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Research paper

Characterization of an immune-evading doxycycline-inducible lentiviral vector for gene therapy in the spinal cord

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ABSTRACT

Gene therapy is a powerful approach to promote spinal cord regeneration. For a clinical application it is important to restrict therapeutic gene expression to the appropriate time window to limit unwanted side effects. The doxycycline (dox)-inducible system is a widely used regulatable gene expression platform, however, this system depends on a bacterial-derived immunogenic transactivator. The foreign origin of this transactivator prevents reliable regulation of therapeutic gene expression and currently limits clinical translation. The glycine-alanine repeat (GAR) of Epstein-Barr virus nuclear antigen-1 protein inhibits its presentation to cytotoxic T cells, allowing virus-infected cells to evade the host immune system. We developed a chimeric transactivator (GARrtTA) and show that GARrtTA has an immune-evading advantage over "classical" rtTA in vivo. Direct comparison of lentiviral vectors expressing rtTA and GARrtTA in the rat spinal cord shows that the GARrtTA system is inducible for 6 doxycycline-cycles over a 47 week period, whereas with the rtTA-based system luciferase reporter expression declines during the 3rd cycle and is no longer re-inducible, indicating that GARrtTA provides an immune-advantage over rtTA. Immunohistochemistry revealed that GARrtTA expressing cells in the spinal cord appear healthier and survive better than rtTA expressing cells. Characterization of the immune response shows that expression of GARrtTA, in contrast to rtTA, does not recruit cytotoxic T-cells to the transduced spinal cord. This study demonstrates that fusion of the GAR domain to rtTA results in a functional doxycycline-inducible transactivator with a clear immune-advantage over the classical rtTA in vivo.

1. Introduction

Lentiviral vectors are widely used in pre-clinical gene therapy studies. The originally HIV derived lentiviral vectors are vesicular stomatitis virus surface glycoprotein (VSV.G) pseudotyped replication-deficient viral particles that carry a transgene of choice in a recombinant viral genome. These viral particles have a broad tropism allowing the delivery of genes of interest to a wide range of dividing and non-dividing cell types (Naldini et al., 1996). Due to the stable integration of the transgene in the host genome, long term reliable expression of therapeutic transgenes can be achieved (Blomer et al., 1997; Kordower et al., 1999).

Lentiviral delivery of genes that promote neuron survival (Eggers et al., 2019), stimulate axon regeneration (Andrews et al., 2009) or reduce the inhibitory properties of the neural scar (Bartus et al., 2014; Burnside et al., 2018) is a promising strategy to enhance the regenerative capacity of the mammalian nervous system after traumatic injury. However, uncontrolled expression of therapeutic proteins can be harmful and may limit their therapeutic use (Georgievska et al., 2002; Blits et al., 2004; Tannemaat et al., 2008; Hoyng et al., 2014b) . For example, lentiviral vector-mediated expression of glial cell line-derived neurotrophic factor (GDNF) or nerve growth factor (NGF) were shown to stimulate axonal regeneration in the injured peripheral nerve. However, prolonged expression resulted in local trapping of the regenerating

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axons (Blits et al., 2004; Eggers et al., 2008; Tannemaat et al., 2008; Santosa et al., 2013) and impeded functional recovery (Blits et al., 2003; Hoyng et al., 2014b). Overexpression of brain-derived neurotrophic factor (BDNF) in the injured spinal cord promoted sprouting and reorganization of locomotor circuits (Blits et al., 2003) but continued expression induced spasticity in spinal cord injured rats [(Boyce et al., 2012, Fouad et al., 2015), reviewed in (Smith, 2014)]. Uncontrolled overexpression of NGF following a spinal cord lesion resulted in extensive sprouting of sensory afferents but also induced hyperalgesia and autonomic dysreflexia [(Romero et al., 2000, Cameron et al., 2006), reviewed in (Brown and Weaver, 2012)]. The pro-regenerative effects of sustained lentiviral vector-mediated expression of the neural scar degrading enzyme Chondroitinase ABC (CHABC) are superior to the beneficial effects observed following delivery of the bacterial enzyme by intrathecal injection. While there is no evidence that prolonged CHABC expression has any negative effects (Bartus et al., 2014; James et al., 2015), restricting its expression to an optimal "regenerative time window" is desirable (Burnside et al., 2018). Controlled expression of CHABC would prevent ongoing degradation of the extracellular matrix after axon regeneration and functional recovery has taken place. Therefore, regulatable viral vectors that would allow temporal control over the expression of a therapeutic gene are important for the realization of gene therapy for spinal cord injury.

Currently there are several systems in use for the in vivo regulation of transgene expression. The tetracycline-inducible system is the most commonly used and best studied system for in vivo transgene regulation. Four other systems, including the rapamycin-inducible system and several steroid receptor-inducible systems (tamoxifen, mifepristone, ecdysteroid) have been developed (Gossen and Bujard, 1992; Vegeto et al., 1992; Rivera et al., 1996; Karns et al., 2001; Roscilli et al., 2002; Stieger et al., 2009). There are two versions of the tetracycline-regulatable system. In the originally developed "tet-off" system transgene expression occurs in the absence of tetracycline or its analogue doxycycline and is repressed in the presence of the inducer drug. In the "tet-on" system transgene expression is induced in the presence of tetracycline/doxycycline (Gossen et al., 1995). In the "tet-on" system tetracycline/doxycycline interact with a bacterial transactivator protein that binds to a tet-operator controlled promoter and drives transgene expression (Fig. 1A). The transactivator protein has been improved significantly in recent years, making it more sensitive to the inducer drug and it exhibits less leaky expression in the absence of doxycycline (Urlinger et al., 2000; Zhou et al., 2006; Das et al., 2016a, 2016b). The antibiotic doxycycline is a highly effective inducer, as it is well-tolerated when administered orally or intravenously to humans and it easily penetrates various tissues, including the brain and spinal cord (Sloan and Scheinfeld, 2008).

Although many transgenes have been successfully expressed in vivo using the tetracycline-inducible system (Das et al., 2016a, 2016b), experiments in rodents (Ginhoux et al., 2004; Markusic et al., 2010) and pre-clinical studies in non-human primates (Favre et al., 2002; Latta-Mahieu et al., 2002; Ginhoux et al., 2004) showed that long-term repeated induction of transgene expression failed due to cellular and

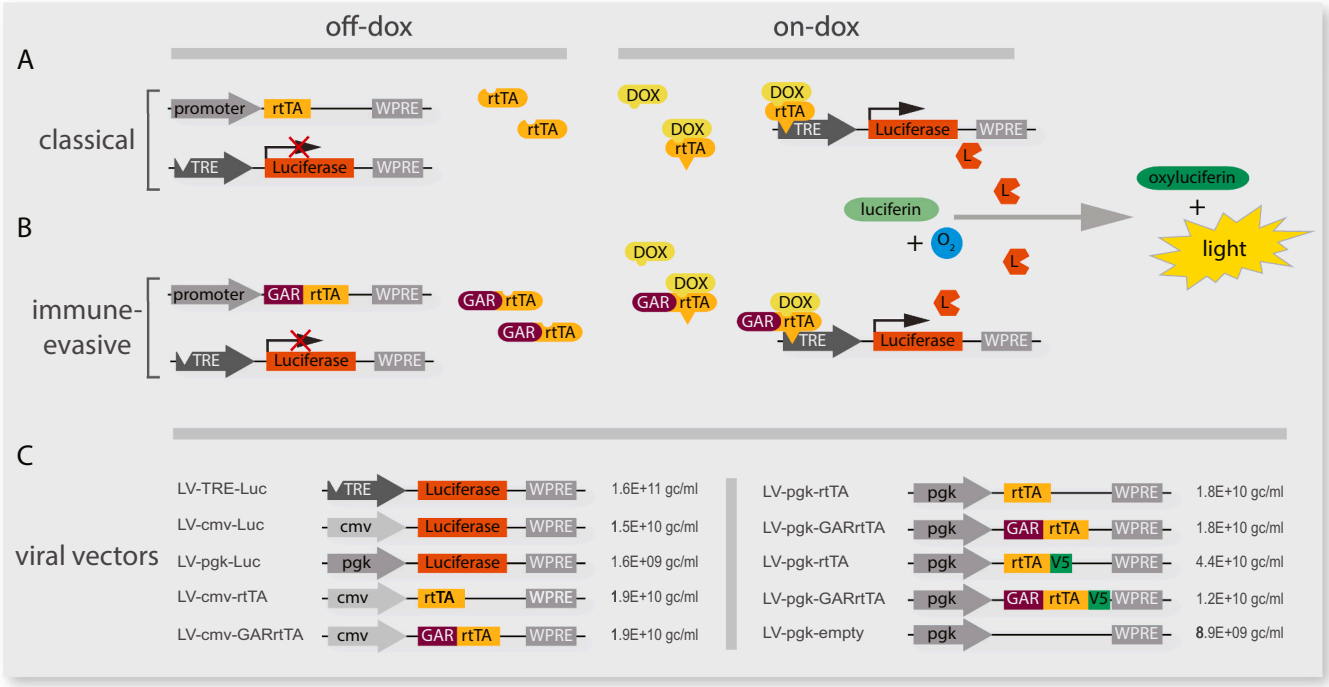

Fig. 1. *Schematic representation of the doxycycline inducible system and the viral vectors with their titers.* (A) The classical doxycycline inducible system is composed of two co-applicated viral vectors. One vector contains a constitutive active promoter that drives a reverse tetracycline-controlled transactivator (rtTA). The second vector carries Tet responsive elements + a minimal CMV (TRE) to drive transgene expression (Luciferase). In the absence of doxycycline (off-dox) there is no transgene expression. Transgene expression can only occur when doxycyline (DOX) is present (on-dox) and binds to rtTA. This induces a conformational change upon which the rtTA-DOX complex can bind to the TRE promoter and activate Luciferase transgene expression. Luciferase can convert the luciferin substrate into oxyluciferine and during this process photons (light) are emitted. (B) In the immune-evasive doxycycline inducible system the rtTA is fused to the Glycine-Alanine rich repeat domain (GAR) of the EBNA-1 protein of the Epstein-Barr virus. This immune-evasive transactivator (GARrtTA) can bind to doxycycline and activate transgene expression by binding to the TRE promoter. (C) The panel shows lentiviral constructs in which transgene expression is driven by the cytomegalovirus (cmv) major immediate-early promoter (cmv), the phosphoglycerate kinase promotor (pgk) or the tetracycline responsive element with a minimal cmv (TRE). LV-TRE-Luciferase construct consists of a firefly luciferase under the control of a tetracycline responsive element (TRE) which in the presence of dox can be activated by the transactivator protein (rtTA or GARrtTA). The transactivator is the $S2^2$-variant of the transactivator protein fused to 3 minimal F-type activator domains of VP16 (Zhou et al., 2006). GARrtTA expresses the same transactivator N′-terminally fused with a Glycine-Alanine rich repeat (GAR). In all constructs the transgene is followed by a Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) to increase viral vector mediated transgene expression.

humoral immune responses directed at cells expressing the transactivator (Favre et al., 2002; Latta-Mahieu et al., 2002; Chenuaud et al., 2004; Le Guiner et al., 2007). This currently limits the use of this system for in vivo experiments and in a clinical setting.

To hide the immunogenic properties of the rtTA (reverse tetracycline-controlled transactivator) from the immune system we generated a fusion protein of the classical rtTA with the long Gly-Ala rich repeat (GAR) of the Epstein-Barr virus Nuclear-Antigen-1 protein (EBNA-1) (Zaldumbide et al., 2010). The GAR in EBNA-1 prevents antigenic peptide presentation to major histocompatibility complex class I (MHCI) molecules thereby preventing T-cell mediated destruction of EBNA-1 expressing cells during the latency phase of the Epstein-Barr virus life cycle (Levitskaya et al., 1995; Blake et al., 1997; Levitskaya et al., 1997; Munz et al., 2000). This new GAR carrying transactivator (GARrtTA, Fig. 1B) retains its transactivator activity and has an immune-evasive advantage over rtTA in a bioassay for human antigen presentation (Hoyng et al., 2014a, 2014b).

In this study, we compared the long-term performance of the classical rtTA to the new immune-evasive GARrtTA in the adult rat spinal cord. To monitor dox-induced transgene expression we measured luciferase (Luc) expression controlled by a promotor containing a tetracycline responsive element (TRE) (Agha-Mohammadi et al., 2004) embedded in a lentiviral vector (LV-TRE-Luc). LV-TRE-Luc was co-injected with lentiviral vectors carrying rtTA or GARrtTA where expression was controlled by either the cytomegalovirus (cmv) promoter (LV-cmv-rtTA or LV-cmv-GARrtTA) or mouse phosphoglyceratekinase (pgk) promoter (LV-pgk-rtTA, or LV-pgk-GARrtTA). Luciferase activity was quantified by in vivo bioluminescence imaging during multiple alternating cycles of no-dox/dox supplied to the rats via their diet for up to 47 weeks. We show that LV-pgk-GARrtTA/LV-TRE-Luc, in contrast to LV-pgk-rtTA/LV-TRE-Luc, remains inducible and stable for at least 6 dox-cycles over a long term period. Characterization of the immune response by FACS sorting of immune cells shows that expression of GARrtTA, in contrast to rtTA, does not recruit cytotoxic T-cells to the transduced spinal cord. Together this shows that fusion of the GAR domain to rtTA results in a doxycycline-inducible transactivator with an immune-advantage over the classical rtTA.

2. Materials and methods

2.1. Lentiviral vectors

Second generation lentiviral transfer plasmids [(Naldini et al., 1996, Hendriks et al., 2007), here referred to as pLV)] were generated, and lentiviral vectors (LV) were produced and titred as described previously (Hoyng et al., 2014a). pLV-pgk-Luc, pLV-pgk-rtTA, and pLV-pgk-GARrtTA were generated by replacing the cmv promoter with the pgk promoter in pLV-cmv-Luc, pLV-cmv-rtTA, and pLV-cmv-GARrtTA respectively. To determine the survival of cells expressing the transactivators, constructs were generated with N-terminal V5-tagged rtTA and GARrtTA to allow for reliable immunohistochemical detection of transduced cells. The transactivator (rtTA) used in the experiment is the rtTA-V16 variant developed by viral evolution, as described previously (Zhou et al., 2006). The tetracycline/doxycycline inducible promoter element (TRE) in pLV-TRE-Luc is a variant designated p8teto-36ΔI embedded in a minimal cmv promoter (Agha-Mohammadi et al., 2004). Fig. 1C summarizes the viral vector constructs and provides the batch titers used in this study.

2.2. Animals

A total of 68 adult female Wistar rats (Harlan, Netherlands) were used. Animals were housed under standard conditions with regular or doxycycline (6 g/kg, TD.09282, Harlan) enriched food and water ad libitum. All experimental procedures were performed in accordance with the European guidelines for the Care and Use of Laboratory Animals (86/609/EEC) and the local committees for animal welfare and experimentation.

2.3. Surgery

Animals were anesthetized using isoflurane (Isoflo, Abbott, The Netherlands). After opening the dorsal skin and musculature the spinal cord was fixed in place (Ruitenberg et al., 2002) and exposed by a laminectomy at thoracic level 10 (T10). Two times 1 μl lentiviral vector was injected (with rostral and caudal injections about 2 mm apart and 0.8 μm deep, 0.2 μl/min) in the spinal cord using an automatic microinjection device (Harvard apparatus, Inc., Holliston, MA). After injection the muscles (sutures, 6–0 Vicryl) and skin (wound clips) were closed. The animals received per-operative and post-operative (for 3 days) analgesia (0.03 ml/100 g subcutaneous Temgesic, buprenorphine hydrochloride; Schering-Plough, Weesp, the Netherlands). Wound clips were removed after 7 days.

2.4. In vivo imaging

Animals were anesthetized using isoflurane (Isoflo, Abbott, The Netherlands), hair was shaved off the back above the injected area and a 2 min background measurement was performed on an IVIS 200 imaging system (Caliper Live Sciences, Hopkinton, MA, USA). After the background measurement 400 μl luciferin solution (375 mg/ml phosphate buffered saline; Regis Technologies, Morton Grove, IL, USA) was injected in the tail vein. Subsequently luciferase activity was measured in 3 consecutive two minute long recordings. For each measurement, a region of interest (ROI, fixed size for all measurements) was centered over the area with the highest bioluminescence signal. Of the 3 recordings per animal per time point the measurement with the highest signal was used for further analysis. Average bioluminescence per group per time point was plotted ± SD.

2.5. Histology

Animals received a lethal dose of pentobarbital (0.11 ml/100 g, I.P. Sanofi Sante, Maassluis, The Netherlands) and were subsequently transcardially perfused with saline, followed by 4% paraformaldehyde in phosphate buffer. The spinal cord was dissected, cryoprotected overnight in 25% sucrose in PBS and snap frozen in dry-ice cooled isopentane. Series of sagittal sections (20 μm) were cut on a cryostat and mounted on microscope slides (Superfrost Plus, Menzel-Gläser, Braunsweig, Germany). For immunohistochemistry the sections were washed with PBS, blocked with blocking buffer (5% fetal calf serum in Tris buffered saline (TBS) with 0.1% Triton X-100) and incubated overnight at 4 °C with primary antibodies (Luciferase, NeuN, GFAP and V5; details in Table 1) diluted in blocking buffer. The next day, the sections were washed 3 times in TBS and incubated for 2 h with fluorescent labeled secondary antibodies diluted in blocking buffer.

Table 1
Primary antibodies.

Antigen	Dilution	Company	Catalog #
Luciferase	1:100	Bio-connect	CR2029RAP
NeuN	1:250	Chemicon	MAB377
GFAP	1:500	Dako	Z033401
V5	1:500	Invitrogen	R960–25
CD45 BUV395	1:250	BDBioscience	740,258
CD11b V450	1:300	BDBioscience	562,108
CD43 PE	1:150	Miltenyi Biotec	130–107-721
CD45RA FITC	1:250	BDBioscience	561,886
CD3 BV605	1:250	BDBioscience	563,949
CD4 PE-Cy7	1:200	BDBioscience	561,578
CD8 APC	1:150	Miltenyi Biotec	130–119-664
RP-1 BV786	1:200	BDBioscience	743,058
Live/Dead NIR	1:500	Biolegend	423,105

Following a final wash in TBS supplemented with DAPI the sections were coverslipped using Mowiol. Images where taken on a Leica SP5 confocal microscope. In two sections of each animal we outlined three circular regions of interest (ROI) in the white matter of the injected spinal cord segment. In these ROI we measured the GFAP positive area and expressed this as percentage of the total ROI area. Using ImageJ software, the number of debris particles where counted in a 1.55 μm^2 ROI in three sections containing the virus injection per animal. Subsequently we calculated the average number of particles per μm^2 . For the V5-tagged transactivator quantification every tenth section was visually inspected and the sections with the most V5 transgene expression were selected for further analysis. The center section, a section 200 μm dorsal and a section 200 μm ventral, were imaged at a Leica SP5 confocal microscope. In ImageJ software a fixed threshold was applied to each image and the surface area of transgene expression was measured. After determining the average expression area per animal the group average was calculated.

2.6. Flow cytometry

To study the differential immune cell response to LV-PGK-rtTA, LV-pgk-GARrtTA or LV-pgk-empty vector control, animals ($n = 6$ per group) were injected with these vectors and 7 weeks later animals were anesthetized with pentobarbital (Euthatal®, 80 mg/kg, i.p.) and transcardially perfused with phosphate buffered saline +2% EDTA. The transduced spinal cord segment (~8 mm) was dissected immediately and kept in ice cold PBS. After mechanical dissociation the tissue was passed through a 70 μm cell strainer (BD Falcon, Germany) and centrifuged at 300*g* at 4 °C. The sample was incubated with Myelin Removal Beads (Miltenyi Biotec, Germany) and passed through a LS column (Miltenyi Biotec, Germany), eluted cells were resuspended in DPBS (Gibco). After cell counts, samples were incubated with anti-rat CD16 and CD32 (1:50, BD Bioscience) for 15 min on ice to block the Fc receptors and specific antibodies were used to identify neutrophils, microglia, macrophages (CD43+ and CD43-), B cells and T cell (CD4+ and CD8+) populations (antibodies detailed in Table 1).

3. Results

3.1. Cmv driven inducible luciferase expression

To compare the performance of the new GARrtTA and the classical rtTA transactivator in the spinal cord of adult rats we performed a side-by-side comparison. The lentiviral vector expressing luciferase under the control of a TRE promoter (LV-TRE-Luc) was co-injected with titer matched lentiviral vectors expressing either GARrtTA or rtTA under the control of a constitutively active cmv promoter (group names: LV-cmv-GARrtTA or LV-cmv-rtTA respectively, Fig. 1) in the adult rat spinal cord (T10 level). The ratio between the vector genomes of LV-TRE-Luc and LV-cmv-GARrtTA or LV-cmv-rtTA was 1:1 and the total number of genomic copies injected was 3.8E+07. One week after the lentiviral vectors were injected measurements of luciferase activity (bioluminescence) using an IVIS 200 imaging system were started (Fig. 2, bioluminescence image of an example rat). Animals injected with LV-cmv-luciferase (LV-cmv-Luc) served as positive control and, as expected, these animals displayed clear luciferase activity immediately after administration of luciferin through the tail vein (Fig. 2C, D).

Example bioluminescence heat maps for a LV-cmv-rtTA and a LV-cmv-GARrtTA animal over the 12 week observation period are shown in Fig. 2A and quantification of the mean bioluminescence per week for each group is shown in Fig. 2B. In the 2 weeks prior to the start of the dox feeding LV-cmv-rtTA and LV-cmv-GARrtTA injected animals showed a mean leaky expression of 3 to 5 times ($p < 0.05$) over background (= bioluminescence measured before luciferin injection). Supplementing the regular diet with dox for a period of 3 weeks (from 3 to 5 weeks) resulted in a significant increase in luciferase activity in both inducible vector groups. At week 3, animals in the cmv-rtTA group showed on average a 122 fold induction (358 times background expression) compared to the non-induced state in week 2 ($p < 0.05$). For the cmv-GARrtTA group a 15 fold increase (78 times background expression) in expression was observed compared to the non-induced leaky expression measured in week 2 ($p < 0.01$). Continuous dox feeding in the following 2 weeks resulted in elevated luciferase activity compared to non-induced expression levels. However, despite continued dox feeding we observed that in both cmv-rtTA and cmv-GARrtTA animals luciferase activity is decreased in week 4 and 5 compared to the first week of induction (week 3). Returning to regular chow without dox resulted in a significant decrease in luciferase expression in week 6, 7 and 8 compared to the peak expression in week 3 (cmv-rtTA: $p < 0.05$; cmv-GARrtTA: $p < 0.01$). A second round of dox administration (week 9–11) to either the cmv-rtTA animals or the cmv-GARrtTA group did not induce a significant increase in luciferase expression compared to week 8 without dox. Taken together, this shows that cmv-rtTA and cmv-GARrtTA are able to induce transgene expression in the adult spinal cord upon dox administration. However, both cmv driven transactivators allow only one round of regulated luciferase expression.

3.2. Comparative analysis of cmv and pgk promoter activity

We noted that in the positive control group injected with the constitutively active cmv-Luc, a gradual decline in luciferase activity occurred over time (Fig. 2C and D). The luciferase activity in the LV-cmv-Luc group started at 27 times above background in week 1, however, it gradually declined to only 6 times above background at the end of the experiment in week 12 ($p < 0.01$) (Fig. 2C and D). Inactivation of the cmv promoter due to e.g. methylation may underlie this decline in transgene expression (Herbst et al., 2012). We hypothesized that the failure to re-induce luciferase expression during the second cycle of dox may be caused by significantly diminished activity of the cmv promoter driving insufficient rtTA and GARrtTA expression. We therefore, replaced the cmv promoter by a pgk promoter (Deglon et al., 2000; Zhao et al., 2011). This mammalian promoter has been shown to work very well in the brain and spinal cord (Delzor et al., 2012; Bartus et al., 2014; Desclaux et al., 2015; Andrews et al., 2016; Nieuwenhuis et al., 2021).

Therefore, we compared luciferase activity when using a pgk promotor to drive gene expression with that of the cmv promotor (pgk-luc vs cmv-luc, Fig. 2C, D). LV-pgk-Luc lentiviral vector was injected at a dose of 1.6E09 gc/ml (3.2E+06 gc per animal total). The constitutively active pgk-Luc directs high levels of luciferase activity in week 1 that, in contrast to the observations with cmv-Luc (used at a 10 fold higher dose than LV-pgk-Luc), further increased in week 2 and 3, reaching expression levels of up to 182 times background ($p < 0.001$) (Fig. 2C and D). After week 3 the luciferase activity gradually starts to decline to 19 times background at week 12. Around week 20 a plateau is reached and is expression maintained at 7 times above background activity until the latest time point studied (47 weeks).

Comparison of pgk-Luc activity to cmv-Luc activity over the first 12 weeks after injection shows that pgk-Luc, with 10 times less viral particles injected, is performing as well as cmv-Luc (ns, $p = 0.10$). At 3 weeks post injection pgk-Luc performs significantly better and, with 10 times less viral particles injected, reaches a 4 times higher level of expression than cmv-Luc at week 3 ($p < 0.05$). The ability of pgk to drive similar or higher levels of luciferase expression, with 10 times less viral particles injected, shows that pgk is a much stronger promoter than the cmv promoter in the adult rat spinal cord.

3.3. PGK driven GARrtTA-induced luciferase expression persists for 6 dox-cycles

Titer matched lentiviral vectors containing the pgk-GARrtTA and pgk-rtTA expression cassettes were co-injected with LV-TRE-Luc (3.6E+07 gc per animal total) into the adult rat spinal cord. After one

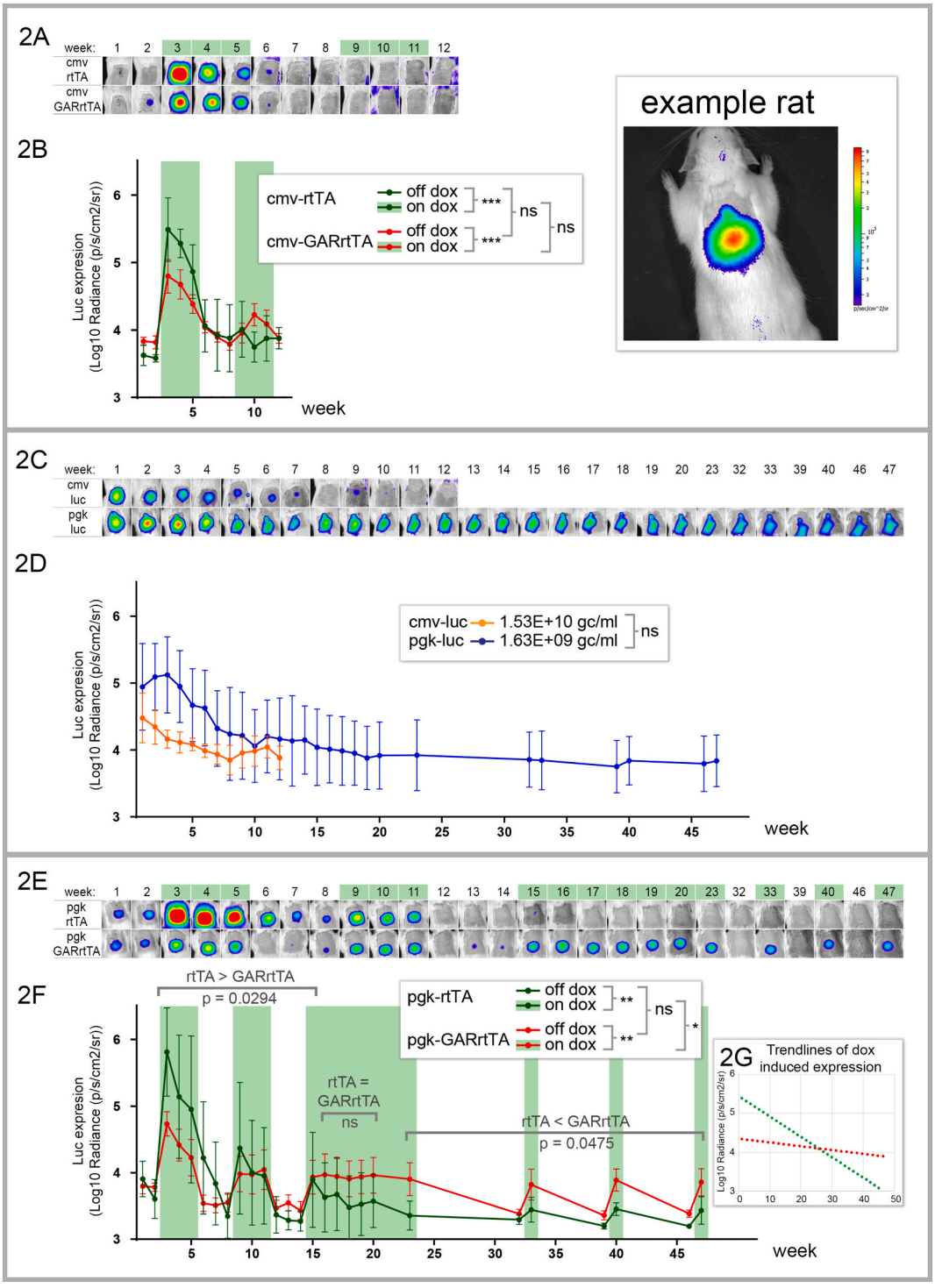

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Fig. 2. *Analysis of luciferase activity in the rat spinal cord over time.* (2A) Representative IVIS derived images (dorsal view) of two individual rats (cmv-rtTA and cmv-GARrtTA) overlaid with a heat map of the bioluminescence signal shows luciferase activity in the presence (dox, green) or absence of doxycycline overtime. (2B) Quantification of the group ($N = 5$) means per time point are plotted in graph. In the first two weeks with dox there is in both groups a very weak luciferase signal detected. Upon administration of dox in week 3 luciferase activity is strongly increased but gradually declines in week 4 and 5. Removal of dox food results in a further decrease of luciferase activity in week 6 to 9. A second round (week 9–11) of dox administration fails to re-induce luciferase activity again.
(2C) Representative images of 2 individual animals injected with the constitutively active cmv-Luc or pgk-Luc group, quantification of group (N = 5) means in graph (2D). From week 1 on high levels of luciferase activity are measured in both groups. Despite a 10× times lower titer injected for the pgk-Luc group the expression levels slightly higher than measured in the cmv-Luc group. In both groups luciferase activity declines overtime, however at 47 weeks post injection pgk-Luc is still expressed above background.
(2E) Representative IVIS derived images (dorsal view) of two individual rats (pgk-rtTA and pgk-GARrtTA) overlaid with a heat map of the bioluminescence signal shows luciferase activity in the presence (dox, green) or absence of doxycycline over time, quantification in graph (2F). A low leaky expression is present in the pgk driven transactivator groups before dox induction (week 1 and 2). Stimulation with dox induces a high level of luciferase expression in week 3 in the pgk-rtTA and the pgk-GARrtTA group. In week 4 and 5 the levels of expression gradually decline but stay well above non-induced levels. A second round of dox induces high luciferase expression again in both groups. Pgk-rtTA animals show a decrease in bioluminescence in the following 2 weeks (week 10 and 11) despite continuous dox feeding. Pgk-GARrtTA mediated luciferase expression is stable over this period. Also in the 3th, 4th and 5th round of induction the level of expression is stable in the pgk-GARrtTA group whilst the levels continue to decline in the pgk-rtTA group. Graphs show group means ± SD, 5 animals per group, *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$, ns = non-significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

week we started measurements of luciferase activity. Bioluminescence heat maps of an individual pgk-rtTA-treated and a pgk-GArrtTA-treated rat show the regulation of luciferase activity in response to dox over time (Fig. 2E). In the first two weeks after viral vector injection and in the absence of dox, the pgk driven transactivator groups showed a leaky expression of 3 to 4 times above background (pgk-rtTA, $p < 0.05$; pgk-GArrtTA, $p < 0.01$) (Fig. 2F). Supplementing the regular diet with dox resulted in a 336 (745 times background) and 11 (48 times background) fold induction in bioluminescence measured in the pgk-rtTA and the pgk-GARrtTA groups respectively in week 3 compared to non-dox week 2 (rtTA, $p < 0.0001$; GARrtTA, $p < 0.0001$). Luciferase activity declined in week 4 and 5 in the pgk-rtTA group to 287 and 240 ($p < 0.0001$) and in the pgk-GARrtTA group to 28 and 18 ($p < 0.0001$) times background on continuous dox feeding during this period. Subsequent removal of dox reduced luciferase expression to 2 (pgk-rtTA, $p < 0.0001$) and 3 (pgk-GARrtTA, $p < 0.0001$) times above background measured in week 8. This shows that pgk driven transactivators effectively induce transgene expression in the adult spinal cord upon oral dox administration.

In contrast to our observations in the cmv driven transactivator experiment, a second round of dox administration to the pgk driven transactivator groups did result in the re-induction of transgene expression. In the first week of the second dox cycle (week 9), the pgk-rtTA group showed a 40 fold (55 times background) increase in bioluminescence after dox administration compared to the non-dox week 8 before. The pgk-GARrtTA group displayed a 3 fold (10 times background) increase in luciferase expression in this period. Continued dox feeding in the next 2 weeks resulted in continuous luciferase activity compared to the non-dox period in both groups, however, in the pgk-rtTA group the bioluminescence levels decreased to 17 and 14 times background. In the pgk-GARrtTA group luciferase activity does not decline and stayed stable at 10–13 times background in week 10–11. Upon removal of dox, in both groups the luciferase iferase activity returned to about 1–3 times background activity.

In the first 2 cycles of dox induction in the pgk-rtTA group the luciferase expression in the 1st week of the 3 week dox cycle is higher than in the following 2nd and 3rd week of that cycle. We therefore decided to prolong the 3rd round of dox feeding to 9 weeks to examine if the expression would further decline or stabilize after the initial drop in luciferase -activity after the first week. In week 15 (first week dox of round 3) the luciferase expression reached 14 times background and then quickly dropped to 4–5 times background in the following 5 weeks (week 16–20) in the pgk-rtTA animals. After 9 weeks (week 23) of continuous dox, luciferase activity declined to 2 times background activity in the pgk-rtTA group. In contrast, continued dox feeding in the pgk-GARrtTA group showed, after the initial 3rd round induction of luciferase expression in the first week (week 15), no decline of luciferase expression in the subsequent weeks. Thus, Luciferase activity in the pgk-GARrtTA group was stable at around 9 times background expression for the 15 to 23 week period (that is for the entire third round of dox administration).

To determine the long term inducibility of transgene expression in the pgk-rtTA and pgk-GARrtTA group, animals received dox-food in week 32, 40 and 47. In the pgk-rtTA group luciferase expression was hardly observed following re-induction (2 times above background). This in contrast to the pgk-GARrtTA group where we observed a consistent and stable 3 to 4 fold (6 to 7 times background) induction of transgene expression in all of these late dox-on phases and a subsequent reduction in expression during dox-off phases.

Together these data show that pgk driven transactivators can be used to induce two rounds of transgene expression which was not possible with cmv driven transactivators. Importantly, the data reveal that pgk-GARrtTA out performs pgk-rtTA in the third dox-cycle and continues to be inducible for at least 47 weeks in three subsequent dox-cycles. Thus, although pgk-rtTA driven expression is initially higher (week 1–15, $p = 0.0294$), the induction continues to decline over time, whereas pgk-GARrtTA induced expression is initially lower but is more stable and consistent and luciferase-expression can be reinduced from 16 weeks onward reaching significance over the 23 to 47 week period ($p = 0.0475$). A comparison of the trendlines of the peak expression of each dox cycle of pgk-rtTA and GARrtTA reveals the steep decline of pgk-rtTA induced expression while pgk-GArrtTA shows a more stable level of induced expression (Fig. 2G).

3.4. Changes in cellular morphology and presence of debris in pgk-rtTA, but not in pgk-GARrtTA treated spinal cords

After 47 weeks immunohistochemistry was performed on sagittal spinal cord sections from the pgk-rtTA and the pgk-GARrtTA injected animals to determine which cell types express luciferase (Fig. 3). In the pgk-rtTA group only a small number of the luciferase-positive neurons was observed. These cells appeared less healthy compared to the pgk-GARrtTA animals. The small number of remaining luciferase expressing neurons in the pgk-rtTA group often exhibited swollen or rounded cell bodies with short processes displaying plasma membrane blebbing (arrows in Fig. 3A, high magnification in insert), whereas luciferase positive neurons in the pgk-GARrtTA group had a normal neuronal appearance with clear normal looking processes (Fig. 3G). In addition to the “unhealthy” neuronal appearance in the pgk-rtTA group, we observed luciferase-positive cellular debris in the spinal cord segments of the pgk-rtTA group (Fig. 3B-F). Cellular debris was present in the gray and white matter and appeared as small aggregates that were autofluorescent in both the green and red fluorescent channel (area marked with asterisks in Fig. 3B-F). The virus injection sites of pgk-rtTA injected animals contain more debris particles than pgk-GARrtTA injected animals (Fig. 3M). Staining for the glial marker GFAP showed that especially in the white matter the areas where debris was present had lost

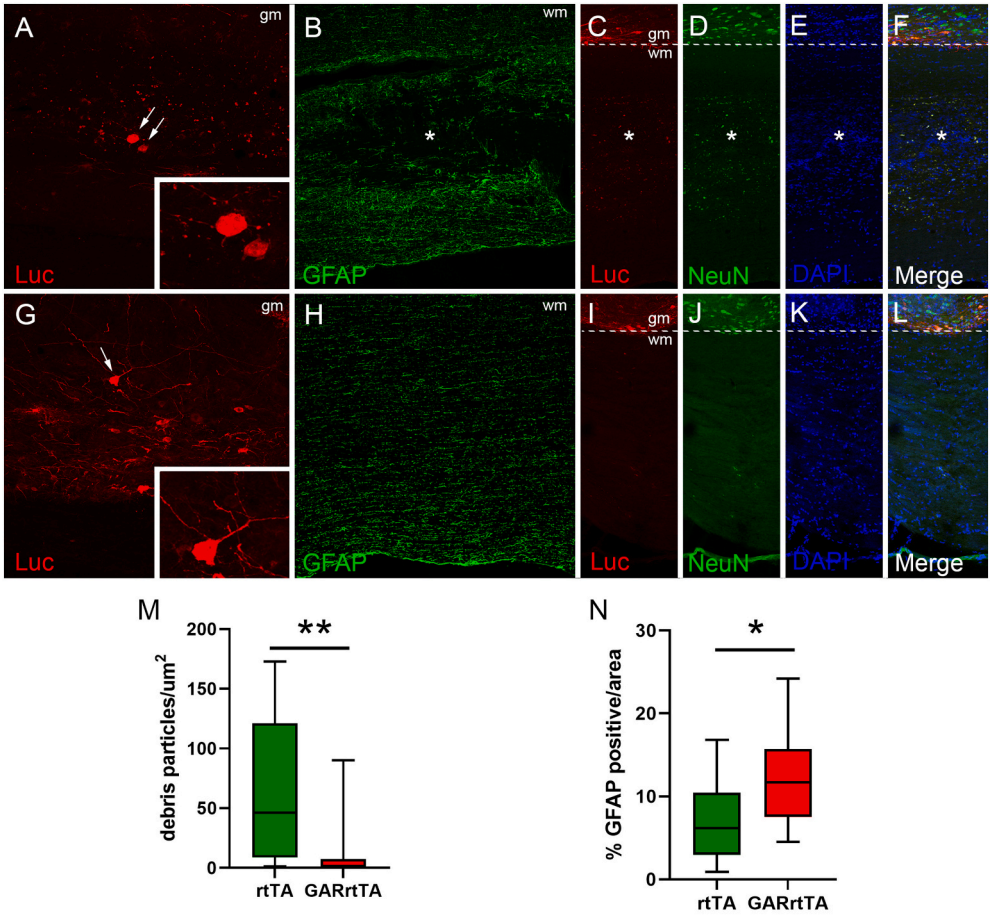

Fig. 3. *Spinal cords of the GARrtTA group appear healthier after 47 weeks of transgene regulation.* Immunohistochemistry on sagittal spinal cord sections of pgk-rtTA (3A-F) and pgk-GARrtTA (3G-L) injected animals reveals luciferase (Luc, red) expression in neurons (NeuN, green) in the gray matter (gm). All animals in the pgk-rtTA group have debris in the white matter (wm) (3A-D, visible in all channels, area marked with *). In the pgk-GARrtTA groups there was no evidence of debris (3G-L). Quantification of the number of debris particles shows that rtTA expression results in more debris particles than GARrtTA expression (3 M). Luciferase expressing neurons in the pgk-rtTA animals often have short processes and a swollen soma (3A, red, arrows, insert shows high magnification) while neurons in the pgk-GARrtTA group appear healthy and have normal processes (3G). White matter areas (wm) in the pgk-rtTA animals frequently showed loss of GFAP expression (3B, green, marked with *). No loss of GFAP expression was observed in pgk-GARrtTA animals without cellular debris in/around the injection site (2H), quantification in 3 N. Box plot shows means, whiskers: min to max, 5 animals per group. * $p < 0.05$, ** $p < 0.01$, Scale bar = 200 μm for 3A, B, G, H, 100 μm for 3I—F, 3I-L. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GFAP positive astrocytes (Fig. 3B). No loss of GFAP positive glia was observed in the spinal cord areas where no cellular debris was observed in the GARrtTA group (Fig. 3H). Quantification confirms the loss of GFAP expression in pgk-rtTA compared to pgk-GARrtTA injected animals (Fig. 3N).

3.5. Survival of GARrtTA expressing cells

Because there are no antibodies available that detect rtTA in tissue sections we generated V5-tagged transactivators to analyze the survival of transduced cells expressing the transactivator in vivo by performing immunohistochemistry using antibodies against the V5-tag (Fig. 4). V5-tagged transactivators are still fully functional in vitro (data not shown). We first performed a study to demonstrate the expression of the transactivators based on detection of the V5-tag in vivo. This revealed that 14 days after injection of LV-pgk-rtTA-V5 or LV-pgk-GARrtTA-V5 numerous V5 positive cells can be detected in the spinal cords of both groups (Fig. 4A, B). Most of the transactivator expressing cells have a neuronal-like appearance and do not co-label with the astrocyte marker GFAP (Fig. 4A, B). Next, the presence of V5 positive cells was studied at 7 and 14 weeks after injection of LV-pgk-rtTA-V5 and LV-pgk-GARrtTA-V5. At 7 weeks there are only a few rtTA positive cells remaining, while there are still many GARrtTA expressing cells present (Fig. 4C vs. 4D). Quantification of the V5-positive area shows an ~11 fold difference between rtTA and GARrtTA (Fig. 3F). Also 14 weeks after transduction there are many more GARrtTA positive cells left in comparison to rtTA positive cells, resulting in a ~ 6 fold difference in the V5-positive area (Fig. 4E, F, quantification in 4G).

3.6. The GAR domain provides immune evasive properties to the transactivator in vivo

To determine if the maintained expression of GARrtTA is associated with a reduced recruitment of immune cells in the transduced spinal cord area we used fluorescence-activated cell sorting (FACS) (Fig. 5). Spinal cords of rats injected with LV-pgk-rtTA or LV-pgk-GARrtTA were collected seven weeks after transduction. This post-injection timepoint was chosen because GARrtTA-induced luciferase expression is stable whereas rtTA-induced luciferase expression is rapidly declining (Fig. 1). In addition to the transactivator groups, an LV-pgk-empty group was included in the experiment to control for a possible immune reaction evoked by the lentiviral particles themselves. In the FACS data (Fig. 5A, summarizing quantification in 5B) we observed no differences in the numbers of resident (microglia) and infiltrating (macrophages Cd43- and CD43+ and polymorphonuclear neutrophils) innate immune cells between groups (Fig. 5B). We also found similar numbers of antigen presenting dendritic cells in the injected spinal cord segment of all three groups. And the number of B-lymphocytes in the transduced spinal cord area was not different between the groups (Fig. 5C). However, seven weeks after lentivirus injection there is a difference in the number of T-cells present in the classical rtTA compared to the GARrtTA expressing spinal cords. Although the trend for an increase in the number of CD4 positive T-cells in the rtTA group is not significantly different from empty and GARrtTA ($P = 0.1970$ and 0.1937 respectively, Fig. 5D) there is a significant increase in the number of CD8 positive T-cells in rtTA transduced spinal cords compared to empty ($P = 0.0171$) and GARrtTA ($P = 0.0121$, Fig. 5E). The number of CD8+ T-cells in the GARrtTA injected group is not significantly different from the empty vector

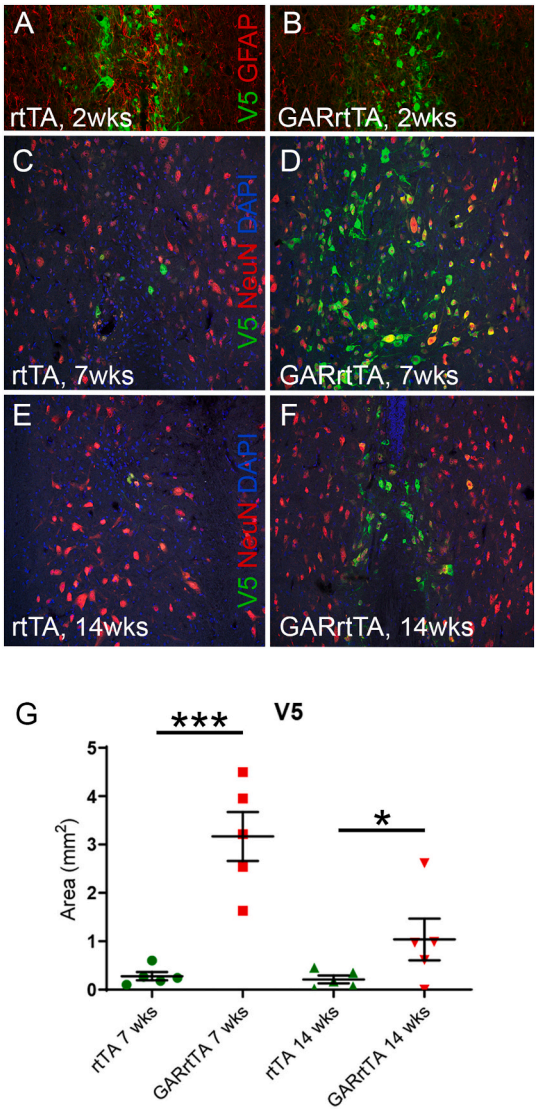

Fig. 4. *Better survival of GARrtTA expressing cells compared to rtTA expressing cells*. Immunohistochemistry for the V5-tag (green) on longitudinal spinal cord sections shows that 2 weeks after virus injection both rtTA-V5 (4A) and GARrtTA-V5 (4B) are equally well expressed. Most transactivator expressing cells are negative for the astrocyte marker GFAP (red, 4A, 4B). The number of rtTA expressing cells is dramatically decreased compared to GARrtTA expressing cells 7 and 14 weeks after injection. Quantification of the V5 positive area in 4F. The majority of the transactivator expressing cells have a neuron-like appearance and double label for the neuron marker NeuN (red, 4C, D, E, F). Graphs show group means ± SEM, 5 animals per group, *** $p < 0.0001$, * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

control injected animals ($P = 0.9403$). Together this immune cell characterization revealed that the fusion of the GAR-peptide to the transactivator prevents the recruitment of cytotoxic CD8+ T-cells to the spinal cord transduction site.

4. Discussion

In this study we characterized the in vivo performance of a doxycycline dependent lentiviral gene switch based on an immune-evasive GARrtTA transactivator driven by a pgk promoter. We show that this gene switch enables reliable regulation and repeated reinduction of transgene expression in the adult rat spinal cord for a period of nearly one year. The addition of the GAR domain to the N-terminus of rtTA results in a transactivator that prevents the recruitment of CD8+ cytotoxic T-cells and significantly improves the survival of transactivator expressing cells in the spinal cord.

In the original doxycycline inducible lentiviral vector system, the immune-evasive transactivator expression was under the control of the human cytomegalovirus (cmv) major immediate-early promoter (Hoyng et al., 2014a). The viral origin of this promoter makes it vulnerable to epigenetic silencing (Mehta et al., 2009; Herbst et al., 2012). This likely explains the failure to re-induce luciferase expression in the spinal cord when cmv is used to direct transactivator expression. To circumvent epigenetic silencing the cmv promoter was replaced by a pgk promoter. The pgk promoter has been shown to be an efficient driver of virally delivered transgene expression in various brain regions (Deglon et al., 2000; Delzor et al., 2012; Andrews et al., 2016; Nieuwenhuis et al., 2021) and in the spinal cord (Bartus et al., 2014). A comparative analysis of the cmv and pgk promoters in the rat spinal cord revealed that a dose of approximately 10 times less viral particles of LV-pgk-Luc was sufficient to reach comparable levels of luciferase expression. Moreover, the levels of pgk-directed transgene expression continued to increase during the second and third weeks, whereas with the cmv promoter, transgene expression had already started to decline after the first week. This corroborates that the pgk promoter is a much stronger transcriptional activator of virally delivered transgene expression than the cmv promoter (Bartus et al., 2014; Nieuwenhuis et al., 2021). Despite being a strong mammalian promoter which is not subject to epigenetic silencing, pgk-mediated transgene expression gradually declines over time after 3 weeks. This decline may have multiple reasons. First, injection of the viral particles with a glass needle may compromise the viability of some cells in the injection area resulting in gradual degeneration of a subset of transduced cells overtime. Second, an immune response evoked by the lentiviral envelope may lead to cell loss in the injected area (Abordo-Adesida et al., 2005; Brown et al., 2007). Finally, the random integration of lentiviral vector DNA in the host genome may interfere with cellular gene expression resulting in the gradual degeneration of a subset of lentiviral vector transduced cells (Cesana et al., 2014).

The ability of dox to reliably re-induce luciferase expression 6 times over a long time course (almost one year) demonstrates the robustness of the pgk driven immune evasive gene-switch. The beneficial effect of adding the GAR-peptide to rtTA becomes clearly visible between 8 and 15 weeks post-injection when luciferase expression induced by GARrtTA surpasses rtTA induced luciferase expression. While rtTA induced luciferase expression continues to decline, GARrtTA mediated transgene expression is stabilizing in the second dox-cycle around week 8 and allows repeated induction of transgene expression for at least 47 weeks. Histological analysis shows that persistent long-term functional activity of GARrtTA is accompanied by with the presence of more and healthier luciferase-positive neurons in the spinal cord which contrasts with the loss of transduced cells, the presence of cellular debris and diminished GFAP expression, observed in the rtTA group. The differential temporal expression profiles of V5-tagged rtTA and GARrtTA suggests that a loss of V5-rtTA expressing cell between 2 and 14 weeks post-injection is responsible for the loss in rtTA-mediated luciferase expression.

In a previous study we demonstrated that lentivirus mediated expression of rtTA, but not GARrtTA, in the contusion injured spinal cord resulted in an increase in gamma-interferon and CD8b expression (Burnside et al., 2018). This is indicative of a T-cell mediated immune response against rtTA expressing cells. The FACS analysis in the current study provides direct evidence that the number of CD8+ T-cells is increased in the spinal cord following expression of rtTA, but not after expression of GARrtTA. This suggests that GARrtTA expressing cells are not presenting transactivator derived antigenic peptides on their cell surface and are therefore not subjected to CD8+ T-cell mediated immunological destruction.

Apart from the earlier mentioned reduced proteasomal degradation of GAR domain carrying proteins, two other potential mechanisms have been hypothesized to mediate the immune evasiveness of the GAR. One

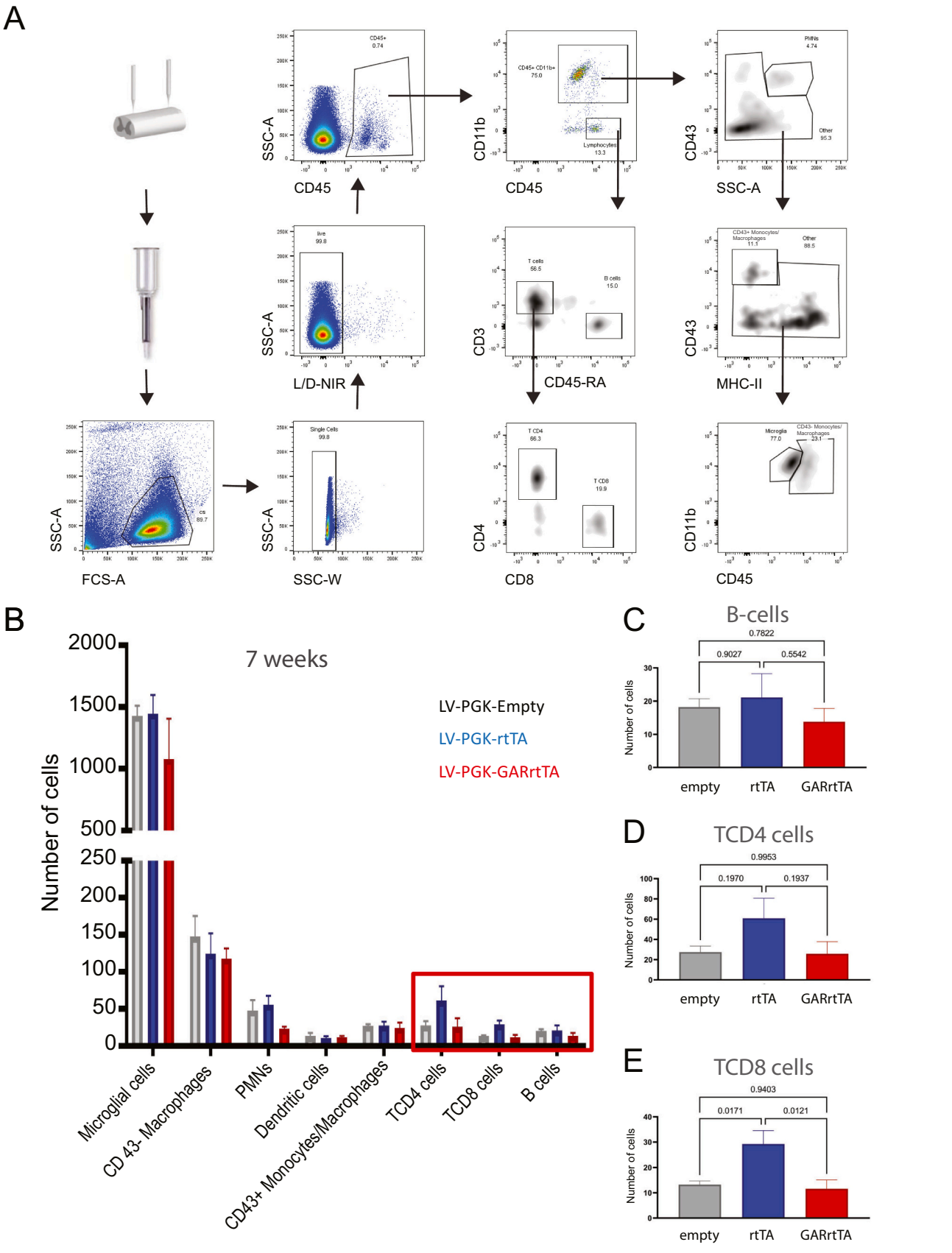

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Fig. 5. *Reduced immune response in pkg-GARrtTA injected spinal cords.* Fluorescence-activated cell sorting for immune cell markers performed on the transduced spinal cord segment 7 weeks after injection of LV-pgk-rtTA, LV-pgk-GARrtTA or LV-pgk-empty control (overview of the procedure in 5A). Summary of the immune cell quantification is shown in 5B. All groups have similar numbers of resident (microglia) and infiltrating (macrophages CD43- and CD43+ and polymorphonuclear neutrophils) phagocytotic cells. No differences in the number of dendritic cells between the group were detected (5B). Closeup graphs of the number of B-cells (5C), CD4+ T-cells (5D) and CD8+ T-cells (5E) measured in the transduced area show that the LV-pgk-GARrtTA group looks very similar to the empty vector control group. While in the LV-pgk-rtTA group significantly higher numbers of CD8+ T-cells are found in the injected spinal cord (5B). Graphs show group ($N = 6$ per group) means ± SEM and p-values.

hypothesis is that secondary G-quadruplex structures that can form in the DNA sequence of GAR act as negative regulators for gene transcription (Siddiqui-Jain et al., 2002; Holder and Hartig, 2014; Pradhan et al., 2020). This is in agreement with the previously observed lower levels of GARrtTA mRNA compared to rtTA mRNA after titer-matched infection of HEK293T cells (Hoyng et al., 2014a). In addition, G-quadruplex structures in GAR mRNA have been shown to reduce initiation of translation (Kumari, 2007, Murat, 2014).

The second hypothesis highlights the *in* cis role of the GAR protein domain in suppression of different translation initiation events in the cell (Yin et al., 2003; Apcher et al., 2009; Apcher et al., 2010; Cardinaud et al., 2010). An N′-terminally positioned GAR domain has been shown to limit both the number and rate of translational initiation throughout the entire mRNA by inhibiting the assembly of upstream initiation complexes. This suppression of antigenic peptide synthesis from all reading frames reduces the processing and subsequent presentation of antigens by MHC class I molecules. Thus, reduced presentation of early translation initiation generated transactivator peptides on the cell surface by MHC class I molecules could help to prevent detection of the GARrtTA transduced cells by the immune system. Both mechanisms may contribute to the increased survival of GARrtTA expressing cells in the spinal cord.

Overall GARrTA performs better than rtTA except during the first dox-cycle where rtTA is a stronger activator of luciferase expression than GARrtTA. The lower induction of luciferase expression by GARrtTA compared to rtTA indicates that the addition of GAR to rtTA renders the new transactivator less efficient, affects its biosynthesis or a combination of these possibilities. We previously showed that infection of HEK293T cells with titer-matched LV-cmv-GARrtTA and LV-cmv-rtTA results in lower GARrtTA than rtTA mRNA synthesis (Hoyng et al., 2014a) while the accompanying protein levels were higher for GARrtTA then for rtTA. The higher protein levels may be attributed to the increased stability and reduced proteosomal degradation reported for GAR domain containing proteins (Levitskaya et al., 1997; Sharipo et al., 1998; Dantuma et al., 2000; Ossevoort et al., 2003; Zhang and Coffino, 2004). However, increased GARrtTA protein levels did not lead to a better induction of transgene expression in vitro, indicating that the GARrtTA protein is more stable but is a less efficient transcriptional activator than rtTA (Hoyng et al., 2014a). The lower induction of luciferase observed in the first dox cycle is therefore most likely due to the reduced efficiency of GARrtTA compared to rtTA.

Although this study does not provide insight in the possible mechanisms by which the GAR domain may reduce the efficiency of rtTA the addition of the GAR domain (resulting in a three times larger protein, 248 vs. 678 amino acids) may cause steric hindrance or reduce the binding affinity of the rtTA to doxycycline and/or to the TRE. Increasing the dox concentration has been shown not to compensate for the loss of GARrtTA efficiency in vitro (Hoyng et al., 2014a). Increasing total GARrtTA protein levels does improve dox induced transgene expression, suggesting that rtTA binding to TRE is affected by the addition of GAR. This could be due to a less stable or incomplete conformational change of GARrtTA upon dox binding or a lower affinity of the GARrtTA-dox complex to the tet-responsive elements in the promoter. Alternatively, the GAR domain could interfere with the recruitment of additional transcription factor components to the inducible promoter resulting in reduced transgene expression.

For a wide range of future gene therapy applications it will be advantageous that the expression of the transgene can be restricted to the therapeutic window. This requires that in time transgene expression can be switched “on” and “off” at will. The doxycycline-transactivator system offers this possibility, however, the immunogenicity of the transactivator is currently a major limitation for the use of this system in a clinical setting. Protection from the adaptive immune response could be particularly important for applications in neurological disorders where there is a large inflammatory component e.g. traumatic brain and spinal cord injuries. But also in conditions where there is neuronal degeneration and death, not only would this lead to loss of the transgene expression as the transactivator containing cells get removed by T cells, but this would exacerbate the cell loss in these conditions. Here we show that the addition of the GAR peptide to the original bacterial-derived transactivator successfully mimicked the immune-evasive strategy of the Epstein-Barr virus EBNA1 protein thereby preventing the immune-mediated loss of the transactivator expressing cells in vivo. In contrast to the classical rtTA, this “stealth” GARrtTA offers the opportunity to reliably control the expression of a therapeutic transgene in vivo.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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