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Rapid Communication

CD8$^+$ T-cells mediate immunopathology in tick-borne encephalitis

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ABSTRACT

Epidemics of tick-borne encephalitis involving thousands of humans occur annually in the forested regions of Europe and Asia. Despite the importance of this disease, the underlying basis for the development of encephalitis remains undefined. Here, we prove the key role of CD8$^+$ T-cells in the immunopathology of tick-borne encephalitis, as demonstrated by prolonged survival of SCID or CD8$^{-/-}$ mice, following infection, when compared with immunocompetent mice or mice with adoptively transferred CD8$^+$ T-cells. The results imply that tick-borne encephalitis is an immunopathological disease and that the inflammatory reaction significantly contributes to the fatal outcome of the infection.

Introduction

Tick-borne encephalitis (TBE), a disease caused by Tick-borne encephalitis virus (TBEV), is an increasing public health problem in northern and central Eurasia, where thousands of human encephalitis cases and numerous deaths are reported annually (Gritsun et al., 2003a). TBEV is a single-stranded, positive-sense, enveloped RNA virus, a member of the Tick-borne flavivirus (TBFV) group, that together with the Mosquito-borne flavivirus (MBFV) group and the No-known vector (NKV) group comprise the genus *Flavivirus* within the family *Flaviviridae* (Thiel et al., 2005). In humans TBEV may produce a variety of clinical symptoms, including fever and acute or chronic progressive encephalitis (Gritsun et al., 2003a,b), with or without a fatal outcome. As with other viral infections, the virulence of the circulating strain and the immunological status of the infected individual may contribute to the severity of the disease (Růžek et al., 2008). Recent studies on the molecular basis of pathogenesis, mostly performed with the MBFV group, established that apoptosis and immune-mediated tissue damage may determine the outcome of flavivirus infections (reviewed in Chambers and Diamond, 2003; King et al., 2007). However, the mechanism by which viruses in the TBFV group induce encephalitis is not completely understood. In this study we provide direct evidence for the immunopathology of TBE using different mouse strains to model TBEV infections. We address this issue by analyzing the role of specific T-cell subpopulations, i.e. CD4$^+$ and CD8$^+$ T-cells, in the recovery and/or immunopathology of TBE in mice.

Results

To assess the contribution of immunopathology in the development of encephalitis, we directly examined the role of two subpopulations of T-cells, i.e. CD4$^+$ and CD8$^+$, following infection of different strains of mice with TBEV.

Groups of mice with severe combined immunodeficiency (SCID), and control immunocompetent (Balb/c) mice, were inoculated subcutaneously with 100 pfu of the prototype neurovirulent TBEV strain Hypr. Morbidity, mortality, and mean survival times (MST) were then recorded. The clinical signs emerged virtually at the same time in both groups, i.e. approximately on the 8th or 9th day post-infection (p.i.). The mice showed clinical signs of hunching, ruffling of fur and hind-limb paralysis. Mean survival time of the immunocompetent Balb/c mice was 10.4±3.3 days; however, SCID mice survived significantly longer (MST of 13.9±0.8 days, $p<0.05$; Fig. 1A).

Analogously, CD8$^{-/-}$ knockout mice derived from a C57Bl/6 genetic background, exhibited prolonged mean survival times following TBEV infection (MST of 12.73±2.10 days) when compared with immunocompetent C57Bl/6 mice (7.45±1.37, $p<0.001$; Fig. 1B).

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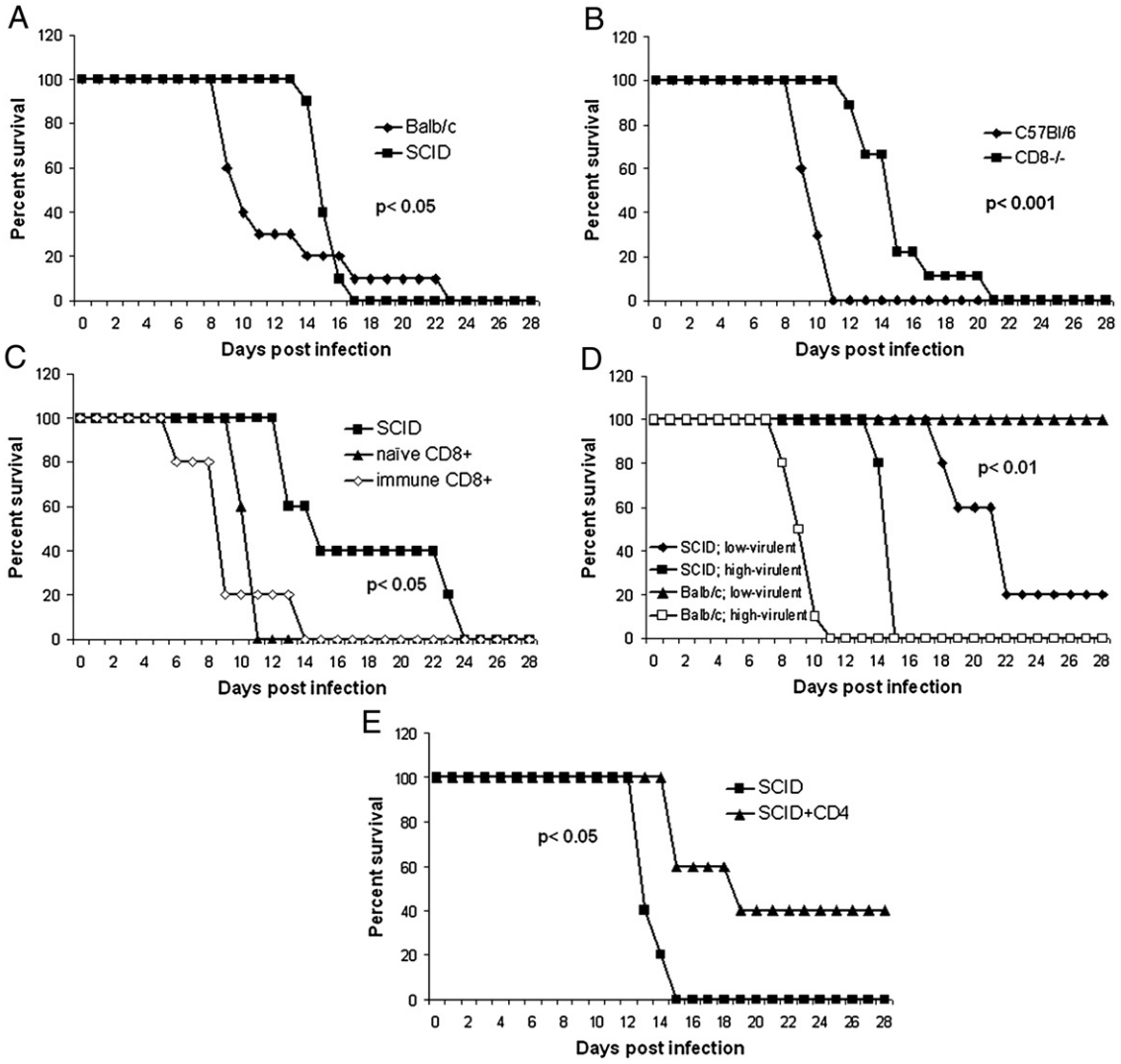

Fig. 1. Survival of mice infected with TBEV. Each animal was inoculated subcutaneously with a 100 pfu of strains Hypr (A–C and E), 263-TR (high-virulent) or 263 (low-virulent) (D). The mouse strains used were Balb/c, SCID, C57Bl/6 and C57Bl/6 $CD8^{-/-}$ as indicated.

To examine the precise role of $CD8^+$ T-cells in the pathogenesis of TBE, $CD8^+$ T-cells from control immunologically naïve as well as immune Balb/c mice, were isolated and adoptively transferred to SCID mice as described in Materials and methods. Adoptive transfer of immune as well as naïve $CD8^+$ T-cells led to significantly shorter MST following TBEV infection in comparison with SCID mice (MST of 15.0±3.8 days). Mice that received $CD8^+$ T-cells from immunised mice survived slightly shorter (MST of 7.0±2.4 days) than mice receiving $CD8^+$ T-cells from naïve mice (MST of 9.3±0.5 days), but the difference was not statistically significant although reproducible.

We compared the infection of SCID and Balb/c mice caused by two TBEV strains (263 and 263-TR) differing in virulence (Fig. 1D). Following subcutaneous inoculation, strain 263 is completely non-neuroinvasive in Balb/c mice, whilst 263-TR is highly virulent. Although 263-TR virus causes 100% mortality in both strains of mice, the MST was notably longer in SCID mice. On the other hand, 80% of the SCID mice infected with strain 263 developed lethal encephalitis although their mean survival time was much longer (19.0±2.9 days) compared with mice infected with the neuroinvasive strain (13.4±0.5 days, $p<0.01$; Fig. 1D).

Besides $CD8^+$ T-cells, we also evaluated the role of $CD4^+$ T-cells in TBEV infection, using the adoptive-transfer approach (Fig. 1E). $CD4^+$ T-cells negatively selected from spleens of naïve Balb/c mice were adoptively transferred intraperitoneally to SCID mice (2×10^6 cells/mouse) and after one week, the mice were subcutaneously inoculated with strain Hypr. The adoptive transfer of $CD4^+$ T-cells to SCID mice led to a significantly prolonged MST (18.5±4.9 days) and increased survival after infection in comparison with infected SCID mice (13.5±0.7 days; Fig. 1C).

We then compared the growth of TBEV in the organs of, Balb/c, C57Bl/6, $CD8^{-/-}$, and SCID mice following subcutaneous inoculation of 100 pfu of the virus. Comparison of the viral load in the blood, spleen and brains of $CD8^{-/-}$, C57Bl/6 and Balb/c mice was not significantly different. However, although SCID mice exhibited prolonged survival following infection, the viral loads in the blood (days 3 and 8 p.i.) and spleen (days 6 and 8 p.i.) were much higher compared with the other strains of mice (Figs. 2A, B) and in brain compared to Balb/c mice (day 6 p.i.; Fig. 2C). Moreover, in SCID mice, the high viremia increased with time; in contrast, low viremia in all other mouse strains was detected on day 3 p.i. and low titers of virus were also detected in serum on day 8 p.i. in $CD8^{-/-}$ and C57Bl/6 mice (Fig. 2A). In brains, there were no statistically significant differences in the viral load in TBEV infected Balb/c, $CD8^{-/-}$ and C57Bl/6 mice (Fig. 2C).

To understand the cellular basis for the more rapid progress of the infection in immunocompetent mice in contrast with SCID or $CD8^{-/-}$ mice, we examined brain tissues for histopathological changes. Marked levels of infiltrates were observed in the vicinity of menin-

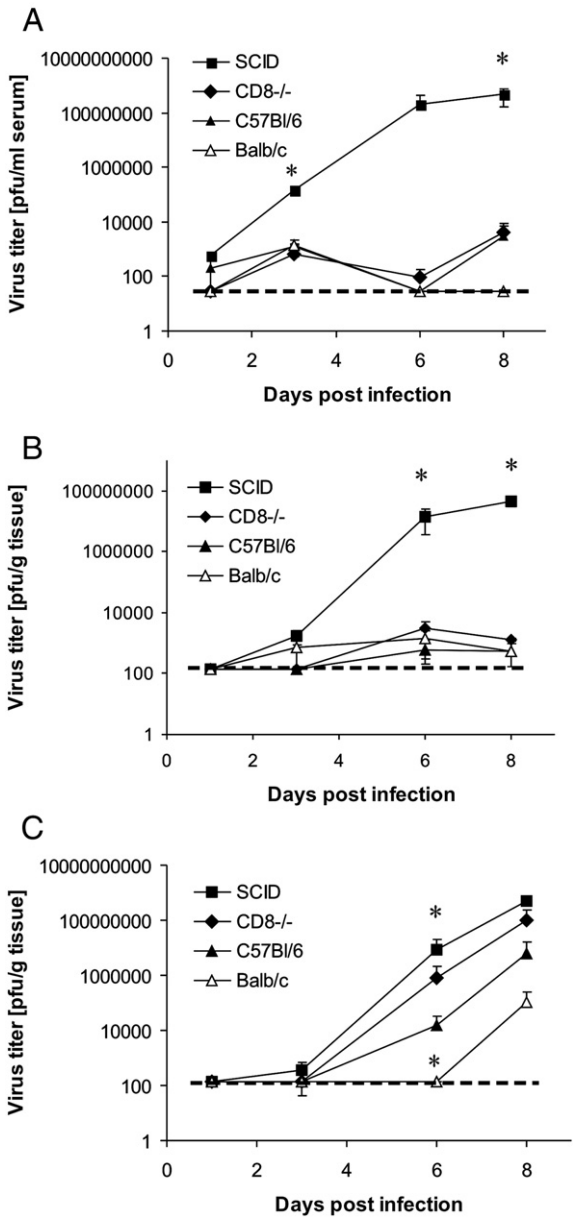

Fig. 2. TBEV burden in serum (A), spleen (B), and brains (C) of SCID, CD8$^{-/-}$, C57Bl/6, and Balb/c mice subcutaneously inoculated with 100 pfu of TBEV strain Hypr. Tissue and serum specimens were titrated individually by plaque assay on PS cells. For each time point the titers are the average of three mice. The dashed line indicates the limit of sensitivity of the assay. Asterisks indicate the differences that were statistically significant ($p<0.05$); SCID versus all other mouse strains (A and B), or SCID versus Balb/c (C).

geal vessels in both immunocompetent strains of mice (Balb/c and C57Bl/6) and to a lesser extent also in CD8$^{-/-}$ mice (Fig. 3). In infected Balb/c mice (Fig. 3E), the infiltrates were diffusely distributed and accompanied with signs of oedema. Diffuse/focal microgliosis and neuronal necroses were also seen. Predominantly necrotic lesions were found in the granular layer of the hippocampus (*stratum granulosum*); in this case, partial substitution of the granular layer with necrotic cells containing pycnotic nuclei was observed (Fig. 3K).

In infected C57Bl/6 mice, some of the perivascular infiltrates formed perivascular cuffs containing mononuclear infiltrating cells and histiocytes (Fig. 3F). Diffuse microgliosis, neuronal necrosis (Fig. 3H) and karyorrhexis of glial cells (Fig. 3I) were observed. The alterations observed in stratum granulosum of hippocampus (unicellular necroses) were similar to those observed in TBEV-infected Balb/c mice. Cell infiltrates were also in the ventricular system (around the plexus chorioideus).

In the case of CD8$^{-/-}$ mice infected with TBEV, histological investigation revealed moderate infiltrates of lymphocytes (Fig. 3G) and isolated hemorrhages (Fig. 3L).

The brain tissue of infected SCID mice was only slightly affected; only occasional isolated hemorrhages or proliferated microglial cells were seen.

There was no difference in the histology of brains of control uninfected Balb/c, C57Bl/6, CD8$^{-/-}$ and SCID mice (Figs. 3A–D).

Immunofluorescence investigation of the brain sections revealed that most of the infiltrating cells were CD8$^+$ T-cells (see Supplementary data). Very few infiltrating CD4$^+$ T-cells were detected in the brain sections of infected Balb/c or C57Bl/6 mice and virtually no lymphocytes were detected in brains of uninfected mice. In TBEV infected CD8$^{-/-}$ mice, lymphocytic infiltrates comprised CD4$^+$ T-cells. No immunoreactivity was observed in infected and control SCID mice.

Discussion

In flavivirus encephalitis, three possible mechanisms of brain tissue destruction have been postulated. Firstly, the virus itself causes direct neuronal damage; secondly, the neuronal death is caused by virus-induced inflammatory response; and finally, a combination of both, i.e. neuronal damage and immunopathology is responsible (Chambers and Diamond, 2003; King et al., 2007).

Recent data on MBFV indicate that a major cause of encephalitis in mice following infection is the detrimental effect of the host immune response. Transgenic mice lacking functional CD8$^+$ T-cells demonstrated extended survival and decreased mortality when infected with West Nile virus (WNV), in comparison with control wild-type mice (Wang et al., 2003). Similarly, for Murray Valley encephalitis virus (MVEV), the lack of perforin or Fas ligand molecules, that mediate effector activity of cytotoxic T-cells, protected mice against the development of encephalitis and fatal infection (Andrews et al., 1999). Generally, the immunopathology in flavivirus encephalitis seems to be mediated primarily by CD8$^+$ T-cells. On the other hand, the role of virus specific CD4$^+$ T-cells in flavivirus encephalitis is not well understood, although some experimental data indicate a requirement of such cells in protection against acute infection (Chambers and Diamond, 2003). An absence of CD4$^+$ T-cells in mice with genetic or acquired deficiency resulted in persistent WNV infection in the CNS, ultimately leading to uniform mortality. Moreover, adoptive transfer of WNV-primed CD4$^+$ T-cells significantly improved the survival of CD4$^{-/-}$ mice after WNV infection (Sitati and Diamond, 2006).

No corresponding data in relation to apoptosis or immune-mediated pathology have been reported for TBEV. Therefore, the underlying basis of the development and progress of TBE is still largely undefined.

Here, we addressed the contribution of the host immune system to the development of TBE by a combination of experimental approaches. Prolonged survival of SCID or CD8$^{-/-}$ mice in contrast with immunocompetent Balb/c and C57Bl/6 mice following infection with a neurovirulent TBEV strain indicated the detrimental effect of the host immune response in the development of the disease. Subsequently, we investigated the role of two subpopulations of T-cells, i.e. CD8$^+$ and CD4$^+$ T-cells, in the immunopathology of TBE using the adoptive transfer approach. The experiments demonstrated that the immunopathology is primarily mediated by CD8$^+$ T-cells, whereas CD4$^+$ T-cells confine the development of TBE. The helper CD4$^+$ T-cells play a protective role although the mechanism for this is not yet clear; it is probably based on CD4$^+$-mediated secretion of IFN-γ and other proinflammatory cytokines and/or stimulation of macrophage-like cells. CD4$^+$ T-cells are believed to control viral infections through the activation of B- and CD8$^+$ T-cell responses, production of inflammatory

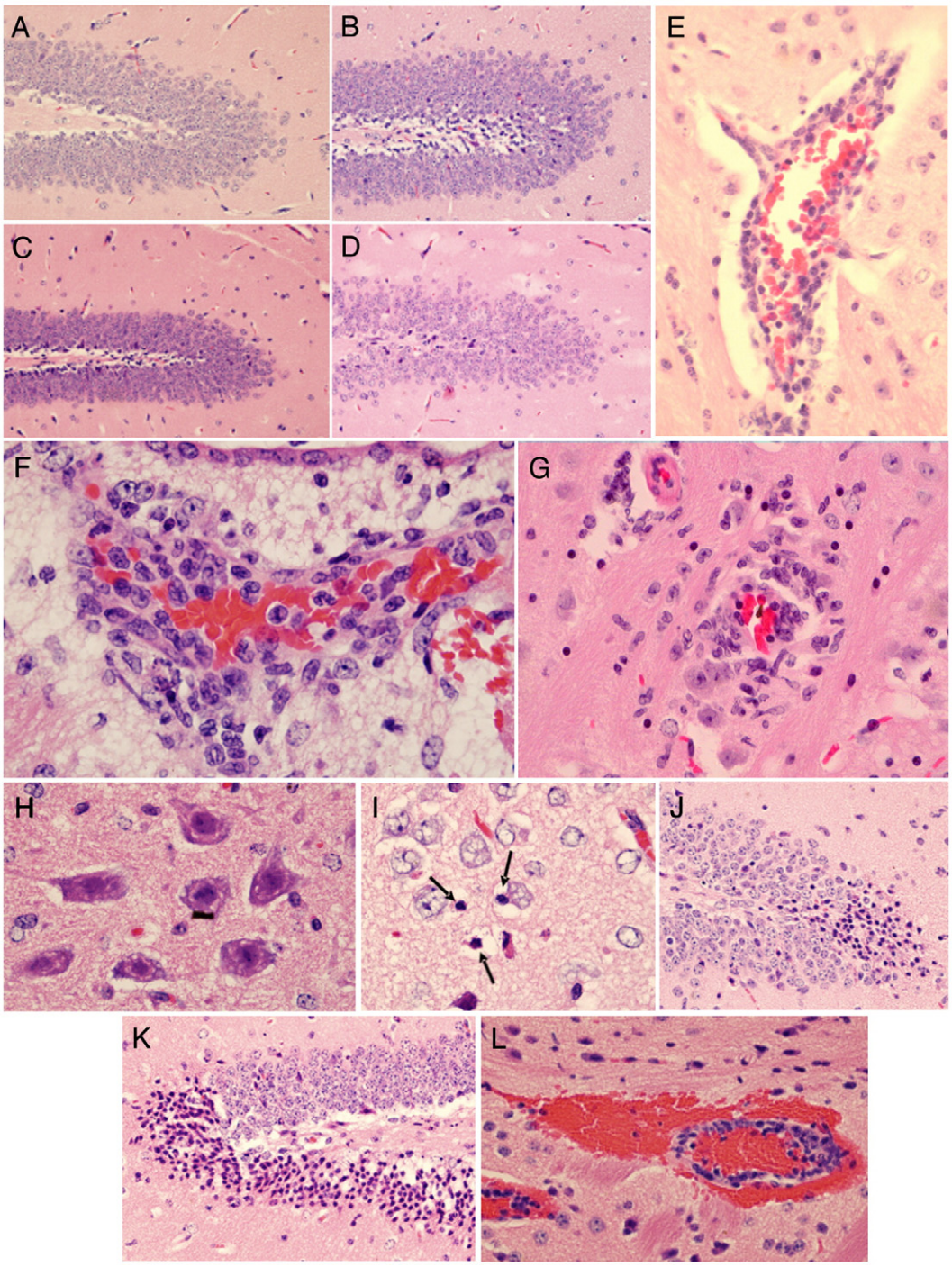

Fig. 3. Histology of TBEV-infected brains in parasagittal sections, hematoxylin–eosin staining. Brain sections of uninfected Balb/c (A), C57Bl/6 (B), $CD8^{-/-}$ (C) and SCID (D) mice. Expanding leukocyte infiltrations in TBEV-infected Balb/c (E), C57Bl/6 (F) and $CD8^{-/-}$ (G) mice. Neuronal necrosis (H) and karyorrhexis of glial cells (arrows) (I) observed in brains of C57Bl/6 mice. Partial substitution of granular layer with necrotic cells containing pycnotic nuclei in C57Bl/6 (J) and Balb/c (K) mice. Hemorrhagia and perivascular cuff in $CD8^{-/-}$ mice (L).

and antiviral cytokines, direct cytotoxic effects on infected cells and promoting memory response (Sitati and Diamond, 2006).

Our results showing an immunopathological effect of $CD8^+$ T-cells and the role of $CD4^+$ T-cells in confining the infection, correlate with some of the observations on WNV (Wang et al., 2003; Sitati and Diamond, 2006) and confirm the previous suggestions by Gelpi et al. (2006b). These suggestions were based on the observation that prominent inflammatory infiltrates and cytotoxic T-cells were present in close contact with morphologically intact neurons in human post-mortem brain tissues, thus indicating a key role for cytotoxic T-cells in the development of TBE. Similarly, in another study, transferred splenocytes shortened the incubation period of the disease implying a pathogenic role for the immune system in TBE (Semenov et al., 1975).

We also performed a histopathological investigation of the brains of all mouse strains used in this study. Investigation of moribund Balb/c and C57Bl/6 mouse brains revealed encephalitis associated with inflammatory cell infiltration, in accordance with previously published observations in mice (Osetowska and Wróblewska-Mularczyk, 1966), hamsters (Simon et al., 1966), monkeys (Simon et al., 1967), and humans (Gelpi et al., 2006a,b). Necrotic neurons were also observed. Immunofluorescence staining of $CD4^+$ and $CD8^+$ T-cells in the brain sections revealed lower levels of $CD4^+$ and predominance of

$CD8^+$ T-cells in the infected immunocompetent mice. The same result has been reported previously in WNV infections (Liu et al., 1989; Wang et al., 2003), but in brains of TBE infected patients, both $CD4^+$ and $CD8^+$ T-cells were present (Gelpi et al., 2006b).

Moreover, the comparative data of viral growth in the strains of mice used here, support the concept of a role for immunopathology in the development of TBE. Following infection of mice with 100 pfu of TBEV, viral loads in the blood and spleens were significantly higher in SCID mice compared with the other mice. Although SCID mice exhibited prolonged survival after TBEV infection and only minor histopathological changes in the brains were observed, high viral brain loads were detected in these mice, and they were significantly higher compared with Balb/c mice (day 6 p.i.; Fig. 2C). Therefore, it seems that the viral load did not determine the survival time or the pathology. Interestingly, no substantial differences were seen in the viral load in the blood, spleen and brains of $CD8^{-/-}$ and C57Bl/6 mice, suggesting that the $CD8^+$ T-cells have only a little role in TBEV clearance. This contrasts with the data on WNV (Shrestha and Diamond, 2004), where the absence of $CD8^+$ T-cells led to higher virus load in the CNS, and increased mortality.

Although we have demonstrated an immunopathological basis for encephalitis caused by TBEV infection, the role of direct damage of neurons or apoptosis remains unknown and these factors also need to be investigated. Apoptosis, as a damaging mechanism of virus-induced neuronal death in experimental mice has been demonstrated for other flaviviruses, e.g. mosquito-borne Yellow fever virus (YFV), Japanese encephalitis virus (JEV) and WNV (Yasui, 2002; Shrestha et al., 2003). However some exceptions have also been reported, for example in the case of MVEV, it was shown that less than 0.1% of mouse neurons develop apoptosis (Andrews et al., 1999). Moreover, ultrastructural investigation of mouse brain neurons indicated that neuronal dysfunction rather than morphological destruction occurs during JEV infection (Hase et al., 1990).

TBEV induces both apoptosis and necrosis in human neuroblastoma and glioblastoma cell lines (Růžek et al., manuscript in preparation) and also in mouse and monkey brain neurons (Isaeva et al., 1998; Kamalov et al., 1998), but prominent signs of neuronal apoptosis were not seen in postmortem brain tissues from human TBE patients, as demonstrated by anti-caspase 3 immunohistochemistry and TUNEL assay (Gelpi et al., 2006b). In addition, TBEV was also isolated from the brains of healthy animals in wild and Syrian hamsters in the laboratory indicating that propagation of TBEV in brain tissue is not necessarily accompanied by apoptosis (reviewed in Gritsun et al., 2003b).

Evidence that direct damage of CNS also occurs in TBEV infected mice was supported by our experiments in which two different TBEV strains differing in neuroinvasiveness were studied. A strain 263 is completely non-neuroinvasive for mice, whereas strain 263-TR is highly neuroinvasive. However, both of these viruses are equally neurovirulent following intracranial inoculation. These differences were attributed to a point mutation in an active centre of the virus serine protease. This mutation delays virus propagation at the site of inoculation, thereby gaining time for adaptive immune responses to develop and limit virus spread into the CNS (Růžek et al., 2008). We compared 263 and 263-TR in SCID and Balb/c mice (Fig. 1D). Although the 263-TR virus causes 100% mortality in both strains of mice, the MST was notably higher in SCID mice supporting the hypothesis of a pathogenic effect due to an immune response (Fig. 1D). However, comparison of 263-mediated infection in Balb/c and SCID mice supports the other hypothesis implying direct damage of the CNS due to virus replication. These observations imply that in the absence of an immune response even the attenuated, less invasive virus, 263 eventually reaches and damages the CNS; this corresponds to the neurovirulent properties of the 263 strain observed following intracranial virus inoculation. Together, these two comparisons indicate that in normal mice, with fully developed immune systems, both factors, i.e. direct virus damage to the CNS and an immune response contribute to the clinical outcome.

Currently, there is no specific therapy for TBE other than supportive measures. However, the administration of tetracycline hydrochloride, a compound with an anti-inflammatory effect, during experimental TBEV infection in mice led to significantly decreased mortality rates (Atrasheuskaya et al., 2003). Moreover, tetracycline hydrochloride acted as an immunomodulator, which was able to reduce manifestations of inflammation response during TBE infection in humans; this action led to quicker recovery from clinical symptoms and, consequently, to a faster recovery (Atrasheuskaya et al., 2003). Our data, showing the immunopathological features of TBE, provide additional support for immunomodulatory therapy in this disease.

In conclusion, our experiments demonstrate that $CD4^+$ T-cells may have some function in the limiting of TBE, whereas $CD8^+$ T-cells play a role in the immunopathology of TBE caused by neurovirulent strains. However, in the absence of an immune response the virus on its own is also capable of causing encephalitis. Our study expands the current knowledge of the antiviral immune response during TBEV infection and the understanding of the pathogenesis of TBE and could provide the basis for a rational therapeutic strategy against this dangerous human pathogen.

Materials and methods

Mice

Balb/c and C57Bl/6 mice were originally obtained from Charles River Laboratoires (Sulzfeld, Germany). CD8α-knockout mice of the C57Bl/6 background ($CD8^{-/-}$; strain B6.129S2-Cd8a^{tm1Mac}) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). SCID mice (C.B17/Icr-scid) of the Balb/c background, originally obtained from Charles River Laboratoires (Sulzfeld, Germany), were housed in plastic cages with sterilized woop-chip bedding situated in flexible film isolators (BEM Znojmo, Czech Republic) with high-efficiency particulate air filters. Sterilized pellet diet and water were supplied *ad libitum*. Balb/c, C57Bl/6 and $CD8^{-/-}$ mice were housed in plastic cages with wood-chip bedding situated in a specific-pathogen free room with a constant temperature of 22 °C and a relative humidity of 65%. Groups of 10 adult mice (6–8-week-old) were used in each experiment.

Viruses

All experiments were performed with representatives of the West-European TBEV subtype. The strain Hypr, a prototype Czech TBEV strain, was originally isolated from the blood of a 10-year-old child diagnosed with tick-borne encephalitis in 1953 in the Czech Republic. A non-neuroinvasive strain 263 was isolated from *Ixodes ricinus* collected by flagging in the Czech Republic in 1987 (Růžek et al., 2008). A highly virulent derivative of the strain 263, designated 263-TR, was obtained after the passage of the original strain in PS cells at 40 °C (Růžek et al., 2008). Low-passage virus strains were used in this study.

Adoptive transfer of $CD4^+$ or $CD8^+$ T-cells

To produce immune $CD8^+$ T-cells, the Balb/c mice, were inoculated intraperitoneally with two doses of 10^3 pfu of the non-neuroinvasive strain 263 (Růžek et al., 2008) at two-week intervals. One week after the second dose, spleens from either immune or immunologically naïve Balb/c mice were removed and single-cell suspensions were derived after mechanical disruption of the spleen tissue through a 40-μm-pore-size filter. Splenocytes were washed three times in RPMI-1640 medium and pure and well-defined populations of $CD4^+$ or $CD8^+$ T-cells were isolated from whole splenocytes by immunomagnetic separation. For isolation of $CD4^+$ T-cells, the Dynal Mouse CD4 Negative Isolation Kit (Invitrogen Dynal AS, Oslo, Norway) was used. $CD8^+$ T-cells were isolated from the suspension using the Dynal Mouse CD8 Negative

Isolation Kit (Invitrogen Dynal AS, Oslo, Norway). The purity of lymphocytes was analyzed by flow cytometry. Samples (0.5×10^6 cells) were incubated with specific monoclonal antibodies against surface antigens CD4 (FITC anti-mouse CD4, clone H 129.19, PharMingen, San Diego, CA, USA) and CD8 (PE anti-mouse CD8α, clone 53–6.7, PharMingen, San Diego, CA, USA). Labeled cell samples were analyzed on an Epics XL Flow Cytometer (Coulter) equipped with a 15-mW argon-ion laser with excitation capabilities at 488 nm. Ten thousand events were measured. The labeled cell population was analyzed using System II software (Coulter). Cell populations with purity >90% were adoptively transferred to SCID mice via intraperitoneal route (2×10^6 cells/mouse). Seven days after the adoptive transfer, mice were infected with 100 pfu of TBEV subcutaneously.

Virus growth in mouse tissues

Groups of adult SCID, $CD8^{-/-}$, C57Bl/6, and Balb/c mice (females, 6–8-week old) were inoculated subcutaneously with 100 pfu of Hypr strain. At the given time point post-inoculations, 3 mice of each group were anesthetized and humanely killed. Specimens of the blood, spleen, and brain were collected. Organs were individually weighed and homogenized, and prepared as 20% suspensions ($w.v^{-1}$) in L-15 medium containing 3% newborn calf serum. The suspensions were clarified by centrifugation at 10,000 g and the supernatant media were titrated by plaque assay on PS cells.

Histopathological investigation

Brains from control and TBEV-infected Balb/c, C57Bl/6, $CD8^{-/-}$ and SCID mice were examined for pathological changes. The mice were infected with 100 pfu of the strain Hypr in 200 μl of L15 medium and control mice were subcutaneously injected with 200 μl of L15 medium. Brains were collected at the 8th day p.i. (Balb/c, C57Bl/6) or the 11th day p.i. ($CD8^{-/-}$, SCID), respectively, and were fixed in 10% neutral buffered formalin and embedded in paraffin. 5-μm parasagittal sections were stained with hematoxylin–eosin.

Immunofluorescence staining

Brain tissues were routinely formalin fixed and paraffin embedded. 5-μm parasagittal sections were then treated to remove paraffin and antigen retrieval was performed by boiling sections in 10 mM citrate buffer (pH 6.0) for 20 min. After blocking, the sections were incubated with either CD4-specific or CD8-specific monoclonal antibodies (kindly provided by Dr. Imtiaz Khan, Dartmouth Medical School, Hanover, NH) for 1 h at room temperature and then with $F(ab)_2$ goat anti-rat FITC-labeled IgG antibody (Serotec; diluted 1:100) for 1 h at room temperature. The sections were visualized using an Olympus BX60 microscope and photographed using an Olympus DP71 digital camera. Examples of typical infiltrates are submitted as Supplementary data.

Statistical analysis

The significant differences in survival time between groups of infected mice were analyzed by 'Survival Analysis' and the statistical difference in virus burden in mouse organs was determined by ANOVA followed by Fischer LSD Post Hoc test. Data without normal distribution were transformed by use of the $X' = \log(X)$ formula. All analyses were performed using Statistica® 7.1 software (StatSoft CR, Praha, Czech Republic). p-values <0.05 were considered significant.

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All of the experimental procedures were done in accordance with the national law on the use of experimental animals, safety and use of pathogenic agents.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.11.023.

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