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Inhibition of caspase-1 prolongs survival of mice infected with rabies virus

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ABSTRACT

Rabies virus infects almost all mammals resulting in lethal disease. To date there is no treatment available for symptomatic rabies and there is an urgent need to develop treatment strategies that would prolong survival, thereby providing a window of opportunity for the host to mount a protective immune response. We hypothesized that both virus and excessive immune response contribute to disease and that interfering with both is necessary to prevent lethal disease. Here, we have inhibited the pro-inflammatory response associated with pyroptosis and showed that inhibition of CASP-1 had a beneficial effect on survival time. Our results confirm that some inflammatory responses may be involved in the pathogenesis of severe disease and the results suggest that effective intervention includes inhibition of virus and host response.

1. Short communication

Rabies encephalitis, caused by any of the Lyssaviruses is the only known infectious disease causing almost 100% mortality to infected individuals. Despite the availability of effective vaccines and the possibility to protect against infection by post-exposure treatment, rabies still accounts for almost 60,000 deaths annually mainly affecting children and young adults in the resource-poor countries [1].

Once neurological symptoms appear in infected individuals only palliative care can be given and death is inevitable. In developed countries sporadic cases are reported, and patients will be typically admitted to intensive care units and put under induced coma, which allows physicians to test experimental treatment protocols. To date, little success has been achieved with such experimental treatments [2].

Monoclonal antibodies and hyperimmune serum have been given intrathecally to rabies patients in an attempt to control infection with passive transfer of antibodies at the site of infection. Alternatively, antivirals such as ribavirin have been used with no improvement in clinical course of disease [3]. The mechanisms of recovery reported in the few patients that have survived clinical rabies are not understood but the hypothesis is that the immune system of the patients cleared the virus [4]. For instance, survivors developed virus neutralizing antibodies, which could have limited the amount of virus, allowing the innate immune response to further inhibit or clear the infection. It is hypothesized that cell death due to virus replication and host responses triggered as a result of virus replication both play a role in the pathogenesis of severe rabies. It is clear that neutralizing antibodies are the principal modality of protective immunity. However, the role of innate and T cell immunity in clearing RABV from the CNS or pathogenesis are still poorly understood [5].

Rabies virus (RABV) is an exclusively neurotropic virus that inhibits an efficient interferon antiviral response, but there is no evidence that the virus causes cell death such as necrosis or apoptosis of infected neurons. The role of the inflammatory response is

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contradicting since in some lethal cases of rabies, severe inflammation was observed and in some cases no evidence of inflammation was reported [6,7]. Experimental infection in mice also supports the notion that rabies infection is not associated with an overt inflammatory response. Previous studies in our laboratory have demonstrated the activation of pyroptosis during RABV infection [18]. Pyroptosis is an inflammation-induced form of cell death. In this study we investigated whether pyroptosis is a relevant pathogenic mechanism in the mouse model and whether inhibition of pyroptosis could mitigate rabies disease in mice.

Adult female mice of different genetic backgrounds (BALB/c, C57BL/6) were infected via the intramuscular route in the right hind leg with a lethal dose of wild type silver-haired bat rabies virus (SHBRV-18, 10^6TCID$_{50}$/mouse; a kind gift of Dr. B. Dietzhold, Jefferson University, USA). Control mice were inoculated with an equivalent dose of betapropiolactone (BPL)-inactivated virus. Mice were monitored for clinical signs of rabies and killed by euthanasia when humane end-points were reached (ruffled hair, hunched back and hind-leg paralysis) (Fig. 1a). Virus infection was confirmed with detection of infectious virus in brain samples and RABV antigen in formalin -fixed brain tissue as previously described [8] (Fig. 1b).

To demonstrate activation of the pyroptotic pathway during RABV infection in mice, we tested for up-regulation of key molecules of pyroptosis (Caspase-1, IL-1β and IL-18) on the mRNA level in brain samples. Briefly, brain samples were collected at the time of euthanasia from SHBRV-18-infected mice, weighted and stored as 10% homogenate in DMEM medium (Lonza, Basel, Switzerland) with 10% penicillin and streptomycin (Lonza) at −80 °C. RNA was isolated using the MagNA Pure LC 1.0 system and High Pure RNA isolation kit (Roche, Mannheim, Germany) according to the manufacturer's procedures and quantified using NanoDrop ND-1000 UV–VIS spectrophotometer (NanoDrop Technologies, Wilmington, USA). mRNA was transcribed to cDNA using Oligo(dT)$_{12\text{-}18}$ Primer (Invitrogen, Carlsbad CA, USA) and Superscript III reverse transcriptase (Invitrogen) according to the instructions of the manufacturer. Gene expression 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 as relative to the housekeeping gene β–actin (Applied Biosystems) following the formula $2^{-\Delta Ct} * 10^5$ [9] where $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\beta\text{–actin}}$ and were corrected for RNA quantity. As shown in Fig. 2, in both mouse strains, CASP-1 and IL-1β were significantly elevated in mice that had received infectious virus compared to the mice that received BPL-inactivated virus ($P < 0.05$; Mann-Whitney, non-parametric test). IL-18 was significantly elevated only in C57BL/6 mice that received infectious virus ($P = 0.004$).

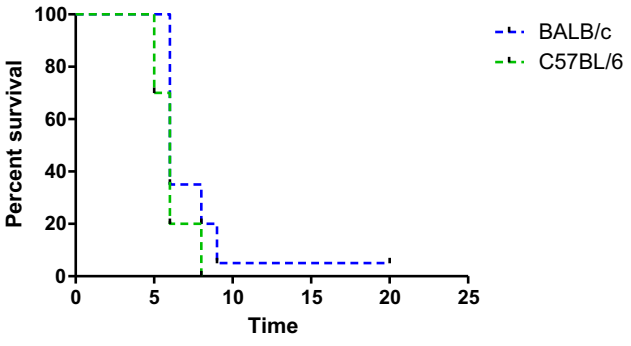

Fig. 1a. Survival curves of BALB/c and C57BL/6 mice after intramuscular inoculation of 10^6 TCID$_{50}$ of SHBRV-18 virus.

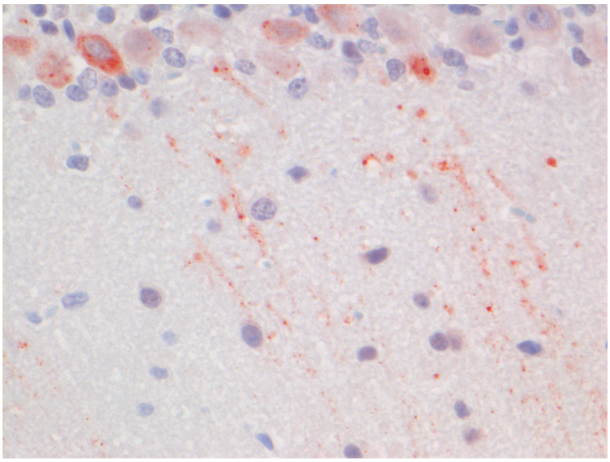

Fig. 1b. RABV antigen was detected in all mice that succumbed to rabies in formalin fixed brain tissues after intramuscular inoculation with SHBRV-18. Antigen was detected in all areas of the brain (cerebellum, cortex, brain stem) and in the spinal cord (see Ref. [7] for detailed method description). One representative picture from a positive cerebellum from a BALB/c mice is shown here.

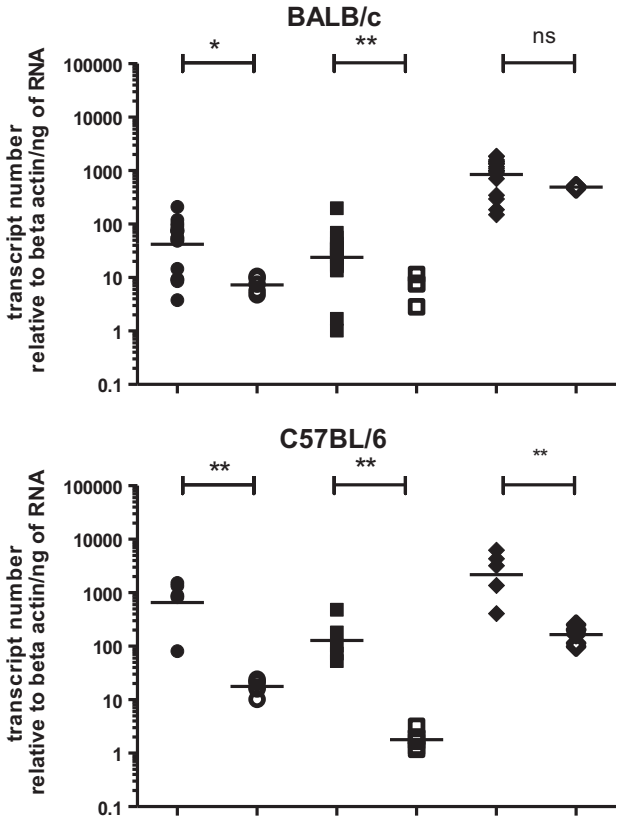

Fig. 2. Upregulation of pyroptotic markers after infection with SHBRV-18 of BALB/c (upper panel) or C57BL/6 mice. ●: CASP-1, ■: IL-1β, ◆: IL-18. Open symbols represent mRNA levels of the respective markers in mice that received BPL-inactivated virus. Statistical differences were calculated with a two-tailed Mann-Whitney test *: $p < 0.05$, **: $p < 0.01$, ns: not statistically different.

Pyroptosis, an essentially pro-inflammatory pathway of cell death could be responsible not only of neuronal loss during rabies but also neuronal dysfunction as has been described for other neurodegenerative diseases [10]. Since mRNA of key pyroptotic molecules was up-regulated in SHBRV-18 infected mice, we sought to

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investigate the effect of pyroptotic inhibitors on the course of clinical rabies in the C57BL/6 mouse model. Groups of 8-week-old female C57BL/6 mice (n = 10) were inoculated intramuscularly in the right hind leg with 10^6 $TCID_{50}$/mouse of SHBRV-18 virus. Five days after inoculation, treatment was initiated with CASP-1 inhibitor (100 ng/mouse of Ac-YVAD-cmk (InvivoGen, Toulouse, France)) or IL-1β inhibitor (200 μg/mouse of Kineret®, Sobi Inc., Stockholm, Sweden). Treatment was given intracranially in 10 μl volume on days 5, 7 and 9 post infection. Control mice (n = 7 per group) received PBS instead of infectious virus on day 0 and the same treatments as infected mice on days 5, 7 and 9. Ten mice received only infectious virus and three mice received only PBS intracranially on day 5, 7 and 9.

All virus-infected mice succumbed to rabies within two weeks post infection and virus was isolated from all infected mice (treated and non-treated). As expected, viral titers in the brain and spinal cord did not differ between treated and non-treated mice (Fig. 3) since the inhibitors used in this study would not influence viral replication. As shown in Fig. 4a, inhibition of CASP-1 significantly prolonged median survival time for 1.5 days compared to non-treated mice (p = 0.0371, Log-rank Mantel-Cox test). On the mRNA level, all key molecules of pyroptosis were suppressed to nearly baselines levels (CASP-1 and IL1β) or even lower (IL-18) compared to infected mice that did not receive treatment (Fig. 4b). Only TNF-α was not completely suppressed to baseline levels, indicating that additional to pyroptosis, more inflammatory pathways are likely to be activated during rabies. Inhibition of IL-1β did not have a significant effect on survival of infected mice compared to non-treated mice (Fig. 4a, p = 0.146, Log-rank Mantel-Cox test). However, Kineret® suppressed the expression of CASP-1 and IL-1β to baseline levels whereas IL-18 expression was down-regulated to even lower than baseline levels (Fig. 4b). Similarly to the group of mice that received Ac-YVAD-cmk, the Kineret® group had reduced expression of TNF-α compared to non-treated mice but not as low as baseline levels.

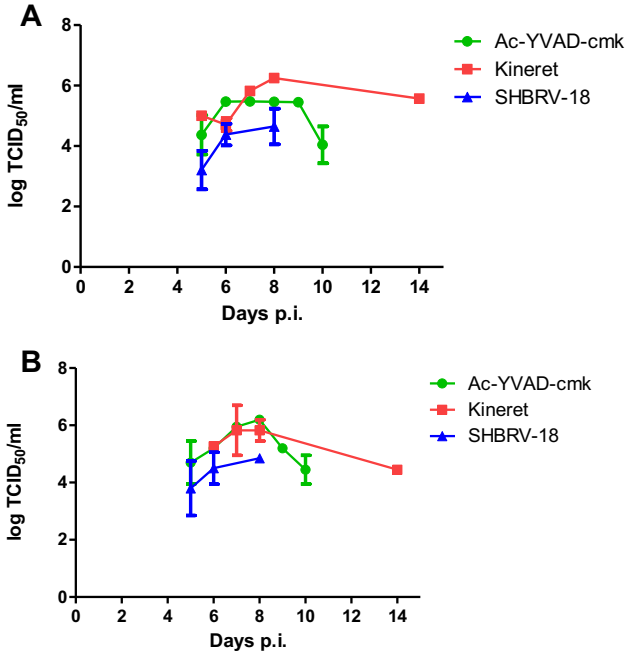

Fig. 3. Infectious viral titers recovered from mice that were euthanized when reached humane end points of rabies virus infection in (A) the brain and (B) spinal cord. Green: SHBRV-18 infected mice that received Ac-YVAD-cmk treatment, red: SHBRV-18 mice that received kineret and blue: non-treated SHBRV-18 infected mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

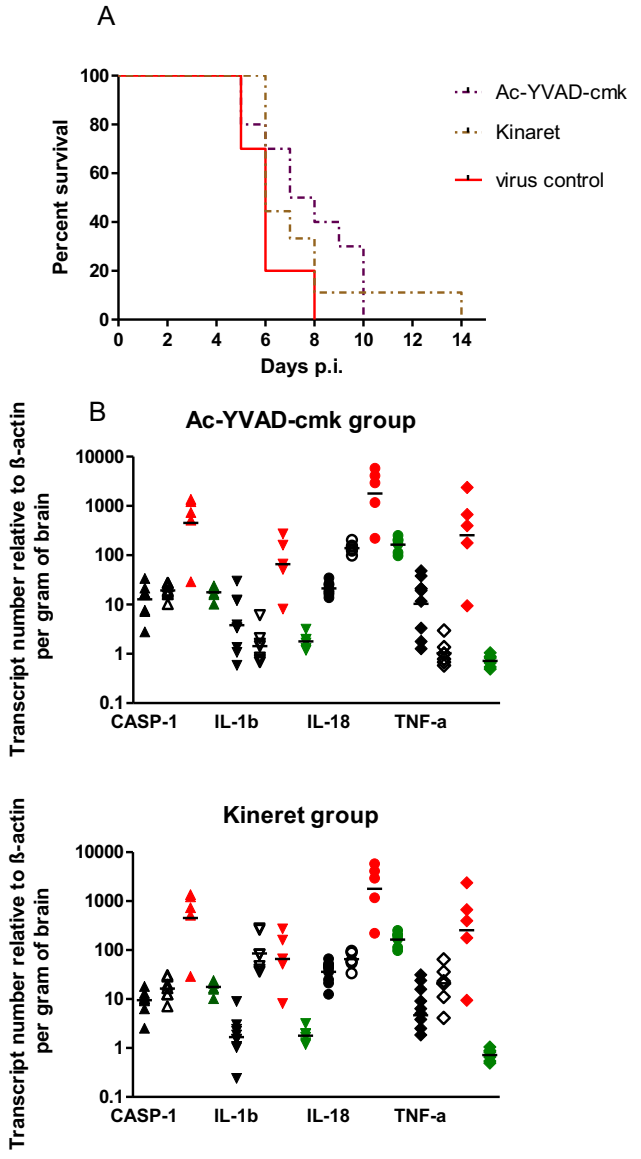

Fig. 4. Inhibition of pyroptosis in SHBRV-18 infected C57BL/6 mice. (a) survival curves after infection and treatment of mice with the respective molecules. (b) mRNA levels of key pyroptotic markers in mice treated with the respective molecules. Open symbols represent the groups of mice that received treatment only (no virus infection). Red symbols represent the group of mice that received SHBRV-18 only and no treatment and green symbols represent the group of mice that received PBS instead of virus or treatment and are considered baseline values for the respective molecules. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Mouse brains were formalin fixed, paraffin embedded and stained with hematoxylin-eosin for histological examination. Histopathological screening of the brains that were collected from the mice revealed that infected animals had minor visible abnormalities. Occasionally, multifocal mild to moderate lymphocytic meningoencephalitis was seen. Neither cytoplasmic eosinophilic inclusion bodies (Negri bodies) nor necrosis were seen. However, virus antigen was detected in the brains of infected mice as previously described [8]. Based on morphological criteria, virus antigen expression was restricted to neurons. Virus antigen was seen as 3- to 5-μm-diameter red granules, mostly in the cytoplasm of neuronal cell bodies, but also arranged in narrow rows in neuronal

processes. Occasionally rabies-antigen positive neurons showed signs of degeneration as demonstrated by loss of Nissl substance with shrinkage. Karyopyknosis and karyorrhexis without infiltration of inflammatory cells was present, although not abundant.

To evaluate the effect of treatment on the number and distribution of neurons expressing virus antigen, we compared virus antigen expression in 12 selected areas of the brain (Table 1). To exclude the time of death as a factor determining virus distribution we only looked in mice that were euthanized at day 8 post inoculation. For this time point we only had one mouse each in the virus control and Ac-YVAD-cmk treated groups and two mice from the Kineret® treated group.

The general trend at day 8 p.i. was that primary and secondary motor areas in layer 5 and the motor area of the midbrain were the most affected since RABV antigen-positive neurons were abundantly detected in these areas. In contrast, scarce positive neurons were seen in the hippocampus, the caudoputamen, and the sensory related colliculi of the midbrain.

To rule out the possibility that a lower proportion of virus-positive neurons was due to virus-induced loss of neurons, neurons were stained with a neuron marker (NeuN), and the total number of neurons in two areas with abundant infection (primary and secondary motor area layer 5) was compared between infected and non-infected mice. There were no significant ($P > 0.05$) differences in the number of neurons between infected and non-infected mice (Mann-Whitney non parametric *t*-test, data not shown).

Only supportive care to mitigate the severe symptoms can be offered to rabies victims until coma and death occur. A small number of patients (n = 5) have recovered from rabies with severe neurological sequelae [11]. Therefore, any treatment that could increase the chances of survival or improve the clinical course of rabies would be beneficial to the thousands of rabies patients. The use of classical antiviral approaches has proven unsuccessful for rabies [2]. In this study, we have shown that therapy, focusing on inhibition of the host response rather than the virus might be useful to treat rabies.

Pyroptosis, an essentially proinflammatory pathway of cell death could be responsible not only of neuronal loss during rabies but also has a detrimental effect on neuronal function with the release of pro-inflammatory cytokines. Pyroptosis was shown to be triggered by for instance chikungunya virus and HIV [12]. We have shown that intervention early in the pyroptotic pathway (inhibition of CASP-1) can prolong survival of mice, whereas intervention with downstream products of the activated pathway (inhibition of IL-1β) did not influence survival time of mice. Since these molecules act as host response modulators rather than antivirals, infectious virus titers were not influenced by the treatment approach suggesting that prolongation of survival in the Ac-YVAD-cmk treated group was indeed due to inhibition of some of the pro-inflammatory pathway and not due to interference with viral replication. Nevertheless, all mice eventually succumbed to rabies, indicating that treatment with CASP-1 inhibitor alone is not sufficient to completely abolish detrimental inflammatory host responses. It is possible that additional inflammatory pathways activated during rabies contribute to pathogenesis and therefore, a combination of different host-response inhibitors might increase the chance of survival after rabies. In addition, inhibiting virus replication as well as host responses might be more beneficial than focusing on host response only.

Our observations that inhibition of CASP-1 could prolong survival time of mice could have been an effect of timing. It is generally accepted that once symptoms occur in rabies patients then death is inevitable. Therefore, in our study we chose to treat mice only at the moment that we observed signs of rabies and not earlier. In our mouse model, there is only a short window of virus being present in the brain and development of signs (typically less than 12 h). It is therefore possible that treatment with CASP-1 inhibitor started too late to be able to control the disease. In addition, within our study protocol, we choose to intervene only for a short period of time (three intracranial inoculations every other day). It is possible that survival rates could have increased if treatment was given more often or for longer period of time. In addition,

Table 1
Virus distribution and abundance (0–5) score of mice that died on day 8.

Virus infected Group	Area scored: Primary motor area layer 2/3	Primary motor area layer 5	Secondary motor area layer 2/3	Secondary motor area layer 5	Prelimbic area layer 2/3	Prelimbic area layer 5	Hippocampus	Caudoputamen	Midbrain; motor related …	Midbrain, sensory related colliculi	Periaquaductal grey	Midbrain, reticular nucleus	Average score
No treatment	3[a]	5	3	5	1	3	1	2	5	0	5	5	3
Ac-YVAD-cmk	0	4	0	4	0	4	0	0	5	0	5	2	2
Kineret	0	5	1	3	na	1	0	1	3	1	4	1	2
	0	1	0	2	1	0	0	0	1	0	3	1	1

[a]0 = no virus; 1 = about less than 20% neurons infected; 2 = about 20–40% neurons infected; 3 = about 40–60% neurons infected; 4 = 60–80% neurons infected; 5 about over 80% neurons infected.

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repeated injection of the respective drugs intracranially may have contributed to death of the animals and administration of drugs parentally would be the preferred route.

It should be noted that our observations should be interpreted with caution. We have used the mouse model to demonstrate the presence of excessive host response that could have a detrimental effect on clinical outcome. However, this remains a model to study disease and the observed responses to infection may be species dependent or even virus strain dependent. For instance, inflammatory response in the brains of dogs infected with rabies virus were subtle [13] whereas mice had different transcriptomic profile when infected with attenuated or highly pathogenic rabies virus [7]. Earlier studies have suggested that immune-mediated cell death, may differ between animal species and inflammatory-immune response accounted for early death in rabies [14–17]. Nevertheless, we have used transcriptomic data to identify pathways that could play a role in pathogenesis of rabies in mice [18] and tailor our proposed intervention strategies to mRNA profiles.

In conclusion, we consider these preliminary experiments promising for the design of innovative treatment strategies against rabies. We propose that CASP-1 inhibition should be included in future designs of combination therapy, given more frequently to increase the chances of survival during rabies.

Ethical statement

Animal experiments were performed under Polish approved license: the IInd Ethical Commission for Experiments in Animals, Lublin, Resolution No. 10/2014 dated March 28, 2014 following EU, Dutch and Polish guidelines for animal experimentation.

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