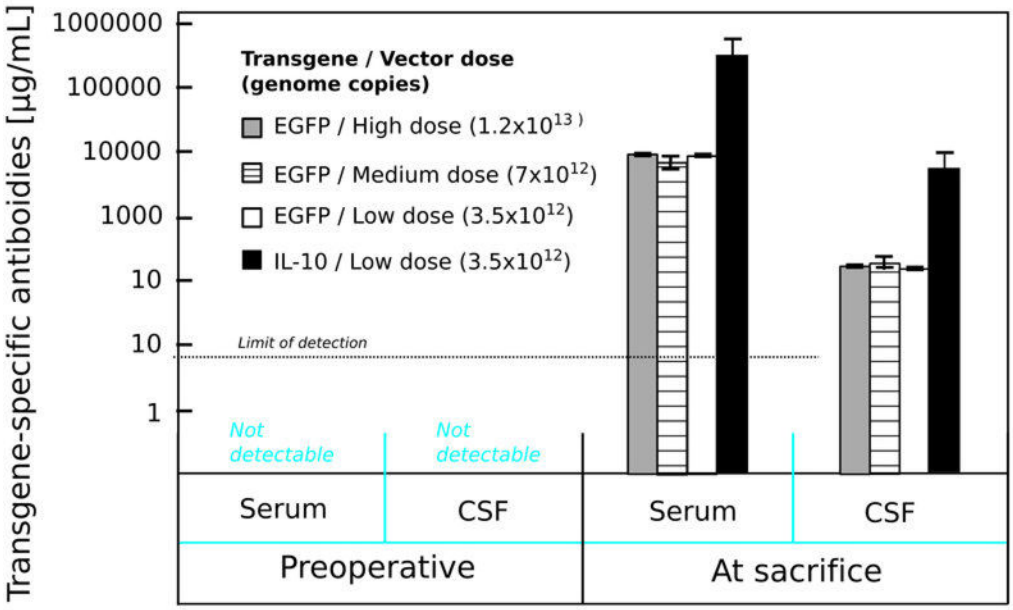

Figure 3. Quantification of serological immunity against xenogeneic transgenes at the end of the experiment

EGFP- and hIL-10 specific antibodies were detectable at the time of sacrifice in both the CSF and sera. Antibody titers were higher in the sera compared to the CSF and the anti-hIL-10 antibodies tended to reach higher levels than the anti-EGFP antibodies. No antibodies could be detected prior to the vector administration and anti-hIL-10 antibodies were found in animals receiving AAV8/EGFP only, confirming the specificity of the assay. The antibody titers were measured by ELISA, whereby antibodies of all isotypes were adsorbed to a plate surface and resulting EGFP/hIL-10 binding capacity standardized to a known concentration of the respective IgG control. Error bars: SEM

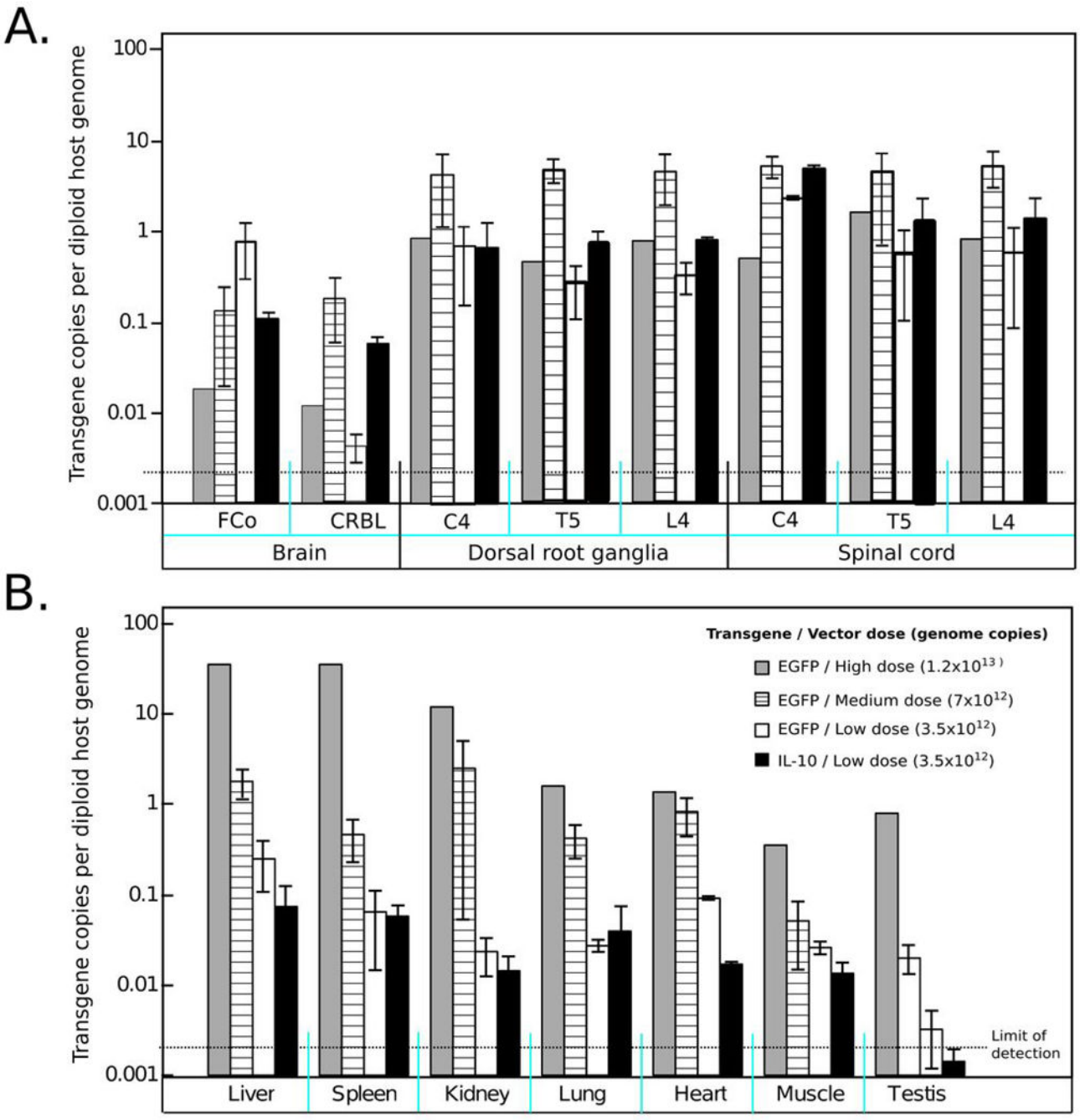

Figure 4. Biodistribution of vector genomes

IT administration of AAV8 resulted in a distribution of the vector within the IT space (Panel A) and suggested its systemic spread, with predilection for the liver and spleen (Panel B). Presence of vector genomes was determined by quantitative PCR using primers specific to either EGFP or human hIL-10. The results were normalized to host diploid genome. Lower limit of detection was determined as the 95% confidence interval of results obtained in independent negative controls (dogs not receiving the AAV). Error bars: SEM.

Table 1

Experimental animals.

Animal ID	Survival time [days]	Transgene	Vector dose [GC]
1668707	28	EGFP	1.2×10^{13}
1919637	27	EGFP	7×10^{12}
1922689	27		
1935977	26	hIL-10 + EGFP	3.5×10^{12} each
1937287	39		