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The effect of selected molecules influencing the detrimental host immune response on a course of rabies virus infection in a murine model

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ABSTRACT

Rabies is invariably fatal, when post-exposure prophylaxis is administered after the onset of clinical symptoms. In many countries, rabies awareness is very low and the availability of post-exposure prophylaxis, as recommended by WHO guidelines, is very limited or non-existent, probably as a consequence of high cost. Therefore, new concepts for rabies therapy are needed. Innate immune mechanisms involving the production of pro-inflammatory cytokines and chemokines, activated after rabies infection, are thought to be involved in the neuropathogenesis of rabies. These mechanisms can contribute to a detrimental host response to the rabies virus (RABV) infection. The use of inhibitors of cytokines/chemokines are supposed to extend the survival of a sick individual. Inhibitors of TNF-α, IL-6 and MAPKs were used in RABV inoculated mice to define their influence on the survival time of rabid mice. The study demonstrated that all inhibitors extended mice survival, but at different rates. A log-rank test confirmed the statistically significant survival of mice treated with TNF-α ($p = .0087$) and MAPKs inhibitors ($p = .0024$). A delay in the time of onset of rabies was also recorded, in mice given TNF-α and MAPKs inhibitors. The highest virus load was found in the spinal cord and the lowest in the cortex, regardless of the experimental group. Significant TNF-α ($p \leq .0001$) and IL-6 ($p \leq .0001$) gene upregulation was observed in mice, as a consequence of RABV infection. Regarding MAPKs pathways, there was significant upregulation of the caspase 3 ($p = .012$, $p = .0026$) and Mcl-1 ($p = .0348$, $p = .0153$) genes, whereas significant downregulation of the cytochrome *C* ($p \leq .0001$), Bcl2 ($p = .0002$, $p = .0007$) and JNK3 ($p = .042$) genes. Rabies pathogenesis is multifactorial, involving both virus and host influences on the course of the infection.

1. Introduction

Rabies has been known as a disease for around 4000 years. Despite the accumulation of broad knowledge regarding the pathogenesis, control and prophylaxis of rabies, it still threatens humans. Although rabies is preventable by vaccination, according to WHO statistics, the RABV causes 59,000 (95% CI: 25–159,000) human deaths annually [1]. The disease is invariably fatal, when rabies post-exposure prophylaxis (PEP), which is a combination of active and passive immunization, fails to destroy the virus, when administered after the onset of clinical symptoms and no effective treatment is available. There are thirteen well-documented survival cases of rabies, in humans, of which only 5 patients did not receive any PEP [2]. In spite of the many years of research, the mechanism of dysfunction/disturbance of CNS in rabies remains unclear and therefore needs investigation, for a better understanding of pathogenesis in the development of a rabies therapy. Once the RABV (RABV) enters the CNS, it induces the host's innate and adaptive immune responses [3,4]. Immune mechanism(s) involving the production of pro-inflammatory cytokines and chemokines, activated after rabies infection, are thought to be involved in the neuropathogenesis of rabies [4]. During RABV infection, activation of mitogen-activated protein kinases (MAPKs) has also been reported [5]. Cytokines, as well as MAPKs attributable to inflammation, may have both a positive and detrimental effect on the host [6,7]. Novel approaches of several neurodegenerative diseases, involving the implementation of inhibitors of inflammatory mediators, are currently under investigation. Inhibitors TNF-α and IL-6 have already been shown to be potential drugs for the treatment of diseases accompanied by an inflammatory response [8,9]. TNF-α is pro inflammatory cytokine and Infliximab (Remicade) inhibits

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its functional activity documented in a wide variety of *in vitro* bioassays. *In vivo*, Infliximab rapidly forms stable complexes with TNF-α, a process that parallels the loss of TNF-α bioactivity. The effect is to reduce the extent of inflammatory response/inflammatory lesions to the infection [10]. Tocilizumab (RoActemra) blocking cell receptor IL-6 was approved for treatment of inflammatory diseases [11]. Whereas, MAPKs regulate diverse cellular functions. They relay extracellular signals to intracellular responses. The eukaryotic cells possess multiple MAPKs pathways, which coordinately regulate gene expression, mitosis, metabolism, survival, apoptosis and differentiation [12]. Sorafenib tosylate as known multikinase blocker may reduce cell response to RABV infection, limiting the scope of pathology within CNS.

The aim of this study was to investigate the effect of selected inhibitors influencing the detrimental host immune response, on a course of the RABV infection in mice, as potential therapeutic molecules. We hypothesized that the application of drugs in a RABV infected animal, decreasing the detrimental inflammatory response stimulated by TNF-α, IL-6, and MAPKs pathways, may extend the survival of a sick animal, providing enough time for the host immune system to clear RABV from the brain. This hypothesis was tested *in vivo*.

2. Material and methods

2.1. Host response inhibitors

Three host response inhibitors were selected as candidates to inhibit the activity of pro-inflammatory mediators. A TNF-α inhibitor - infliximab (Remicade, Janssenn Pharmaceuticals) - chimeric human-murine IgG1 MoAb, produced in murine hybridoma cells by recombinant technology; an IL-6 inhibitor- tocilizumab (RoActemra,Hoffmann-LaRoche Ltd) - humanized IgG1 MoAb against the human interleukin 6 receptor, produced in Chinese hamster ovary; a MAPKs inhibitor - sorafenib tosylate (Nexavar, Bayer Health Care Pharmaceuticas).

Doses of the drugs were calculated for mice from the dose recommended for humans [13–15].

2.2. Virus and animals

Virus: The Silver Haired Bat RABV-18 (SHBRV-18) was obtained from EMC Rotterdam, as a BHK21/BSR cell culture fluid, at a titer of $10^{6.63}$ $TCID_{50}$/ml.

Animals: The experiment was carried out under the Local Ethics Committee for Experiments on Animals in Lublin, approval No. 10/2014. Six-week-old mice, female, C57BL/6 (Harlan Laboratories, U.S.) were randomly assigned to experimental groups, as shown in Table 1, and acclimatized for one week, in laboratory conditions. Subsequently, mice were intramuscularly (IM) inoculated in the left hind leg, with 50 μl of the SHBRV-18 strain ($10^{6.63}$ $TCID_{50}$/ml) on day 0. From 5dpi, the mice were treated with drugs, as shown in Table 1.

For all experimental procedures, mice were anaesthetized with a mixture of ketamine and xylazine. Mice were monitored twice a day and disease progression was evaluated by scoring clinical signs and mortality, as previously described [16]. All mice were sacrificed, according to a schedule of humane end-points. Samples of different parts of the CNS (i.e. the spinal cord, cortex and cerebellum/brainstem) were collected immediatelly after euthanizing of mice for virus titration and molecular studies.

2.3. Virus titration and virus copies determination (qRT-PCR)

Virus titre (VT) was determined for 20% homogenates of the CNS samples, according to the previously described method [17] using BHK cells in a concentration of 4×10^5 cells/ml.

Table 1
Experimental groups and drug administration.

Drug and dose/Administration route	Experimental groups								
	Infected/treated (virus + drug)		Virus control		Drug control		Negative control mice	Mock-infected mice	
	No. of mice/Inoculation day	Drug admin. d.p.i.	No. of mice 10	Admin. Day 0	No. of mice	Admin. d.p.v.i.	No.of mice 5	No of mice/administration route	
Remicade [100 μg] i.c. in 20 μl/mouse	10/0	5,6,7	–	–	7	5,6,7		5/ i.m. + i.c. Eagle	
RoActemra [100 μg] i.c. in 20 μl/mouse	10/0	5,7,9	–	–	7	5,7,9			
Sorafenib [1.2 mg] p.o. in 100 μl/mouse	20/0	5,6,7,…,12	–	–	7	5,6,7,…,12		5/i.m. + p.o. DMSO	

The number of virus N gene copies was measured by qRT-PCR (Mx3005P, Stratagene) on extracted RNA, using a QIAamp Viral RNA Mini Kit (Qiagen). Quantitative RT-PCR was performed, using a QuantiTect Probe RT-PCR Kit (Qiagen) with a known RABV as a standard. Briefly, the reaction mix contained: 12.5 μl of Master Mix, 9 μl of Rnase free water, 1 μl of primer JW12 (10 μM) (ATGTAACACCYCTACAATG) and 1 μl of primer N165-146 (10 μM) (GCAGGGTAYTTRTACTCATA), 0.25 μl of TaqMan SHBRV-18 probe (10 μM) (ACAAGATTGTATTCAAAGTCAATAATCAG), 0.25 μl of enzyme and 1 μl of RNA. The profile for qRT-PCR was: 50 °C for 30 min, 95 °C for 15 min and 45 cycles at 95 °C for 15 s and 55 °C for 1 min.

2.4. Determination of the expression of selected genes

Comparison of the expression level of the selected genes was done between a virus control group and infected and treated groups. For groups treated with TNF-α, IL-6 and MAPKs inhibitors, the level of expression for the *tnfα* gene, the *il6* gene and for MAPKs: *bcl2*, *mcl1*, *jnk3*, *cycs* and *casp3* genes were estimated, respectively. cDNA was synthetized in two steps. A mixture of 5 μl of total RNA, 6 μl of ddH_2O and 1 μl of the OLIGO(dT) primer (Invitrogen) per sample, were incubated at 65 °C for 10 min. A mixture from the first step; 4 μl of the 5x1st strandbuffer, 1 μl of DTT (0,1M), 1 μl of the RNAse inhibitor (RNaseOUT, Invitrogen), 1 μl of SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) and 1 μl of dNTPs (10 mM) was incubated at the following conditions: 25 °C for 5 mins, 50 °C for 45 mins and 70 °C for 15 mins. Real-time PCR was performed on a final volume of 20 μl (7μl of Rnase-free water, 10 μl of TaqMan® Universal PCR Master Mix and 1 μl of primers and a probes mix for each gene -Thermo Fisher Scientific and 2 μl of cDNA). All thermal cyclings were carried out with thermal profiles, as follows: 50 °C for 2 mins, 95 °C for 10 mins and 40 cycles at 95 °C for 15seconds, 60 °C for 1 min, with QuantStudio 6, Life Technologies. The copy number of mRNA of selected genes was normalized to the mRNA copy number of the GAPDH gene, in the presence of a calibrator sample and was calculated using the ΔΔCt method [18].

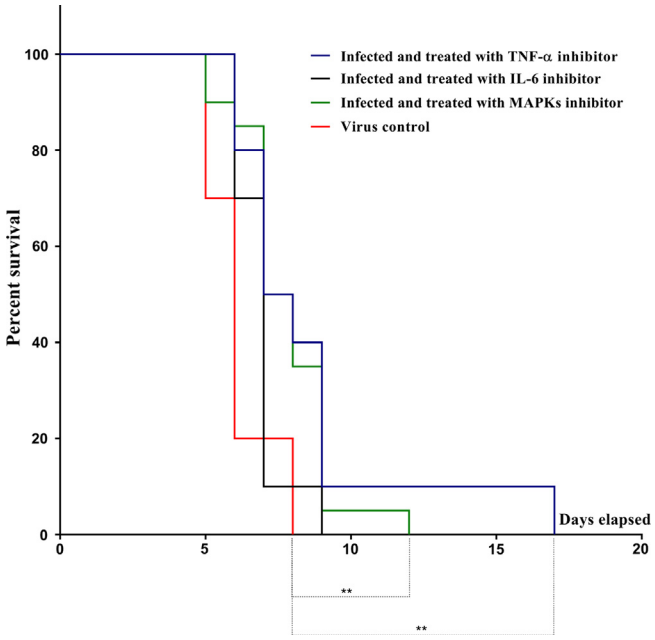

Fig. 1. Percentage survival of particular experimental groups, during the course of the study (red line – virus control, n = 10; blue line – infected and treated with the TNF-α inhibitor, n = 10; black line – infected and treated with the IL-6 inhibitor, n = 10; green line – infected and treated with the MAPKs inhibitor, n = 20). Significant differences were calculated with a log rank test (α = 0.05; $^{**}p \leq .01$).

2.5. Statistical analysis

Statistical analysis was done using the R studio package and GraphPad 7 Prism. Results of survival time were analyzed using a log – rank test. Results from the virus titration, determination of virus copies and estimation of the levels of expression of selected genes were first analyzed using a Shapiro – Wilk test, to evaluate if they fit a normal distribution. In the case of normal distribution, results from two groups were compared, using a Mann-Whitney *U* test, Student's *t* test or Welch's *t* test. All tests were performed at a level of significance of α = 0.05 with **** indicating a p value ≤.0001, *** a p value ≤.001, ** a p value ≤.01 and * a p value ≤.05. A p value of less than 0.05 was considered statistically significant.

3. Results

To assess the survival of the infected and treated groups in comparison to the virus control group, the percentage of surviving animals was plotted against time (Fig. 1). All drugs extended survival time, but at a different rate. The log-rank test confirmed statistically significant survival in groups of mice treated with TNF-α (p = .0087) and MAPKs (p = .0024) inhibitors. The TNF-α inhibitor extended mice survival to an average of 221 hpi, SEM = 25.5 (over 9 dpi) and the MAPKs inhibitor to an average of 191hpi, SEM = 7.821 (almost 8 dpi) relative to an average survival time for the virus control group of 159 hpi, SEM = 7.281 (six and half dpi). One out of ten mice in the treated with TNF-α inhibitor group survived for up to 418 hpi (over 17 dpi). The IL-6 inhibitor extended survival to 222 hpi–over 9 dpi (average survival 177hpi, SEM = 6.18–over 7 dpi) however, this difference was not statistically significant relative to the virus control group.

A delay in time of the onset of the signs of rabies was recorded in groups infected and treated with the TNF- α inhibitor (p = .00078) and the MAPKs inhibitor (0.01).

3.1. Virus titration/qRT-PCR

To investigate the course of the SHBRV-18 infection, VT was performed on 20% brain homogenate CNS samples from the

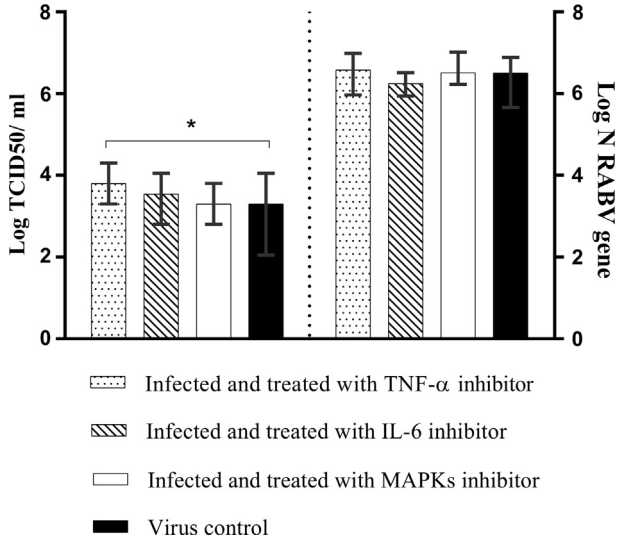

Fig. 2. Comparison of virus titer determined in BHK cells and rabies N gene copies, measured by qRT-PCR in all experimental groups (black bars – virus control, n = 5; dotted bars – infected and treated with the TNF-α inhibitor, n = 10; hatched bars – infected and treated with the IL-6 inhibitor, n = 10; white bars – infected and treated with the MAPKs inhibitor, n = 20). Significant differences were calculated using a Mann – Whitney *U* test (α = 0.05; $^{*}p \leq .05$). Data is presented as median and 95% CI.

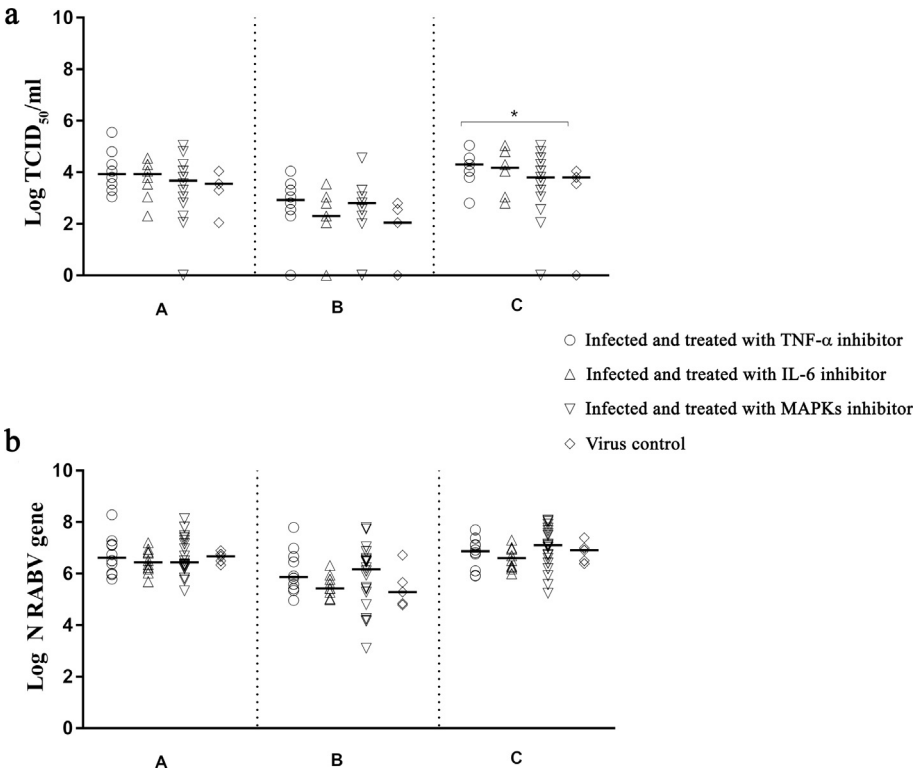

Fig. 3. SHBRV – 18 in different parts of the CNS: A – cerebellum/brainstem, B – cortex, C – spinal cord; based on the comparison of virus titer determined in BHK cell (a) and rabies N gene copies measured by qRT-PCR (b) in relation to different experimental groups of mice (rhomb – virus control, n = 5; circle – infected and treated with the TNF-α inhibitor, n = 10; triangle – infected and treated with the IL-6 inhibitor, n = 10; inverted triangle – infected and treated with the MAPKs inhibitor, n = 20). Significant differences were calculated using a Mann – Whitney *U* test, Student's t – test and Welch's *t* test dependent on a normality test ($\alpha = 0.05$; $^{*}p \leq 0.05$). Data is presented as median.

infected and treated groups and the virus control group. The highest VT was found in CNS samples in the infected and treated with the TNF-α inhibitor group (Fig. 2). The difference was significantly higher in the infected and treated with the TNF-α inhibitor group, in comparison to the virus control group (p = .02). No statistical differences were recorded in a number of the SHBRV-18 N gene, for all experimental groups (Fig. 2).

Regarding the virus load in different parts of the CNS, the highest VT was recorded in the spinal cord and the lowest in the cortex, regardless of the experimental group (Fig. 3a). The difference was statistically significant (p = .044) between the infected and treated with the TNF-α inhibitor group and the virus control group (Fig. 3a).

The result of SHBRV-18 N gene copies determination showed a very similar tendency as the VT, but no significant differences were found within different parts of CNS and between experimental groups (Fig. 3b).

In all experimental infected and treated groups and the virus control group, the highest VT was recorded in the cerebellum/brainstem and the spinal cord, in comparison to the cortex, regardless of experimental group (Fig. 4a). The same tendency as for VT was recorded for the SHBRV-18 N gene copies number in different parts of the CNS, in reference to all experimental groups (Fig. 4b). The highest number of N gene copies was observed in the spinal cord: $10^{7.6}$ copies in infected and treated with the MAPKs inhibitor group, $10^{6.7}$ in the infected and treated with the TNF-α inhibitor group, $10^{6.4}$ in the infected and treated with the IL-6 inhibitor group, in comparison to the $10^{6.8}$ N gene copies in the virus control group (Fig. 4b). Interestingly, the highest number of N gene copies in the cortex was recorded in the infected and treated with the MAPKs inhibitor group, which seems to be the result of the survival time recorded in this group. The number of N gene copies in the spinal cord was significantly higher than in the cortex, in the virus control group (p = .03) and in the group infected and treated with: the TNF-α inhibitor (p = .035), the IL-6 inhibitor (p = .0001) and the MAPKs inhibitor (p = .001). A significant difference in the number of the N gene was also observed between the cortex and cerebellum/brainstem, in the infected and treated with the MAPKs inhibitor group (p = .043) and the infected and treated with the IL-6 inhibitor group (p = .0003) (Fig. 4b).

3.2. mRNA gene expression of selected markers relevant to the inhibitor used

Regarding the TNF-α gene, there was highly significant upregulation in the virus control group, in comparison to the drug control group ($p \leq 0.0001$; Fig. 5). The level of expression of the TNF-α gene in the infected and treated with the TNF-α inhibitor group was slightly lower than in the virus control group, but the difference was not significant. The graph indicates that the upregulation of the *tnfα* gene is clearly related to the SHBRV-18 infection.

Regarding the *il6* gene, its expression was upregulated in the infected and treated with the IL-6 inhibitor group, in comparison to the virus control group (p = .0165) and the drug control group ($p \leq .0001$) (Fig. 5). There was also highly significant upregulation of *il6* gene expression in the virus control group, in comparison to the drug control group ($p \leq .0001$) (Fig. 5).

The *casp3* gene was upregulated in the infected and treated with the MAPKs inhibitor group (p = .012) and in the virus control group (p = .0026) relative to the drug control group.

The *cycs* gene has been downregulated in the virus control group. Significantly higher expression of the *cycs* gene was observed in the infected and treated with the MAPKs inhibitor group, in comparison to the virus control group (p = .0002). Interestingly, a highly significant tendency of downregulation of

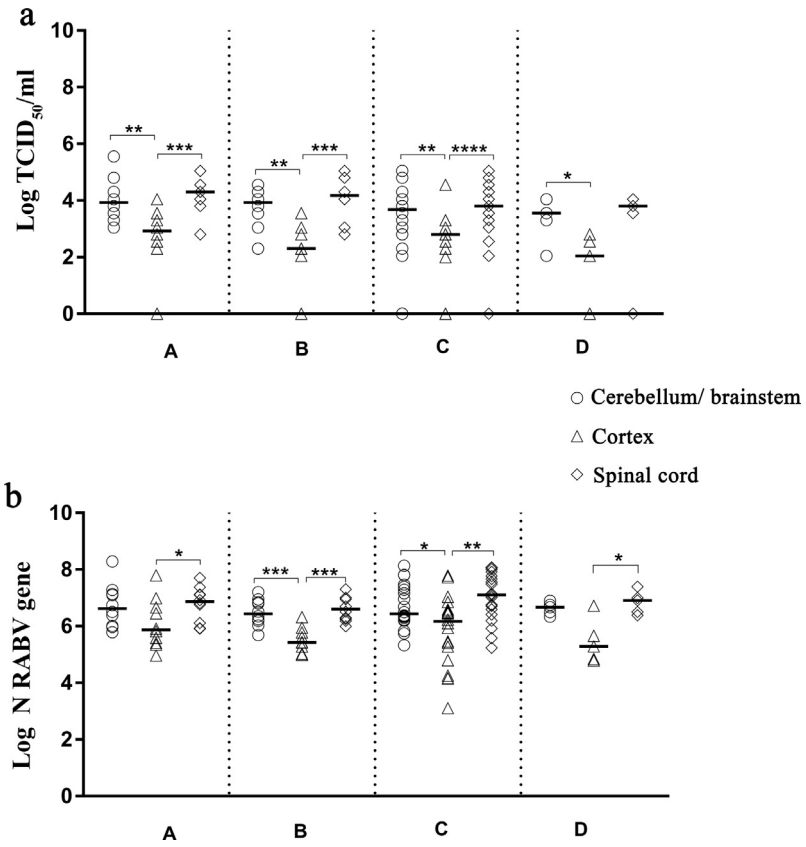

Fig. 4. The comparison of virus titer determined in BHK cells (a) and rabies N gene copies measured by qRT-PCR (b) in different parts of CNS within experimental groups: A – infected and treated with the TNF-α inhibitor, n = 10, B – infected and treated with the IL-6 inhibitor, n = 10, C – infected and treated with the MAPKs inhibitor, n = 20, D – virus control, n = 5 (circle – cerebellum/brainstem; triangle – cortex; rhomb – spinal cord). Significant differences were calculated using a Mann – Whitney *U* test, Student's t – test and Welch's *t* test dependent on a normality test (α=0.05; $^{****}p \leq 0.0001$; $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$). Data is presented as median.

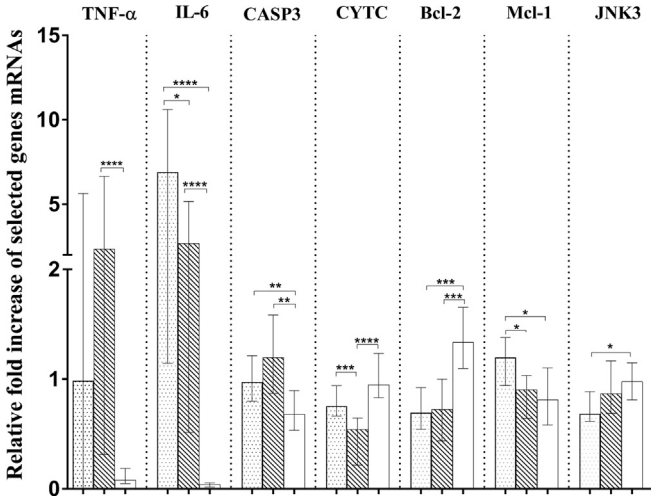

Fig. 5. mRNA levels in experimental groups (dotted bars – infected and treated with the respective TNF-α/IL-6/MAPKs inhibitor; hatched bars – virus control; white bars – drug control). TNF-α mRNA was measured for virus control (n = 5), infected and treated with the TNF-α inhibitor (n = 10) and drug control – mice treated with the TNF-α inhibitor (n = 7). IL-6 mRNA was measured for virus control (n = 5), infected and treated with the IL-6 inhibitor (n = 10) and drug control – mice treated with the IL-6 inhibitor (n = 7). CASP3, CYTC, Bcl-2, Mcl-1 and JNK3 mRNA was measured for virus control (n = 5), infected and treated with the MAPKs inhibitor (n = 20) and drug control – mice treated with the MAPKs inhibitor (n = 6). Significant differences were calculated using a Mann – Whitney *U* test, Student's t – test and Welch's *t* test dependent on a normality test (α = 0.05; $^{****}p \leq .0001$; $^{***}p \leq .001$; $^{**}p \leq .01$; $^{*}p \leq .05$). Data is presented as median and 95% CI; n – number of mice.

the *cycs* gene was recorded in the virus control group, in comparison to the drug control group ($p \leq .0001$) (Fig. 5).

Regarding the *bcl2* gene, downregulation was recorded in the infected and treated with the MAPKs inhibitor group ($p = .0002$) and in the virus control group ($p = .0007$) when compared to the drug control group (Fig. 5). Upregulation of the *mcl1* gene was recorded in the infected and treated with MAPKs group, in comparison to the virus control group ($p = .0348$) and the drug control group ($p = .0153$) (Fig. 5).

JNK3 expression was downregulated in the infected and treated with the MAPKs inhibitor group and in the virus control group, in comparison to the drug control group, however, statistical significance was observed between the infected and treated with the MAPKs inhibitor group and the drug control group ($p = .042$) (Fig. 5).

Fig. 6 shows the mRNA expression of genes (markers) in reference to the particular experimental group and in relation to the relevant part of the CNS. Significantly higher ($p = .0025$) expression of TNF-α mRNA was observed in the cerebellum/ brainstem in the virus control group relative to the drug control group. The same tendency was recorded when testing the spinal cord ($p = .007$) (Fig. 6a).

Expression of IL-6 mRNA was highly upregulated in the cerebellum/brainstem in the infected and treated with the IL-6 inhibitor group ($p \leq .0001$) and in the virus control group ($p = .03$) relative to the drug control group. The same tendency was observed in the cerebellum/ brainstem samples in the infected and treated group, when compared to the virus control group ($p = .0008$) (Fig. 6b). Upregulation of IL-6 mRNA in the spinal cord of the infected and treated with the IL-6 inhibitor group, in comparison

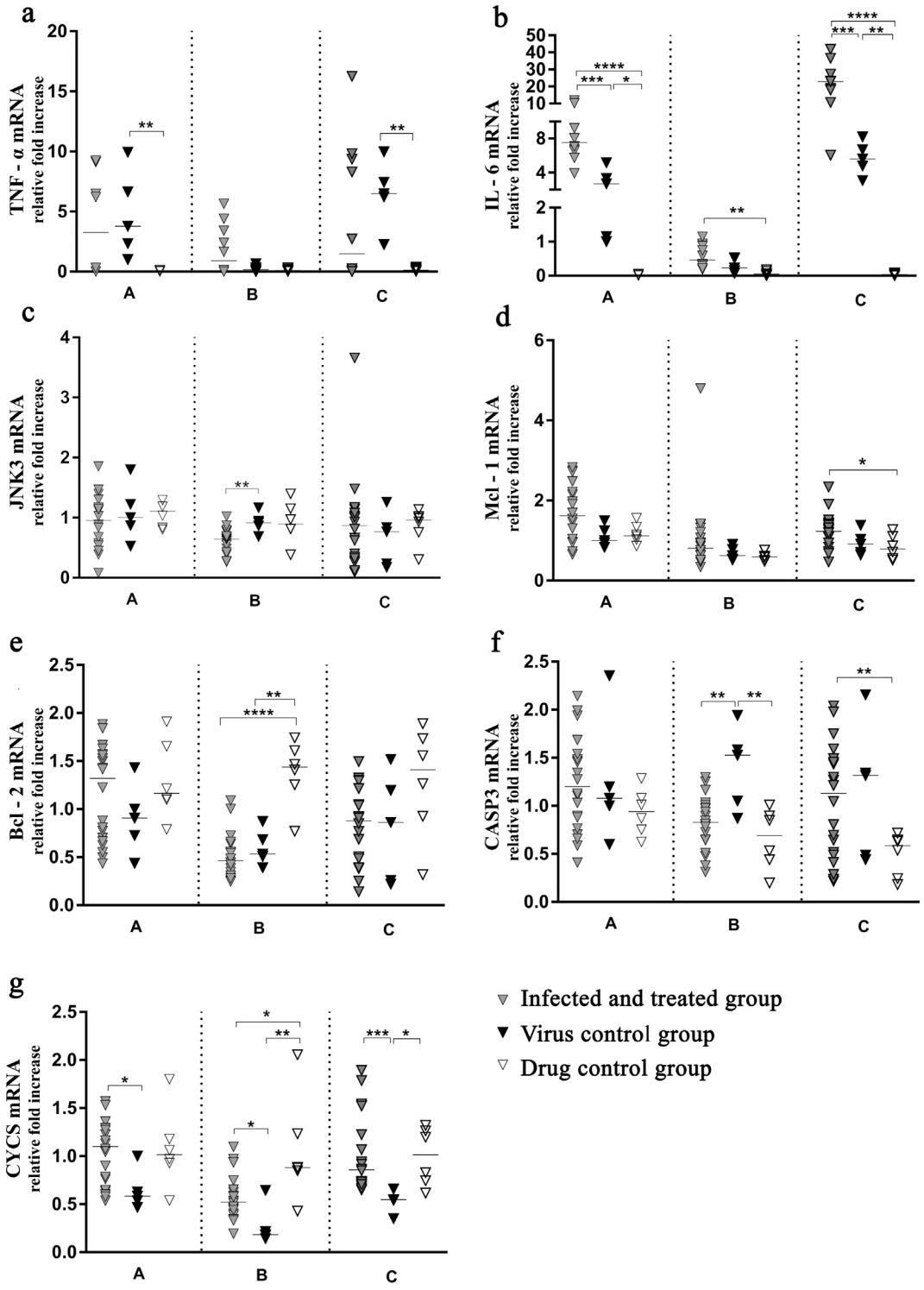

Fig. 6. (a–g): Comparison of mRNA levels within different parts of the CNS (A – cerebellum/ brainstem; B – cortex; C – spinal cord) between experimental groups: (grey triangle – infected and treated with the respective TNF-α/IL-6/MAPKs inhibitor, n = 10; black triangle – virus control, n = 5; white triangle - drug control, n = 7). Significant differences were calculated using a Mann – Whitney *U* test, Student's t – test and Welch's *t* test dependent on a normality test ($\alpha = 0.05$; $^{****}p \leq .0001$; $^{***}p \leq .001$; $^{**}p \leq .01$; $^{*}p \leq .05$). Data is presented as median.

to the virus control group (p = .00062) and the drug control group ($p \leq .0001$) and an increase of expression in the virus control group, in comparison to the drug control group (p = .0029) was shown. Upregulation of IL-6 mRNA was also recorded in the cortex in the infected and treated with the IL-6 inhibitor group, in relation to the drug control group (p = .0012) (Fig. 6b).

Regarding expression of JNK3, downregulation was found in the cortex of the infected and treated with the MAPKs inhibitor group, in comparison to the virus control group (p = .0042). No significant differences in the cerebellum/brainstem and the spinal cord between experimental groups of animals were observed (Fig. 6c).

Referring to Mcl-1 expression, the only significant upregulation ($p = .04$) was noticed in the spinal cord of the infected and treated with the MAPKs inhibitor group, when compared to the drug control group (Fig. 6d).

The expression of Bcl-2 mRNA in the cortex was downregulated in the infected and treated with the MAPKs inhibitor group ($p \leq .0001$) and the virus control group ($p = .0014$) relative to the drug control group (Fig. 6e).

Regarding Caspase 3 mRNA, a highly significant level of expression was recorded in the cortex of the virus control group, in comparison to the infected and treated with the MAPKs inhibitor group ($p = .0011$) and the drug control group ($p = .0093$). Upregulation in the spinal cord of the infected and treated with the MAPKs inhibitor group, in comparison to the drug control group ($p = .0024$) was observed (Fig. 6f).

Cytochrome *C* mRNA expression was upregulated in the cerebellum/brainstem ($p = .0096$), in the cortex ($p = 0.0154$) and

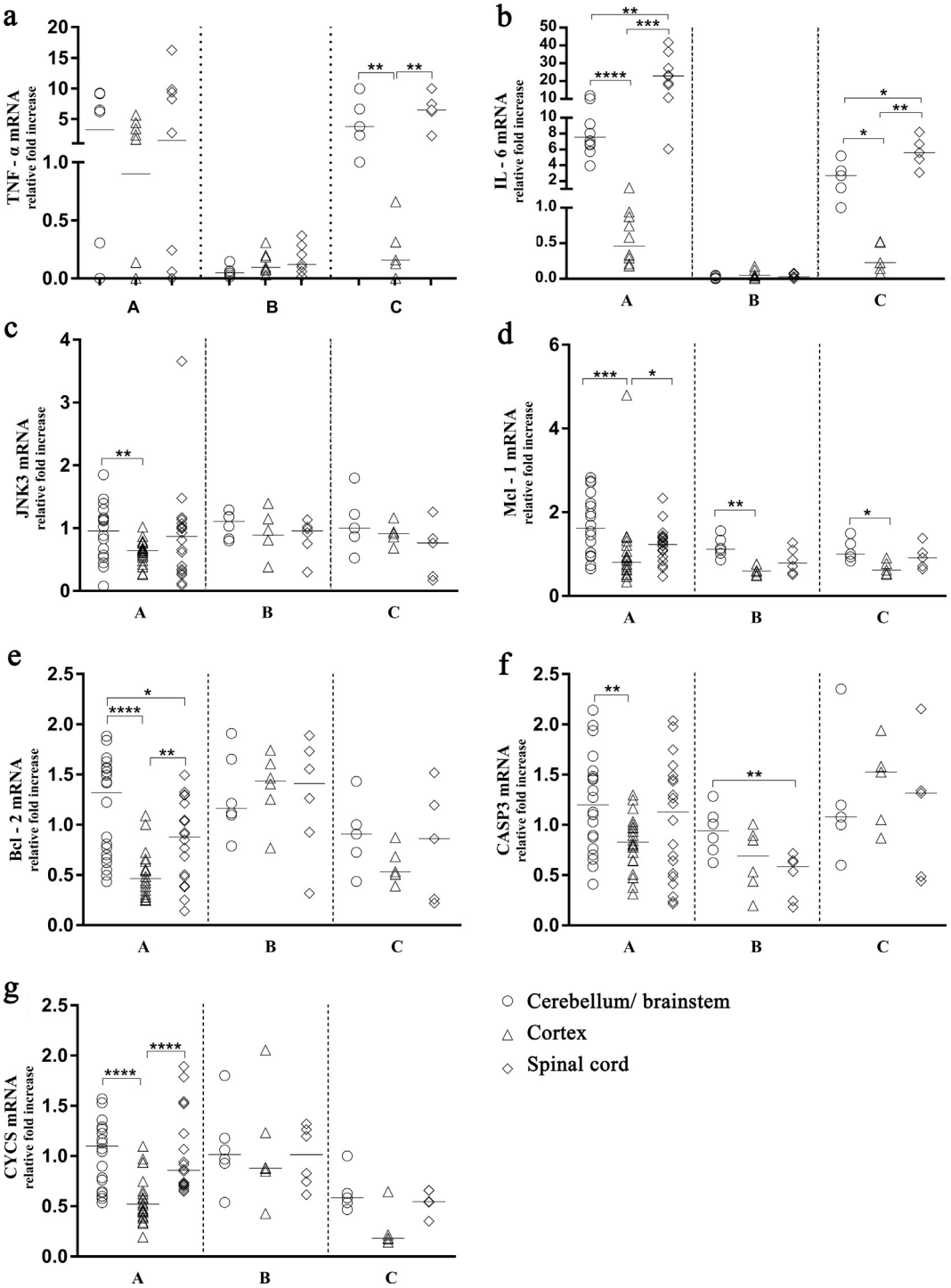

Fig. 7. (a–g): Comparison of mRNA levels within experimental groups: (A – infected and treated with the respective TNF-α/IL-6/MAPKs inhibitor, n = 10; B – drug control, n = 5; C – virus control, n = 5) between different parts of the CNS: (circle – cerebellum/brainstem; triangle – cortex; rhomb – spinal cord). Significant differences were calculated using a Mann – Whitney *U* test, Student's t – test and Welch's *t* test dependent on a normality test ($\alpha = 0.05$; $^{****}p \leq .0001$; $^{***}p \leq .001$; $^{**}p \leq .01$; $^{*}p \leq .05$). Data is presented as median.

in the spinal cord (p = .0002) of the infected and treated with the MAPKs inhibitor group, in comparison to the virus control group. A decrease of *cycs* mRNA expression in the virus control group compared to the drug control group was observed in the cortex (p = .0087) and the spinal cord (p = .0136). There was also down-regulation of *cycs* gene expression in cortex samples of the infected/treated group, in comparison to the drug control group (p = .0459) (Fig. 6 g).

Comparative study of mRNA levels in the CNS of markers relevant to the inhibitor used, with regard to the experimental groups, are shown in Fig. 7.

For TNF-α, statistically significant upregulation of the virus control group in the cerebellum/ brainstem (p = .0079) and the spinal cord (p = .0073) relative to the cortex was recorded (Fig. 7a).

There were significant differences for IL-6 mRNA levels between various parts of the CNS; i.e. significantly higher expression was found in the cerebellum/brainstem ($p \leq .0001$) and the spinal cord (p = .0001) than in the cortex of the infected and treated with the IL-6 inhibitor group. Statistically significant upregulation was also found in the spinal cord, compared to cerebellum/brainstem samples of the infected and treated group (p = .0015). The IL-6 mRNA level in the virus control group was higher in the cerebellum/brainstem (p =.0359) and the spinal cord (p = .0032) than in the cortex. Moreover, within the virus control group, the concentration of IL-6 mRNA was significantly different between the cerebellum/brainstem and the spinal cord (p = .0311) (Fig. 7b).

With regard to the selected MAPKs markers, there was one significant difference in JNK3 mRNA expression level - infected and treated group: the cerebellum/brainstem, in comparison to the cortex (p = .0073) (Fig. 7c). The expression of Mcl-1 was significantly higher in the cerebellum/brainstem (p = .0006) and the spinal cord (p = .0132) in comparison to the cortex of the infected and treated with the MAPKs inhibitor groups. A significant difference was also recorded in mRNA between the cerebellum/brainstem and the cortex of the drug control group (p = .0015) and the virus control group (p = .0157) (Fig. 7d). Regarding Bcl-2, statistically significant differences were noticed in the infected and treated group between all analyzed CNS parts (the cerebellum/brainstem in comparison to the cortex $p \leq .0001$; the cerebellum/brainstem in comparison to the spinal cord p = .0227; the cortex in comparison to the spinal cord p = .0037) (Fig. 7e). The expression of CASP-3 mRNA was upregulated in the cerebellum/brainstem in comparison to the cortex of the infected and treated group (p = .0034). There was also a significant difference in expression of CASP-3 mRNA between the cerebellum/brainstem and the spinal cord of the drug control group (p = .0077) (Fig. 7f). Cytochrome *C* mRNA level differed significantly in the infected and treated with the MAPKs inhibitor group, in the cerebellum/brainstem ($p \leq .0001$) and the spinal cord ($p \leq .0001$) when compared to the cortex (Fig. 7 g).

4. Discussion

Verifying the hypothesis that the excessive innate host immune response plays a detrimental role in RABV infection, contributing the invariably fatal course of infection was the main goal of these experiments. The study was planned and designed to gain information on how RABV changes the levels of activity of selected host immune modulators and if inhibition of their action can influence the extension of the survival time of mice, SHBRV-18 inoculated and treated with the relevant drug. Inhibitors of cytokine (TNF-α and IL-6) as well as MAPKs have previously been shown as effective inhibitors against detrimental host response in the course of neurodegenerative and autoimmune diseases [19,20]. Other studies have also demonstrated some therapeutic potential after the inhibition of pro-inflammatory cytokine expression in the CNS after RABV infection [21,22].

In vivo experiments with TNF-α and multi MAPKs inhibitors clearly extended mice survival infected with SHBRV-18. It should be highlighted that administration of the TNF-α inhibitor took place intra-cranially. This means of administration avoids the blood brain barrier that would probably interfere when delivered iv or im. The MAPKs inhibitor, administered per os extended the survival of SHBRV-18 infected mice even more efficiently than the TNF-α inhibitor. This could be the effect of a peripheral action/influence of the multi MAPKs inhibitor on progression of the RABV infection. However, it should be stressed that the treatment was introduced at 5 dpi, when the first clinical signs in some mice appeared, which means that RABV has already reached the brain. Nevertheless, from the Fig. 1 graph, the therapeutic window can clearly be seen – the space between the survival curves of mice treated with inhibitors and RABV control mice. There was not only significant extension of survival, but also a delay of the onset of clinical signs of the disease. The difference in survival time between infected and treated with Remicade and virus control was 62 h (>2.5 days) while Sorafenib prolonged mice survival for 32 h. One mouse treated with Remicade, that survived until 418 hpi, exhibited clinical signs of rabies for 81 h. Drugs were tested on separate groups of mice. Further experiments are needed with the combination of both inhibitors utilized in the same animals. Considering that only three doses of the TNF-α inhibitor extended mice survival by 61 hpi, this could be an indication of extending the time of the host response inhibitor administration, or to increase the dose of the drug, or to apply both; i.e. to increase the dose and extend the time of administration.

Regarding the RABV load in the CNS, the highest titre was recorded in mice treated with the TNF-α inhibitor. The difference was statistically significant when compared to the titre in the virus control group. This effect could be related to extended mice survival when treated with the TNF-α inhibitor. Despite the extended survival of mice treated with the MAPKs inhibitor, the virus load in the CNS was the same as in virus control mice. This could be explained by Roberts et al. [23] who demonstrated the inhibitory effect of Sorafenib on RABV replication. No significant differences in N gene copies, in mice treated with inhibitors, in comparison to virus control mice, were recorded. This may suggest that virus assembly and N gene synthesis do not progress at the same rate and are not influenced by the inhibitors applied.

Invariably, significantly lower virus titres and lower N gene copies in the cortex than in other parts of the CNS were seen, regardless of the experimental group of animals, including the virus control group. Considering the virus and the N gene load in the CNS, and in comparative analysis referring to the experimental groups, it could be suggested that the inhibitors applied have not influenced the course of virus infection and through influencing the host innate immunological response, they extended the survival time of mice treated with these inhibitors. Experiments combining inhibitors of the host detrimental immune response and inhibitors of virus replication would be a good option for further study, in rabies treatment.

RABV infection highly upregulates genes involved in innate immune and antiviral responses, especially those related to IFNα/β signalling pathways, inflammatory cytokines and chemokines, including TNF-α, IL-6 and IL-10 [3]. Measuring mRNA levels for TNF-α and IL-6 has allowed estimation of the innate immune response to viral infection. Parameters were compared for mice infected and treated, virus and drug controls. This study has shown high upregulation of TNF-α and IL-6 in mice infected with SHBRV-18, both in the virus control group and mice infected and treated with cytokine inhibitors. Highly significant differences between infected groups and drug controls clearly indicate the influence of virus infection on the excessive detrimental host immune response. The results confirm previous data demonstrating upregulation of TNF-α and IL-6 during lyssavirus infection [24,25].

MAPKs regulate diverse cellular functions, among them apoptosis based on external stimuli like viral infection [5]. Extracellular signal-regulated kinase 1 and 2 (ERK 1 and 2), c-Jun N-terminal kinase (JNK 1-3) and p38 families are well-known. JNK 1 and 2 are ubiquitous, whereas JNK3 is relatively restricted to the brain [26]. JNK are activated in response to inflammatory cytokines and are involved in cytokine production, the inflammatory response, stress induced and developmentally programmed apoptosis, actin reorganization, cell transformation and metabolism [5]. Results have shown upregulation of the caspase 3 and Mcl-1 markers in mice of infected and treated and virus control groups, and downregulation of the cytochrome *C*, Bcl-2 and JNK3 genes at the same time, in comparison to the drug control group. Analysis of gene expression levels of particular markers can be related to the host response to viral infection. As suggested by Yin et al. [27] different pathogenic mechanisms exist between different RABV strains. Our results support previous data on no or minimal contribution of apoptosis on the mechanism of SHBRV-18 pathogenesis [28,29]. Increased expression of the Mcl-1 gene encoding the anty-apoptotic protein, as well as downregulation of pro-apoptotic markers, like cytochrome *C* and JNK-3 could suggest that apoptosis is not essential for SHBRV-18 pathogenesis.

In summary, our results provide new data on the influence of pro-inflammatory cytokines and MAPKs inhibitors on mice survival and indirectly on reduction of the detrimental effect of the host immune response after SHBRV-18 infection. Considering the prolongation of mice survival after treatment with TNF-α or MAPKs inhibitors, they may be suggested as potential therapeutics. As rabies pathogenesis is multifactoral, with both host and virus factors being involved, it is crucial to consider a combination therapy, including TNF-a and MAPKs inhibitors, with others molecules proved as effective against RABV infection.

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Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of this paper.

References

[1] WHO. WHO: Weekly epidemiological record. World Heal Organ Geneva 2017;7:77–88.
[2] Weyer J, Msimang-Dermaux V, Paweska JT, le Roux K, Govender P, Coertse J, et al. A case of human survival of rabies, South Africa. South African J Infect Dis 2016;53:1–3. https://doi.org/10.1080/23120053.2016.1128151.
[3] Appolinário CM, Allendorf SD, Peres MG, Ribeiro BD, Fonseca CR, Vicente AF, et al. Profile of cytokines and chemokines triggered by wild-type strains of RABV in mice. Am J Trop Med Hyg 2016;94:378–83. https://doi.org/10.4269/ajtmh.15-0361.
[4] Chopy D, Detje CN, Lafage M, Kalinke U, Lafon M. The type I interferon response bridles RABV infection and reduces pathogenicity. J Neurovirol 2011;17:353–67. https://doi.org/10.1007/s13365-011-0041-6.
[5] Nakamichi K, Saiki M, Sawada M, Takayama-Ito M, Yamamuro Y, Morimoto K, et al. RABV-induced activation of mitogen-activated protein kinase and NF-kappaB signaling pathways regulates expression of CXC and CC chemokine ligands in microglia. J Virol 2005. https://doi.org/10.1128/JVI.79.18.11801-11812.2005.
[6] Chopy D, Pothlichet J, Lafage M, Mégret F, Fiette L, Si-Tahar M, et al. Ambivalent role of the innate immune response in RABV pathogenesis. J Virol 2011;85:6657–68. https://doi.org/10.1128/JVI.00302-11.
[7] Ito N, Moseley GWGW, Sugiyama M. The importance of immune evasion in the pathogenesis of RABV. J Vet Med Sci 2016;78:1089–98. https://doi.org/10.1292/jvms.16-0092.
[8] Hennigan S, Kavanaugh A. Interleukin-6 inhibitors in the treatment of rheumatoid arthritis. Ther Clin Risk Manag 2008;4:767–75.
[9] Ma X, Xu S. TNF inhibitor therapy for rheumatoid arthritis. Biomed Reports 2013;1:177–84. https://doi.org/10.3892/br.2012.42.
[10] Sedger LM, McDermott MF. TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants – past, present and future. Cytokine Growth Factor Rev. 2014;25:453–72. 10.1016.
[11] Tanaka T, Narazaki M, Kishimoto T. Anti-interleukin-6-receptor antibody, tocilizumab, for treatment of autoimmune diseases. FEBS Letters. 2011;585:3699–709.
[12] Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev. 2011;75:50–83.
[13] Guo Y, Lu N, Bai A. Clinical use and mechanisms of infliximab treatment on inflammatory bowel disease: a recent update. Biomed Res Int 2013:2013. https://doi.org/10.1155/2013/581631.
[14] Huynh H, Lee JWJ, Chow PKH, Ngo VC, Bin Lew G, Lam IWL, et al. Sorafenib induces growth suppression in mouse models of gastrointestinal stromal tumor. Mol Cancer Ther 2009;8:152–9. https://doi.org/10.1158/1535-7163.MCT-08-0553.
[15] Alten R, Maleitzke T. Tocilizumab: A novel humanized anti-interleukin 6 (IL-6) receptor antibody for the treatment of patients with non-RA systemic, inflammatory rheumatic diseases. Ann Med 2013;45:357–63. https://doi.org/10.3109/07853890.2013.771986.
[16] Healy DM, Brookes SM, Banyard AC, Núñez A, Cosby SL, Fooks AR. Pathobiology of RABV and the European bat lyssaviruses in experimentally infected mice. Virus Res 2013;172:46–53. https://doi.org/10.1016/j.virusres.2012.12.011.
[17] OIE. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Rabies (infection with RABV) 2013;2.1.17:1–28.
[18] Rao X, Huang X, Zhou Z, Lin X. An improvement of the 2̂(–delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. Biostat Bioinforma Biomath 2013;3:71–85.
[19] Burgess S, Echeverria V. Raf inhibitors as therapeutic agents against neurodegenerative diseases. CNS Neurol Disord - Drug Targets 2010;9:120–7. https://doi.org/10.2174/187152710790966632.
[20] Astrakhantseva IV, Efimov GA, Drutskaya MS, Kruglov AA, Nedospasov SA. Modern anti-cytokine therapy of autoimmune diseases. Biochem Biokhimiiā 2014;79:1308–21. https://doi.org/10.1134/S0006297914120049.
[21] Mehta S, Roy S, Mukherjee S, Yadav N, Patel N, Chowdhary A. Exogenous interferon prolongs survival of rabies infected mice. Virus Dis. 2015;26:163–9. https://doi.org/10.1007/s13337-015-0269-5.
[22] Huang Y, Jiao SZ, Tao XY, Tang Q, Jiao WT, Xiao J, et al. Met-CCL5 represents an immunotherapy strategy to ameliorate RABV infection. J Neuroinflammation 2014;11:13. https://doi.org/10.1186/s12974-014-0146-y.
[23] Roberts JL, Tavallai M, Nourbakhsh A, Fidanza A, Cruz-Luna T, Smith E, et al. GRP78/Dna K Is a target for nexavar/stivarga/votrient in the treatment of human malignancies, viral infections and bacterial diseases. J Cell Physiol 2015;230:2552–78. https://doi.org/10.1002/jcp.25014.
[24] Mansfield KL, Johnson N, Nuñez A, Hicks D, Jackson AC, Fooks AR. Up-regulation of chemokine gene transcripts and T-cell infiltration into the central nervous system and dorsal root ganglia are characteristics of experimental European bat lyssavirus type 2 infection of mice. J Neurovirol 2008;14:218–28. https://doi.org/10.1080/13550280802008297.
[25] Sugiura N, Uda A, Inoue S, Kojima D, Hamamoto N, Kaku Y, et al. Gene expression analysis of host innate immune responses in the central nervous system following lethal CIN COMPARISON TO THE-11 infection in mice. Jpn J Infect Dis 2011;64:463–72.
[26] Bogoyevitch MA, Kobe B. Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. Microbiol Mol Biol Rev 2006;70:1061–95. https://doi.org/10.1128/MMBR.00025-06.
[27] Yin JF, Ding YL, Huang Y, Tao XY, Li H, Yu PC, et al. Comparative analysis of the pathogenic mechanisms of street RABV strains with different virulence levels. Biomed Environ Sci 2014;27:749–62. https://doi.org/10.3967/bes2014.034.
[28] Yan X, Prosniak M, Curtis MT, Weiss ML, Faber M, Dietzschold B, et al. Silver-haired bat RABV variant does not induce apoptosis in the brain of experimentally infected mice. J Neurovirol 2001;7:518–27. https://doi.org/10.1080/135502801753248105.
[29] Sarmento L, Li X, Howerth E, Jackson AC, Fu ZF. Glycoprotein-mediated induction of apoptosis limits the spread of attenuated RABVes in the central nervous system of mice. J Neurovirol 2005;11:571–81. https://doi.org/10.1080/13550280500385310.