Combination drug treatment prolongs survival of experimentally infected mice with silver-haired bat rabies virus

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ABSTRACT

Rabies is a lethal disease in humans and animals, killing approximately 60,000 people every year. Currently, there is no treatment available, except post-exposure prophylaxis (PEP) that can be administered whenever exposure to a rabid animal took place. Here we describe the beneficial effects of a combination treatment initiated at day 4 post infection, containing anti-viral drugs and immune modulators in infected mice. Combination therapy resulted in significant increase in survival time ($P < 0.05$) and significantly lowers viral RNA in the brain and spinal cord ($P < 0.05$). Furthermore, treatment influenced markers of pyroptosis and apoptosis and early inflammatory response as measured by the levels of TNF-α. Morphological lesions were absent in rabies virus infected mice with few signs of inflammation. However, these were not significant between the different groups.

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1. Introduction

Rabies virus (RV) causes deadly encephalitis in animals and humans, for which there is no treatment available to date. Once neurological symptoms appear in exposed individuals, almost 100% of patients die. RV belongs to the family *Rhabdoviridae* and is classified within the genus *Lyssavirus*. Disease is caused by any of the known Lyssaviruses and it is believed that the mechanism of pathogenesis does not differ between the different members of this genus. There is a vaccine available against the prototype RV, which is effective to prevent lethal infection and is the fore being used in combination with hyperimmune serum as post-exposure prophylaxis (PEP) [1]. The vaccine can be effective to a certain extend against other lyssaviruses depending on the virus species and the seed RV used for vaccine production [2]. Despite the availability of effective vaccines and the possibility to apply PEP in exposed individuals, rabies still accounts for more than 60,000

deaths annually, mainly among children and young adults in resource-poor countries [3].

In developed countries where sporadic cases occur, patients will be typically admitted to intensive care units, coma is induced and often experimental treatment protocols are used, but so far with limited success. The antivirals ribavirin and interferon-alpha (IFN-α) administered intrathecally showed no effect on disease course [4] whereas the benefit of antibodies given intrathecally is not known [5] although it might be associated with brain herniation [6]. In a rabbit model of rabies, intrathecal administration of an RV vaccine could increase survival of rabid animals [7]. To date, only a few patients survived infection with RV, but although it is not clear whether the applied interventions contributed to survival, it is believed that the treatment allowed the patients to mount a natural immune response to rabies, as evidenced by development of neutralizing antibodies [8]. Although presence of neutralizing antibodies in the rabies patients is not a guarantee for survival, these observations led to the hypothesis that the immune system could control and clear virus infection under certain (yet to be defined) conditions. Despite the possible involvement of the immune response in recovery from rabies, it

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is conceivable that infection with RV (or related lyssaviruses) triggers an excessive, detrimental host response that needs to be controlled in order for the infected individual to survive [9,10]. However, severe inflammation in human and animal fatal rabies cases is usually not accompanied with extensive leukocyte infiltration in the brain and spinal cord [11]. Nevertheless, high levels of cytokines and chemokines were demonstrated, in absence of leukocyte infiltration, in transcriptomic studies of experimentally infected mice [12]. Recent work in our lab has demonstrated activation of inflammatory responses and activation of the pyroptotic cell death pathway by transcriptomic analysis during RV infection [13]. Furthermore, based on our transcriptomic observations, selected molecules that were upregulated during RV infections were chosen as possible intervention strategies in the mouse model. We have found that inhibitors of molecules such as caspase 1 (key pyroptotic molecule), MAP-kinases and TNF-α significantly prolonged survival of mice infected with a lethal dose of RV either given alone or in combination [14–16].

In this study we describe the effect of combination therapy that targets viral replication and some pathways of the immune response. We have compared the survival time, viral titers and mRNA responses in experimentally infected mice and showed that targeting both viral replication and part of the immune response have a beneficial effect on the outcome of infection.

2. Materials and methods

2.1. Viruses and drugs

The highly pathogenic silver-haired bat rabies virus (SHBRV-18, a kind gift of Dr. B. Dietzhold, Jefferson University, USA) was used in these studies. Viral stocks were generated on mouse neuroblastoma N2a cells and titers were determined by end-point titration on BHK-21 cells and calculated using the Karber method as previously described [17].

A combination of six different compounds was used to formulate the treatment regimen (drugs; Table 1): caspase-1 inhibitor (200 ng/mouse of Ac-YVAD-cmk from Sigma), TNF-α inhibitor (0.1 mg per mouse of REMICADE from Janssen Pharmaceuticals), MAP-kinase inhibitor (SORAFENIB tosylate 0.6 mg/mouse from Bayer Health Care Pharmaceuticas), mouse IFN-α and β (6000 IU each per mouse from PBL Assay Science), favipiravir (T705, 6 mg per mouse from BOC Sciences), ribavirin (3.2 mg per mouse from Tocris Bioscience) and human rabies immunoglobulins (HRIG 0.8 IU per mouse from NIBSC: WHO International Standard, The 2nd International Standard). Mannitol was used as a blood-brain barrier opener (0.5 ml of 25% mannitol from Sigma per mouse).

2.2. Infection experiment

Eight-week-old female C57BL/6 mice were infected via the intramuscular route in the left hind leg with $10^{6,13}$ TCID$_{50}$ of virus per mouse. All mice were weighted every day and scored for signs of disease every two hours with the 4 h night break between midnight and 4 A.M. according to the following scheme [18]: Score 0: no signs. Score 1: ruffled hair and hunched back. Score 2: paralysis of the inoculated leg, spasms. Score 3: paralysis in both hind legs, severe spasms, circular movement, tail paralysis. Score 3 was considered the humane end point of the experiment and mice were euthanized by cervical dislocation when this score was reached.

2.3. Treatment with combination therapy

Two sets of experiments were conducted abbreviated as "survival" and "50% mortality". An overview of the different treatment groups is given in Table 1. Briefly, groups of 8-week-old female C57BL/6 mice were inoculated intramuscularly in the left hind leg with $10^{6.3}$ TCID$_{50}$/mouse of SHBRV-18. Four days after inoculation, treatment was initiated with the combination of compounds (described above) diluted mostly in DMSO (Sigma) or water was injected intraperitoneally in 1 ml volume. Thirty minutes after treatment with the combination of drugs mannitol was given intraperitoneally in the respective groups (Table 1). For the survival experiments,. treatment with the combination of drugs was given on days 4–12. Control groups included mice that were inoculated with virus, but did not receive any treatment or received the treatment combination (with and without mannitol), but received DMEM instead of virus (mock infection).

For the 50% mortality experiments, mice were inoculated with virus (or DMEM) as described above and received the combination treatment (with and without mannitol or no treatment) on days 4, 5, 6 and 7. Then all mice of these groups were euthanized in order to compare the effect of treatment on a pre-determined time point after infection (with mannitol: day 7; without mannitol: day 8).

2.4. Real time PCR

Brain samples were collected at the time of euthanasia from all mice, weighted and stored at −80 °C as 10% homogenate in DMEM medium (Lonza, Basel, Switzerland) containing 10% penicillin and streptomycin (Lonza). RNA was isolated using the MagNA Pure LC 1.0 system and High Pure isolation kit (Roche, Mannheim, Germany) according to the manufacturer's procedures and quantified using NanoDrop™ ND-1000 UV–VIS Spectrophotometer (NanoDrop Technologies, Wilmington DE, USA). Viral RNA was detected using SHBRV-18 specific primers/probe combination as previously described [19] with a modification of the SHBRV-18 probe and the TaqMan™ Fast Virus Master Mix (Applied Biosystems, Foster City, CA, USA) (according to the instructions of the manufacturer in a Applied Biosystems 7500 Fast Real-Time PCR System (TaqMan)). To measure expression level of several molecules involved in the pyroptotic and apoptotic pathways, mRNA was transcribed into cDNA using Oligo(dT)$_{12-18}$ Primer (Invitrogen, Carlsbad CA, USA) and Superscript III reverse transcriptase (Invitrogen) according to the instructions of the manufacturer. The relative level of transcripts was quantified using commercially available TaqMan® Gene Expression Assays of the respective genes (Applied Biosystems, Foster City, CA, USA) and TaqMan® Universal PCR Master Mix (Applied Biosystems). Transcript numbers were expressed relative to the housekeeping gene β–actin (Applied Biosystems) following the formula $2^{-\Delta Ct} * 10^5$ where $\Delta Ct = Ct_{gene\ of\ interest} - Ct_{\beta-actin}$ and were corrected for RNA quantity.

2.5. Histology and immunohistochemistry

For evaluation of microscopic abnormalities and for immunohistochemistry the right side of the mouse brain was placed in 10% neutral-buffered formalin. Brains were cut in six equal parts in coronal plane, which were placed in two cassettes and then embedded into paraffin wax. Paraffin blocks were cut in

Table 1
Overview of the experimental groups of mice that received infection and treatment. Details of the viruses and the drugs used are described in the materials and methods section.

	Survival	50% mortality
Virus only (V)	N = 9	N = 9
Virus + drugs + mannitol (V + D + M)	N = 9	N = 9
Virus + drugs (V + D)	N = 13	N = 13
Drugs + mannitol (D + M)	N = 8	N = 8
Drugs only (D)	N = 8	N = 8

4-µm-thick serial sections and routinely stained with haematoxylin and eosin to detect light microscopic lesions. Immunohistochemistry was performed to detect RV antigen and for the detection of neuron specific antigen in specific cases. Briefly, deparaffinised tissue sections were treated with 0.5% hydrogen peroxide in 85% ethanol to quench endogenous peroxidase activity. After antigen retrieval by incubation with 0.25% Trypsin, non-specific binding was blocked using 20% normal goat serum diluted in phosphate buffered saline (PBS). Slides were incubated with a caprine polyclonal anti-rabies antibody (PA1201; FITC-conjugated; SIFIN, Berlin, Germany; 1:500) at 4 °C overnight. Biotinylated goat-anti-FITC IgG (BA0601; Vector; 1:200) was used as secondary antibody for 30 min at room temperature. Finally, immunolabelling was visualized applying the avidin–biotin–peroxidase complex (ABC) method (PK6100; Vector Laboratories, Burlingame, CA, USA) with 3,3-diaminobenzidine-tetrahydrochloride (DAB) as chromogen followed by slight counterstaining with Mayer's haematoxylin.

Evaluation of inflammatory cell infiltration and antigen labelling was performed using appropriate semi-quantitative scoring systems from 0 to 3. Inflammation was scored on haematoxylin and eosin (H&E) stained paraffin sections according to the number of inflammatory cells in a 400x high power field (0: 0; 1: 1–5; 2: 6–15; 3: >15). Analysis of immunohistochemically stained sections was based on the number of cells positive for rabies virus antigen in a 400x high power field (0: 0; 1: 1–7; 2: 8–15; 3: >15). Telencephalon, hippocampus, di-/mesencephalon, cerebellum, and medulla oblongata were evaluated separately and a mean score was determined for each animal evaluated.

2.6. Statistical analyses

Body weight between the different groups was compared using Bonferroni's multiple comparison test (ANOVA). Survival curves were compared with the Log-rank (Mantel-Cox) test. Viral and mRNA levels were compared with the Mann-Whitney non-parametric test. Inflammation and virus antigen in the brain of infected animals was compared with the Kruskal-Wallis test. All statistical analysis was performed using GraphPad software (Prism 5). Using bonferroni correction the p-value was adjusted for multiple testing, resulting in a significance level of ≤ 0.0036.

3. Results

3.1. Survival mice

We studied the effect of combination therapy on SHBRV-18 infected mice in two different experimental designs. First we studied the effect of the combination therapy on survival of experimentally infected mice. Treatment of infected mice with the drug combination led to weight loss (Fig. 1a), possibly as a result of toxicity. Using ANOVA (Bonferroni's multiple comparison test), we demonstrated that the body-weight means in the V + D + M group and V + D groups were significantly lower compared to the virus control group (P = 0.04). The V + D + M group had the lowest body weight means although not significantly lower than the V + D group (P > 0.05). The control groups (V, D, D + M) did not show any weight loss, suggesting that the treatment alone did not have a significant effect on the body weight of the mice.

Nevertheless, treatment had a positive benefit on prolongation of survival. Median survival time of the V + D + M group was prolonged with 36 h, whereas in the V + D group it was prolonged with 48 h compared to virus control group (Fig. 1b). These differences were significant (p = 0.0156 and p = 0.0093 respectively). To see whether the increased survival time correlated with amount
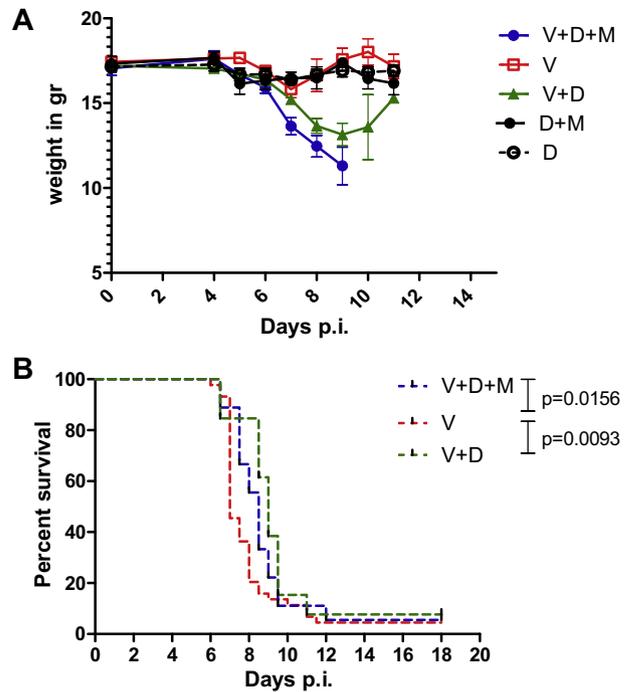

Fig. 1. Measurement of weight (A) and survival curves (B) of mice infected with SHBRV-18, treated with combination therapy and followed for survival. Respective virus and drug controls are also shown. In the weight curves the mean and standard error of the mean are depicted. In the survival curves, mortalities were recorded per 12 h. Survival curves were compared with the Log-rank (Mantel-Cox) test.

of virus in the brain, we compared viral RNA in treated and untreated groups. V + D group of mice had significantly lower RNA in the brain compared to V + D + M group (P = 0.001) and to V group (P = 0.0004) (Fig. 2). In contrast, RNA in the spinal cord was lowest in the V + D + M group compared to virus control (P = 0.002). No difference was measured in the level of RNA in the spinal cord of the V + D group compared to virus control (P > 0.05).

3.2. 50% mortality mice

In a second set of experiments we sought to investigate the effect of treatment (with and without opening the blood-brain barrier (BBB) and the blood-spinal cord barrier (BSCB)) measured on a predetermined time point, by comparing virus titers, expression of
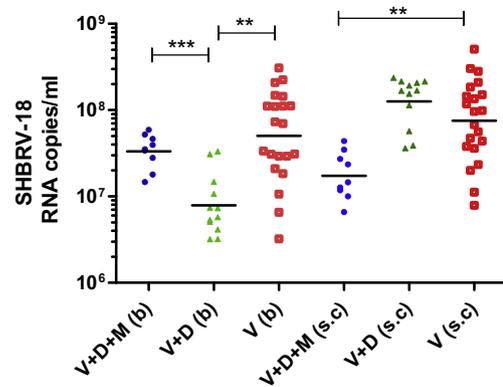

Fig. 2. Viral RNA in the brain (b) and spinal cord (s.c) of treated mice (V + D + M and V + D) compared to virus control groups (V). Mice that were followed for survival are depicted here. Asterisks indicate significant differences (Mann-Whitney non-parametric test).

mRNA of several markers and inflammation in the brain. We assumed that the best time point to compare the effect of treatment was when 50% of the virus control mice had succumbed to rabies infection. To this end, at 7 (with mannitol) and 8 (without mannitol) days post infection (when 50% of the mice in the virus control group had reached the humane end points and were euthanized) all remaining mice were euthanized irrespective of the presence of clinical signs of disease.

First, we measured no significant weight loss in the 50% mortality groups. Next we compared viral RNA titers in the brain and spinal cord of treated mice.

As shown in Fig. 3, viral RNA titers did not differ significantly in the brains of treated mice (V + D + M or V + D) compared to virus control. In contrast, the data suggest that viral RNA in the spinal cord was significantly lower in the V + D + M group compared to virus control (P < 0.0001) (Fig. 3). Furthermore, we investigated the effect of the compounds that we used on host responses. Since our combination treatment included inhibitors of pyroptosis and inflammatory response, we measured the expression of the mRNA of relevant markers of pyroptosis (CASP-1, IL-18, IL-1β, PYCARD), apoptosis (CASP-3, CASP-8, cytochrome C and Bcl-2) and inflammation (TNF-α) (Fig. 4). The data suggest that pyroptosis was inhibited as evidenced by significantly lower mRNA transcripts for CASP-1, IL-18 and PYCARD in the treated groups. However, this was not the case in mice that also received mannitol. Similarly, mRNA transcripts for apoptotic markers were significantly lower

in the groups that were treated without mannitol (Table 2). The inflammatory marker TNF-α was not affected by treatment.

The last parameter that we studied in these groups was inflammation as evident in formalin-fixed brain tissues from the respective mouse groups. No pathological differences were seen in the virus infected and treated mice compared to virus and treatment controls (Fig. 5). However, few perivascular mononuclear inflammatory cells were seen in the virus-infected groups but not in the treatment control groups (Supplementary Fig. 1). Nevertheless, there was no significant difference in the extent of inflammation between infected/treated mice and virus control mice. Rabies virus antigen was detected in moderate to high numbers of neurons in the brain of infected mice (Supplementary Fig. 1). Moreover, distribution of viral antigen in the brain was similar between the groups (Fig. 5).

4. Discussion

We have shown that a combination of treatment including antiviral compounds and host-response modulators can increase survival time of rabies up to 48 h. This was observed in RV-infected mice, when treatment was started four days after infection and given for eight consecutive days. The treatment cocktails included a selection of compounds with antiviral and immunomodulatory effects. We have used IFN-α, IFN-β, ribavirin, and favipiravir as antivirals. We and others have shown that all these compounds can inhibit RV in vitro [18,20]. However, these compounds have not proven effective in the clinic [4]. At the same time we wanted to interfere with a part of the immune response, which has been shown to be elevated in RV-infected mice [13]. Excessive immune activation has also been associated with other neurodegenerative diseases [21,22]. We have selected CASP-1, TNF-α, and MAP-kinase as the immunomodulatory targets. First, we have recently identified increased levels of CASP-1 in RV-infected mice and its inhibition increased survival in infected mice [16]. CASP-1 is involved in induction of an inflammatory form of programmed cell death called pyroptosis [23] and its inhibition could provide neuroprotection in mouse models of neurodegenerative diseases [24]. In our study, the combination treatment excluding mannitol significantly reduced CASP-1 mRNA expression (Table 2 and Fig. 4). This observation suggests that inclusion of a CASP-1 inhibitor in the treatment regimen may have a positive effect on the course of rabies by reducing downstream excessive and possibly detrimental pro-inflammatory responses after rabies infection. Second, TNF-α has been associated with severe rabies and inhibition of TNF signaling, mitigate disease severity [25]. In addition, TNF-α has been implicated in several neurodegenerative diseases, possibly due to its neurotoxic activity [26] through elevated glutamate production resulting in cell damage and death [27], its potential to damage the mitochondria and induction of ROS production [28]. Third, MAPKs regulate important cellular processes such as proliferation, stress responses, apoptosis and immune defense [29]. MAPKs are also involved in production of several cytokines, including TNF-α. Sorafenib is a MAPK inhibitor, which inhibits Ser/Thr kinases, which was recently approved for the treatment of cancer. We postulated that these molecules might be involved in induction of an aberrant immune response responsible for rabies encephalitis. In addition, studies published in this issue by Smreczak et al., have shown that use of REMICADE or Sorafenib can increase survival time of rabies infected mice [14]. Our hypothesis was that by opening the BBB and BSCB we would increase the chances of compounds to reach the infected sites of the brain and thereby inhibit virus replication and/or a deleterious inflammatory response. However, we did not see any significant benefit of using mannitol as a barrier opener in the clinical course of rabies. Although viral titers were slightly
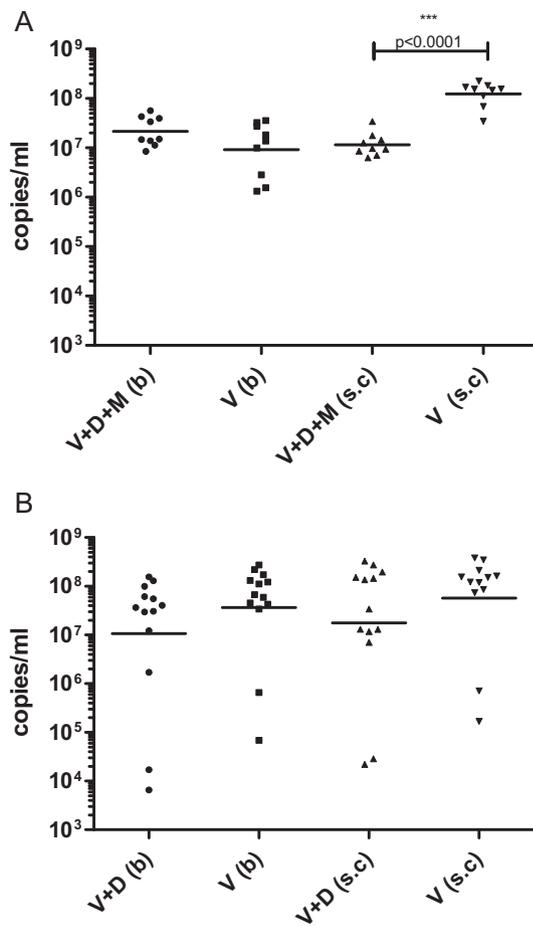

Fig. 3. Viral RNA in the brain (b) and spinal cord (s.c) of treated mice with (A) and without (B) mannitol (V + D + M and V + D) compared to virus control groups (V). 50% mortality groups are depicted here. Asterisks indicate significant differences (Mann-Whitney non-parametric test).
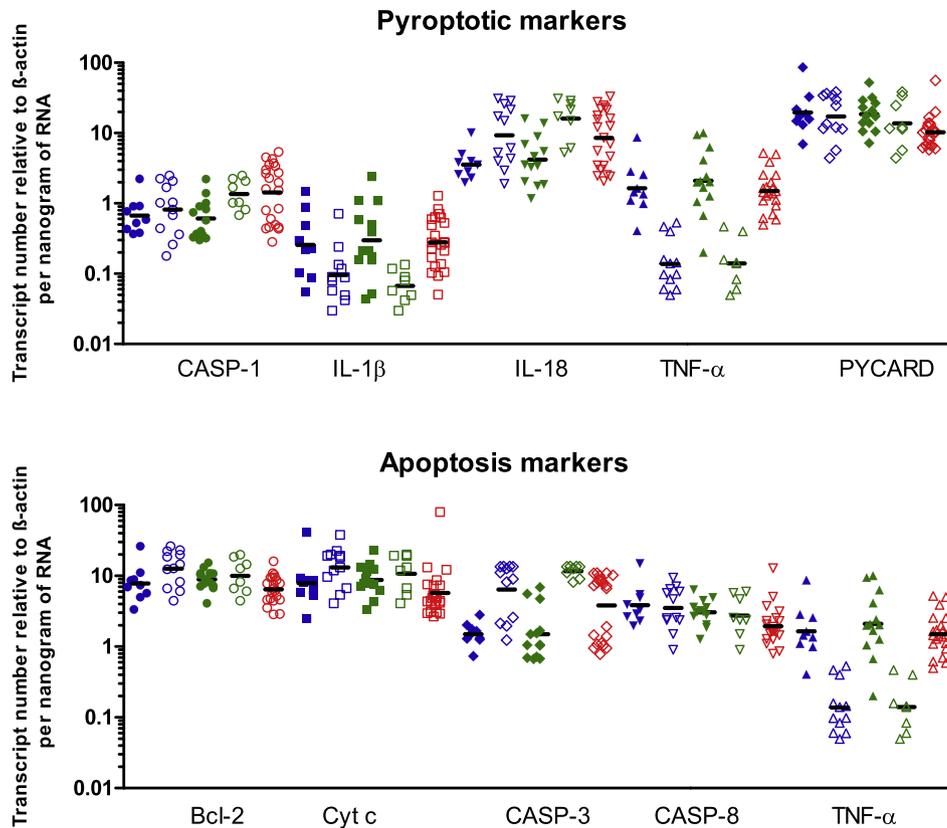
Pyroptotic markers

Apoptosis markers

Fig. 4. Expression of mRNA in the 50% mortality groups of mice. Solid symbols: virus infected and treated mice. Open symbols: mice received only treatment. Blue colors: mice received combination therapy with mannitol. Green colors: mice received combination therapy without mannitol. Red color: virus control (no treatment). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Differential expression of mRNA for different markers in animals infected and treated with the different drug combinations as described in Table 1. Statistical differences are written in bold. Mean mRNA values were compared with the non-parametric Mann-Whitney test.

	CASP-1 V + D + M	IL-1β V + D + M	IL-18 V + D + M	PYCARD V + D + M	
V	0.4994	0.6665	0.6048	0.0400	
D + M	0.0196	1.000	1.000	0.3301	
	Bcl-2 V + D + M	Cyt c V + D + M	CASP-3 V + D + M	CASP-8 V + D + M	TNF-α V + D + M
V	0.3401	1.000	0.0939	0.1135	0.3865
D + M	0.0196	0.0336	0.4140	0.2601	0.0056
	CASP-1 V + D	IL-1β V + D	IL-18 V + D	PYCARD V + D	
V	**<0.0001**	0.9183	**0.0002**	**0.0035**	
D	0.0125	**0.0034**	**0.0027**	0.4915	
	Bcl-2 V + D	Cyt c V + D	CASP-3 V + D	CASP-8 V + D	TNF-α V + D
V	0.0513	**0.0018**	**<0.0001**	**0.0012**	0.5316
D	0.6904	0.5382	**0.0002**	0.6378	**0.0003**

lower in the spinal cord of mice that received mannitol compared to the mice that did not, survival was prolonged in the mice that were treated without mannitol. Our data suggest that mannitol may not have facilitated the entrance of the compounds in the brain in contrast to the spinal cord. It is possible that mannitol may open the BSCB more efficiently than the BBB providing a possible explanation for our observations. It is known that the BSCB shares the same principal building blocks with the BBB, but they are morphological and functional different [30]. Furthermore, regional permeability of the brain after disruption with mannitol

has been shown to be substance-dependent [31]. Therefore it is also possible that the combination of compounds that we used requires different levels of permeability (and subsequently effectiveness) in the brain and spinal cord. It is also possible that repeated administration of mannitol was too toxic for the mice. Alternatively, the diuretic effect of mannitol may have resulted in accelerated clearance of small molecules such as IFN-α, and therefore no measurable antiviral effect.

As seen in Fig. 1a, the mean body weight of RV-infected mice treated with the combination therapy (V + D + M and V + D)
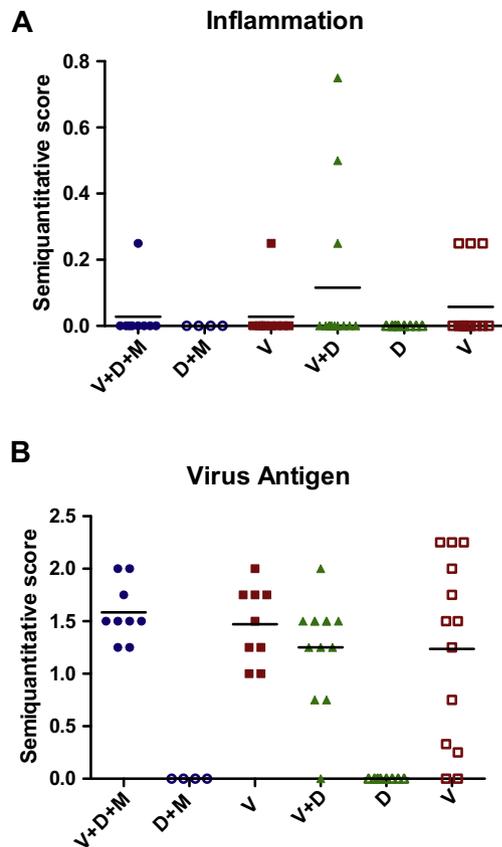
A   Inflammation

B   Virus Antigen

Fig. 5. Inflammation (A) and rabies virus antigen (B) in the brain from 50% mortality groups. Shown are data from infected mice, which received combination therapy with mannitol (7 days post infection) or combination therapy without mannitol (8 days post infection), and from infected but untreated control mice (7 and 8 days post infection, respectively). No significant differences in the degree of inflammation or amount of virus antigen were present between these 4 groups. No inflammation and virus antigen was detected in the respective drug control groups with and without mannitol.

significantly decreased over time compared to the (virus) controls, which could indicate increased toxicity due to treatment. Toxicity due to the treatment regimen (including mannitol) may have counterbalanced the effects of treatment, masking the potential of the combination therapy to treat rabies. On the other hand, we cannot exclude the possible effect of mannitol itself. Mannitol is an osmotic diuretic that may have influenced the weight as it leads to dehydration.

The treatment also affected transcript levels of proteins involved in pyroptosis and apoptosis, however this effect was not seen in combination with mannitol. This loss of measurable effect may also be explained by other mechanisms of action of mannitol. For instance, it was shown that mannitol induces activation of kinases and cell apoptosis [32]. We therefore cannot exclude that mannitol may have had these effects in our study.

It is likely that survival as a read-out parameter was not sensitive enough to measure the effect of treatment. Therefore, we included a 50% mortality as a read-out parameter. The working assumption was that since virus replication was not inhibited an effect would be better evaluated before the system is overwhelmed and the effect of treatment is reversed. Therefore we measured several parameters in all groups when 50% of the mice in the control group died. We did not observe significant differences in the 50% mortality design. Since we used a highly pathogenic rabies strain for these experiments with rapid development of clinical signs in mice, it is possible that this time point was not the best

choice. It is also possible that treating mice for longer periods of time would be necessary to measure a significant effect; in this experimental design, treatment was only given for three consecutive days.

In conclusion, we have seen promising results with the combination therapy that we have used here that warrant further investigations. Future studies, including step-down approaches (i.e. reduce the number of compounds in the combination) should follow up in order to identify best combinations and further optimize treatment strategies against rabies. In addition, more efforts must be deployed in finding better BBB and BSCB openers to enhance delivery of drugs in the brain and spinal cord.

Ethical statement

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.05.065.

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