European Journal of Immunology

Cytolytic effector pathways and IFN-γ help protect against Japanese encephalitis

Maximilian Larena[1], Matthias Regner[1] and Mario Lobigs[1,2]

[1] Department of Emerging Pathogens and Vaccines, John Curtin School of Medical Research, The Australian National University, Canberra, Australia
[2] Australian Infectious Diseases Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, Australia

Japanese encephalitis, caused by infection with the neurotropic flavivirus, Japanese encephalitis virus (JEV), is among the most important viral encephalitides in Asia. While previous studies established an essential role of Ab and type I IFN, it is still unclear if the cell-mediated immune responses, through their direct antiviral effector functions, contribute to protection against the fatal disease. We report here that mice defective in both the granule exocytosis and death receptor pathways of cytotoxicity display increased susceptibility to JEV. The two cell contact-dependent cytotoxic effector mechanisms act redundantly within the CNS to reduce disease severity. We also demonstrate that IFN-γ is critical in recovery from primary infection with JEV by a mechanism involving suppression of virus growth in the CNS, and that T cells are the main source of the cytokine that promotes viral clearance from the brain. Finally, we show by in vivo depletion of NK cells that this innate immune cell population is dispensable for control of JEV infection in the periphery and in the CNS. Accordingly, cell contact-dependent cytolytic and IFN-γ-dependent noncytolytic clearance of virus mediated by T cells trafficking into the CNS help in recovery from lethal infection in a mouse model of Japanese encephalitis.

Keywords: Fas receptor · Flavivirus · Granzyme · Mouse model · Viral encephalitis

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Introduction

To explore the immunological correlates for recovery from primary infection with the medically important neurotropic flavivirus, Japanese encephalitis virus (JEV), we have developed an adult mouse model that resembles the physiological route of virus transmission to the mammalian host by the bite of an infected mosquito [1]. We found that s.c. deposition of a small dose of JEV into the footpad of C57BL/6 (B6) mice resulted in neuroinvasive disease that was lethal in ~60% of animals. Mice staged T-cell-independent IgM and T-cell-dependent IgG responses that became detectable at days 4 and 8 postinfection (pi) respectively, and neutralized virus infectivity in vitro [1]. This early and sustained humoral immune response is considered absolutely essential for survival of infection with JEV and related flaviviruses, because infections of B-cell-deficient (μMT) mice were uniformly lethal [1, 2]. In addition to Ab, type I IFN responses are indispensable in the control of JEV infection [3, 4]. On the other hand, the role of $CD8^+$ T cells in recovery from Japanese encephalitis (JE) remains ambiguous. While JEV infection resulted in extensive activation and migration of $CD8^+$ T cells into the CNS, they did not provide a significant survival advantage based on mortality rates in groups of mice subjected to in vivo depletion of this

Correspondence: Dr. Mario Lobigs
e-mail: m.lobigs@uq.edu.au

immune cell population [1]. Furthermore, genetic deficiency in key molecules (Fas, perforin (Perf), or granzymes (Gzms) A and B) of the cytotoxic effector pathways involved in $CD8^+$ T-cell-mediated clearance of infected cells did not alter susceptibility to JEV. However, viral burden in the CNS was significantly higher in mice either depleted of $CD8^+$ T cells or defective in Fas or Gzms, showing in vivo functionality anti-JEV $CD8^+$ T cells [1]. It remains unclear whether insufficient magnitude of the response per se or associated immunopathology explains why the viral load reduction in the CNS does not translate into a survival advantage. Infection of the CNS with JEV triggers extensive neuronal death induced directly by viral infection as well as bystander damage resulting from proinflammatory cytokine and chemokine release [5–7]; infiltrating $CD8^+$ T cells may thus contribute to both neuropathological events.

Cytotoxic T cells and NK cells utilize two contact-dependent cytolytic mechanisms for induction of target cell death, the granule exocytosis, and Fas–Fas ligand pathways [8]. The former eliminates infected cells through a process in which the pore-forming protein Perf delivers members of a group of proteolytic enzymes, the Gzms, into cells targeted for destruction. The most abundantly released Gzm at the site of the immunological synapse are GzmA and GzmB, which in vitro are proapoptotic molecules. While questions remain on the requirement of Gzm for NK-cell and T-cell-mediated in vivo cytotoxicity [9, 10], the Gzms also possess noncytolytic activities that can contribute to inactivation of intracellular pathogens (reviewed in [11]). The Fas receptor (Fas or CD95) is a transmembrane protein expressed on all nucleated cells, and induces apoptosis upon engagement of Fas ligand expressed on cytotoxic effector cells. The Fas–Fas ligand death pathway is critical in maintaining homeostasis in peripheral lymphoid organs, but also contributes to host defense by eliminating virally infected cells (reviewed in [12]). The requirement for functionality of either or both cell contact-dependent cell death pathways varies between viral disease models (reviewed in [13], and in some cases can also enhance disease severity [14–17].

Cell-mediated immunity against intracellular pathogens also involves the release of cytokines such as IFN-γ and TNF-α from activated T cells, and from cells of the innate arm of the immune response (NK, NKT, and $\gamma\delta$ T cells). The diverse functions of IFN-γ in the control of microbial infections includes activation and polarization of Th cells, upregulation of Fas on infected target cells [18], upregulation of MHC class I (MHC-I) and MHC class II Ag presentation pathways, macrophage activation, and direct antiviral activity that overlaps with that triggered by type I IFN (reviewed in [19]). The requirement for IFN-γ in recovery from murine infections with different flaviviruses is variable: The cytokine plays an essential role in the early protective host response against a virulent North American isolate of West Nile virus (WNV) [20] and mouse-adapted strains of dengue virus [21, 22], but is not required for control of infection with less virulent strains of WNV [23] or that of yellow fever virus [24, 25], and has only a modest protective role in Murray Valley encephalitis virus infection [26]. In human cases of JE, IFN-γ has been associated with a beneficial effect on disease outcome [27].

The aim of the present study was to investigate the role of cell-mediated immune effector functions in recovery from lethal infection in a mouse model of JE. We show that the concerted actions of cell contact-dependent death pathways and IFN-γ protect against JEV by suppression of virus growth in the CNS.

Results

Combined deficiency in Fas–Fas ligand and granule exocytosis increases susceptibility to JE

Previous studies have shown that the mortality rates of $Fas^{-/-}$, $Perf^{-/-}$, and $GzmAxB^{-/-}$ mice infected with 10^3 PFU of JEV, s.c., were similar to that of B6 wild-type (wt) mice, although viral burden in the CNS of the KO mice was higher than that in wt animals [1]. Since cytotoxic leukocytes can trigger lysis of virus infected cells by two major cell contact-dependent pathways, the granule exocytosis and Fas–Fas ligand mechanisms, we investigated here whether loss of functionality of both cytolytic pathways in $Fas^{-/-}xGzmAxB^{-/-}$ mice would increase mortality. Furthermore, investigations on mouse strains deficient in one or more components of the granule exocytosis pathway have indicated that the concerted action of Perf and Gzms is essential for protection against several pathogens [28–30]. To test whether such a requirement for effective control of CNS infection with JEV existed, a mouse strain with a triple KO of Perf, GzmA, and GzmB was used. However, $Perf^{-/-}xGzmAxB^{-/-}$ mice challenged with JEV showed a mortality rate that was comparable to that of wt and $Fas^{-/-}$ mice (Fig. 1A). In contrast, deficiency of both cytolytic effector pathways in $Fas^{-/-}xGzmAxB^{-/-}$ mice caused a significant increase in mortality relative to wt (Fig. 1B). In all groups, moribund mice presented with similar clinical signs starting with generalized piloerection, paresis, and rigidity, and later progressing to severe neurological signs demonstrated by postural imbalance, ataxia, and generalized tonic-clonic seizures. The median time to development of severe signs of encephalitis (when animals were sacrificed) did not differ significantly between groups (12.7 ± 1.5 days for $Fas^{-/-}$, 12.5 ± 1.8 days for $Perf^{-/-}xGzmAxB^{-/-}$, 12.4 ± 0.7 days for $Fas^{-/-}xGzmAxB^{-/-}$, and 12.7 ± 1.6 days for B6 wt mice).

Viral load in serum, spleen, and CNS tissues of wt and $Fas^{-/-}xGzmAxB^{-/-}$ mice infected with JEV was determined at two-day intervals pi. Viremia and viral load in spleen measured by real-time RT-PCR peaked in both groups on day 2 and 4 pi, respectively, and did not differ significantly between the groups (Fig. 2A and B). However, the combined defects in the Fas–Fas ligand and granule exocytosis pathways significantly compromised the ability of mice to control virus growth in the CNS and/or virus entry into the brain. Compared with wt mice, the proportion of $Fas^{-/-}xGzmAxB^{-/-}$ mice showing detectable virus titers in brain was markedly greater, and viral load 40- and 800-fold higher on day 8 and 10 pi, respectively (Fig. 2C). In addition, dissemination of virus into spinal cord was more evident in $Fas^{-/-}xGzmAxB^{-/-}$ mice, with viral titers exceeding those in wt mice by 2 log on day

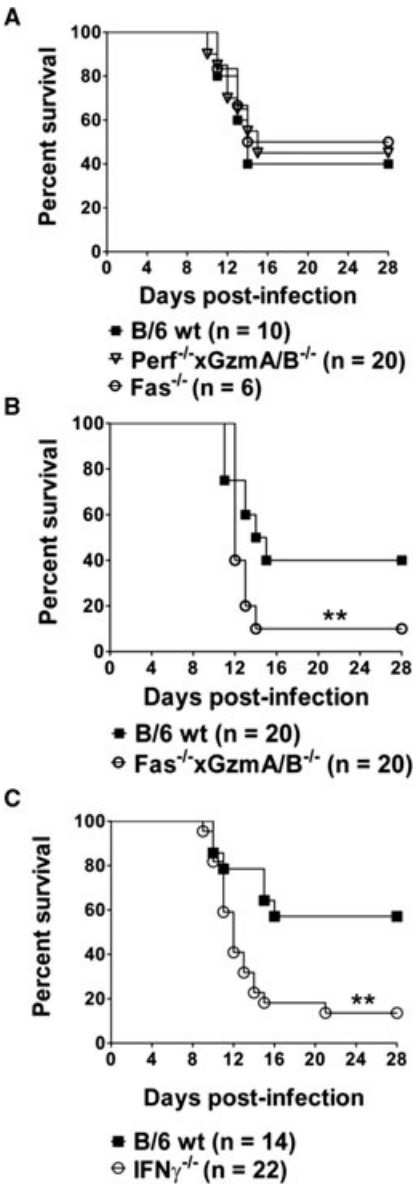

Figure 1. Survival of B6 wt and KO mice defective in cellular antiviral effector functions. Groups of 12-week-old mice were infected s.c. with 10^3 PFU of JEV, and morbidity and mortality were recorded daily. Surviving mice from experiments comparing (A) wt mice against $Perf^{-/-}\times GzmA/B^{-/-}$ and $Fas^{-/-}$ mice, (B) wt mice against $Fas^{-/-}\times GzmA/B^{-/-}$ mice and (C) wt mice against IFN-$\gamma^{-/-}$ mice were monitored for 28 days. Data shown were pooled from two independent experiments. $^{**}p < 0.01$; log-rank test.

10 pi (Fig. 2D). These findings were supported by increased detection of JEV Ag in various regions (cortex, hippocampus, thalamus, cerebellum, and brainstem) of brains of $Fas^{-/-}$xGzmAxB$^{-/-}$ relative to wt mice by immunohistochemical analysis (Supporting Information Fig. 1).

Together, the results show redundancy of the two cell contact-dependent effector pathways of T-cell and NK-cell-mediated cytotoxicity in providing resistance to lethal JEV infection by a mechanism involving suppression of virus growth that was clearly demonstrable in the CNS, but not in extraneural tissues.

IFN-γ is required for protection against JE

A previous study has shown that the combined adoptive transfer of JEV-immune $CD4^+$ and $CD8^+$ T cells, but not individual transfer of either of the two lymphocyte subpopulations, provided recipients with a survival advantage against JE [1]. While the above experiments suggested that cell contact-dependent cytotoxicity was required for the T-cell-mediated protective effect, we also investigated whether release of IFN-γ was important for survival. Using IFN-γ KO mice, we found that lack of the cytokine significantly increased mortality rate relative to wt mice (Fig. 1C), although the median time to development of severe signs of encephalitic disease did not differ significantly between the two groups (11.7 ± 2.3 days for IFN-$\gamma^{-/-}$ and 11.1 ± 2.1 days for wt mice).

A deficiency in IFN-γ resulted in increased CNS viral burden, with viral load in brains of IFN-$\gamma^{-/-}$ mice exceeding that in brain of wt mice by ~15- and ~1000-fold on days 8 and 10 pi, respectively (Fig. 2). IFN-$\gamma^{-/-}$ mice also exhibited more pronounced dissemination of virus into the spinal cord. No difference was found between the two groups in the magnitude and kinetics of viremia, and viral load in spleen.

Collectively, these data indicate that IFN-γ significantly contributed to the control of virus growth in the CNS, but was not required for effective clearance of virus from extraneural tissues.

Intact humoral immune response against JEV in $Fas^{-/-}$xGzmAxB$^{-/-}$ and IFN-$\gamma^{-/-}$ mice

The humoral immune response is critically important in recovery from primary infections with JEV and related flaviviruses [1, 2]. Therefore, magnitude and quality of JEV-specific Ab responses in mice with deficiencies in Fas and Gzms, or IFN-γ, were compared with those in wt mice infected with JEV. $Fas^{-/-}$xGzmAxB$^{-/-}$ and IFN-$\gamma^{-/-}$ mice presented with similar titers and kinetics of the IgM response against JEV relative to wt mice, which was first apparent on day 4 pi and increasing thereafter (Supporting Information Fig. 2A). Moreover, anti-JEV IgG1 and IgG2b Ab responses were similar in the different groups, with the magnitude of IgG2b exceeded that of IgG1 Ab isotype titers at 10 days pi (Supporting Information Fig. 2B and C, respectively). The JEV-neutralizing Ab response was also similar between the groups (Supporting Information Fig. 2D). Accordingly, the ability of $Fas^{-/-}$xGzmAxB$^{-/-}$ and IFN-$\gamma^{-/-}$ mice to mount JEV-specific B-cell immune responses remained unimpaired.

Cellular migration into the CNS

The results thus far indicated that KO mice lacking the ability to trigger the cell contact-dependent death pathways or to secrete IFN-γ displayed significantly increased virus burden in the CNS, but not in extraneural tissues. A role of IFN-γ in leukocyte chemotaxis has been demonstrated (reviewed in [19]). Therefore, we assessed whether an impairment of trafficking of leukocytes into

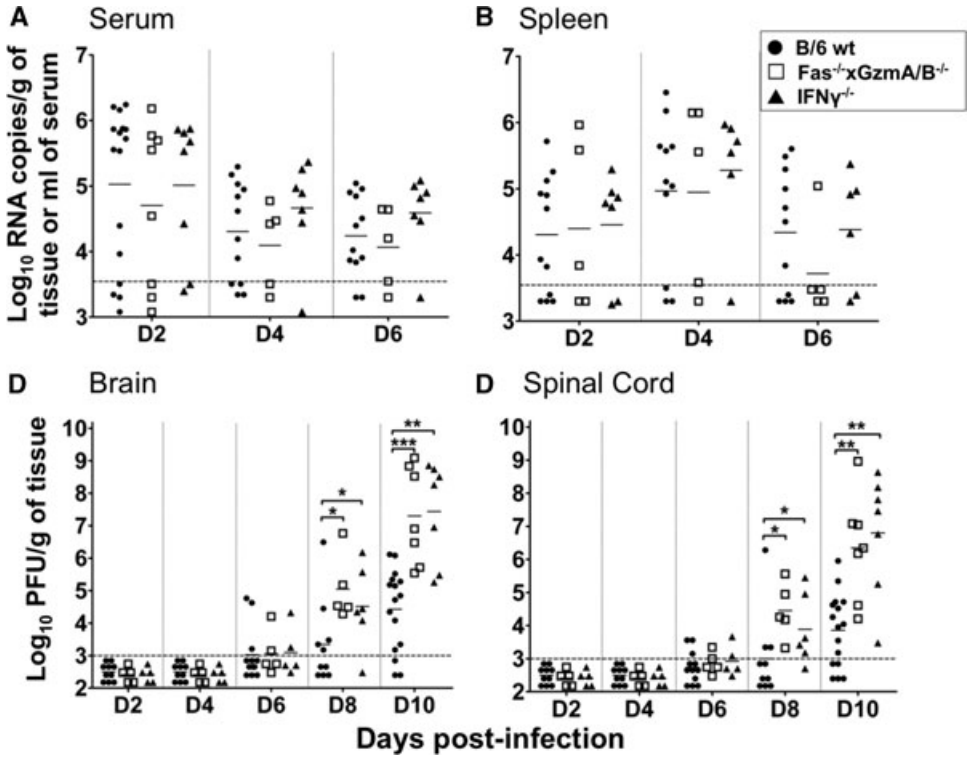

Figure 2. Viral burden in peripheral and CNS tissues. (A–D) The JEV burden in (A) serum, (B) spleen, (C) brain, and (D) spinal cord samples from wt and KO mice infected s.c. with 10^3 PFU of JEV is shown. At the indicated time points, animals were sacrificed, and viral RNA content in serum and spleen samples measured by real-time RT-PCR, while the level of infectious virus in brain and spinal cord samples were measured by plaque-assay. The lower limit of virus detection is indicated by the dotted line. Each symbol represents an individual mouse, and horizontal lines indicate geometric mean titers. Data shown were pooled from two independent experiments performed. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; two-tailed Mann–Whitney test.

the infected brain contributed to poorer control of virus growth and dissemination in the CNS of IFN-$\gamma^{-/-}$ and Fas$^{-/-}$xGzmAxB$^{-/-}$ mice. Leukocytes were isolated from brains of JEV-infected mice and subpopulations quantified by flow cytometry as previously described [31]. IFN-$\gamma^{-/-}$ and Fas$^{-/-}$xGzmAxB$^{-/-}$ mice presented with similar numbers of NK cells, and CD4$^+$ and CD8$^+$ T cells, relative to wt mice at the time of detectable viral burden in brain on day 7 and 10 pi (Fig. 3).

Accordingly, deficiencies in cellular immune effector functions in IFN-$\gamma^{-/-}$ and Fas$^{-/-}$xGzmAxB$^{-/-}$ mice did not alter the ability of NK and T cells to infiltrate the JEV-infected brain. Moreover, the substantially higher viral burden in the CNS later in infection in the KO relative to wt mice did not significantly augment trafficking of these leukocyte subpopulations into the CNS.

Induction of soluble antiviral mediators in NK and CD8$^+$ T cells in the absence of Fas and Gzms

Besides direct cytotoxic action through the Fas–Fas ligand and granule-exocytosis pathways, the antiviral activity of NK and CD8$^+$ T cells is mediated indirectly through the release of cytokines. To investigate whether this latter function of the two leukocytes subpopulations remained intact in Fas$^{-/-}$xGzmAxB$^{-/-}$ mice, intracellular cytokine levels in ex vivo NK and CD8$^+$ cells were measured following JEV infection. At day 4 pi (which corresponds to the peak of the NK-cell response in this infection model; [31]), the percentage of NK cells expressing IFN-γ in spleen of Fas$^{-/-}$xGzmAxB$^{-/-}$ mice did not differ from that in wt mice (5.2% in Fas$^{-/-}$xGzmAxB$^{-/-}$ versus 5.5% in wt mice; Fig. 4A), and MFI of IFN-γ-specific staining was similar between the two groups (data not shown). Likewise, a comparable number of JEV-specific CD8$^+$ T cells were identified in spleen of wt and Fas$^{-/-}$xGzmAxB$^{-/-}$ mice at day 7 pi (Fig. 4A). In addition, the percentage of multifunctional CD8$^+$ T cells, characterized by expression of both IFN-γ and TNF-α in single T cells [32], did not differ significantly between the two groups at day 7 pi (Fig. 4B).

The result shows that activation of the NK-cell response and Ag-specific priming of CD8$^+$ T cells was normal in spleen of Fas$^{-/-}$xGzmAxB$^{-/-}$ mice.

T cells are a critical source of IFN-γ that promotes viral clearance from the CNS

To confirm the protective role of IFN-γ against lethal JE, and to determine its source in the CNS, JEV-immune total splenocytes or purified T cells from wt and IFN-$\gamma^{-/-}$ mice were transferred to

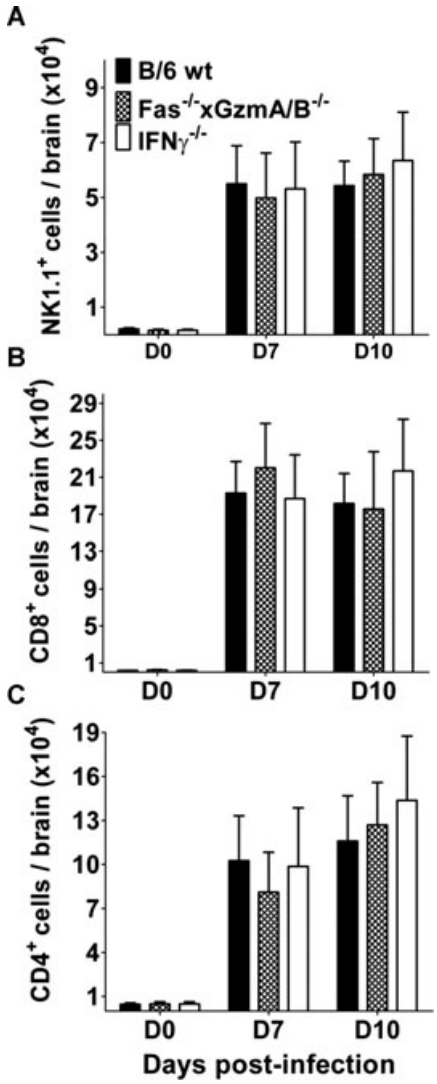

Figure 3. Leukocyte trafficking into the CNS. The kinetics of cell infiltration into brains of 8-week-old wt and KO mice infected s.c. with 10^3 PFU of JEV are shown. Leukocytes were isolated from brains at the indicated time points, and stained with leukocyte subpopulation-specific Ab for identification as (A) NK cells, (B) $CD8^+$ T cells, and (C) $CD4^+$ T cells by flow cytometry. Data are shown as mean + SEM of four samples and are from one experiment representative of two independent experiments.

naive B6 recipients that were subsequently challenged with JEV (Table 1). Consistent with a previous study [1], total immune splenocyte transfer from wt donors completely protected recipient mice from the lethal infection. Likewise, total splenocyte transfer from immune IFN-$\gamma^{-/-}$ mice to B6 recipients resulted in a 100% survival rate. On the other hand, transfer of immune T cells from wt donor mice conferred substantial, albeit partial, protection in wt recipients, in contrast to transfer of immune T cells deficient in IFN-$\gamma^{-/-}$ that showed no protective value. Moreover, viral load in brain and spinal cord of recipients of IFN-$\gamma^{-/-}$ immune T cells at day 10 pi was significantly higher compared to that in mice that received immune T cells from wt donors, and did not differ

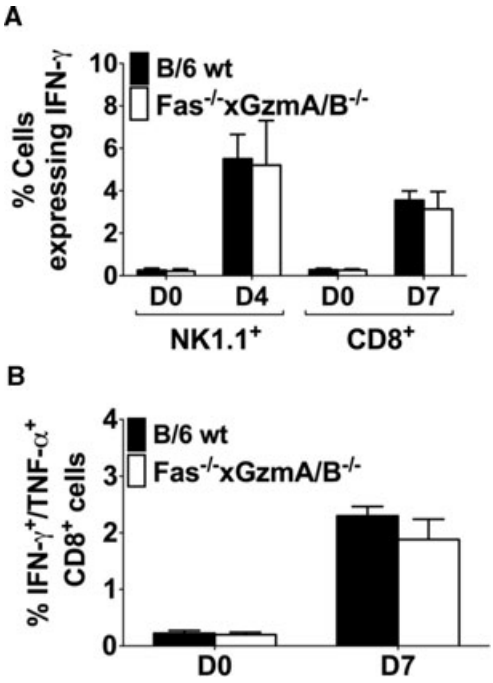

Figure 4. Splenic NK-cell and $CD8^+$ T-cell activation. Eight-week-old wt and Fas$^{-/-}$xGzmA/B$^{-/-}$ mice were infected iv with 10^3 PFU of JEV or left uninfected. (A) Spleens were collected at the indicated time points, and the percentage of $NK1.1^+$ and $CD8^+$ splenocytes expressing IFN-γ were identified by intracellular cytokine staining, where the latter were stimulated ex vivo with a JEV-derived peptide. (B) $CD8^+$ T cells expressing both IFN-γ and TNF-α are shown. Data are shown as mean +SEM of four samples and are from one experiment representative of two independent experiments performed.

from that of a control group that received naïve T cells from wt mice (Fig. 5).

Taken together, these data suggest that the IFN-γ-mediated protection against lethal JE is mediated, at least in part, by immune T cells, and involves control of virus growth in the CNS.

NK cells are dispensable for recovery from lethal JEV infection

NK cells are an important source of IFN-γ, exert cytolytic effector function via the Fas–Fas ligand and granule exocytosis pathways, and traffic into the CNS of JEV-infected mice (Fig. 3A). Accordingly, the beneficial contributions of IFN-γ and cellular cytotoxicity in recovery from JE could have been partly mediated by this innate immune cell population. To address this possible scenario, the effect of in vivo depletion of NK cells on disease severity in the JE mouse model was investigated. The depletion protocol with anti-NK1.1 Ab [33] resulted in >95% depletion of NK cells on days 2 and 7 pi (Fig. 6A). The mortality rate of NK-cell-depleted and mock-treated mice and the average time for development of severe signs of encephalitis (13.0 ± 1.8 days and 12.8 ± 1.5 days respectively) did not differ significantly between the two groups (Fig. 6B).

Table 1. Protective value of IFN-γ expressed from T cells against JEV

Treatment[a)]	Mortality (Number of deaths/total)[b)]	Mean survival time (days) ± SD[c)]
PBS control	67% (6/9)	12.3 ± 1.4
WT donor mice		
Naïve total splenocytes (1×10^7 cells)	75% (15/20; $p = 0.94$)	12.9 ± 1.7
Immune total splenocytes (1×10^7 cells)	0% (0/7; $p = 0.003$)	–
Immune T cells (5×10^6 cells)	28% (5/18; $p = 0.01$)	12.2 ± 1.3
IFN-$\gamma^{-/-}$ donor mice		
Immune total splenocytes (1×10^7 cells)	0% (0/5; $p = 0.01$)	–
Immune T cells (5×10^6 cells)	80% (12/15; $p = 0.10$)	12.7 ± 1.5

[a)] Donor mice were infected iv with 10^3 PFU of JEV or left uninfected, and sacrificed 7 days later for splenocyte collection with or without T-cell enrichment. Cells were transferred to 8-week-old B6 wt mice that were infected a day later with 10^3 PFU of JEV into the footpad. Surviving mice were monitored for 28 days.

[b)] Data shown were pooled from two independent experiments performed. Immune splenocyte treatment groups were compared with the naive splenocyte control group using the log-rank test.

[c)] No significant difference between immune splenocyte transfer and control groups ($p > 0.05$; two-tailed Mann–Whitney test).

To examine whether NK cells were involved in peripheral viral clearance or restriction of virus growth in the CNS, viral burden in serum, spleen, brain, and spinal cord was determined in NK-cell-depleted and mock-treated mice: viremia and viral load in spleen did not show a significant difference between the two groups at early time points pi (Fig. 6C and D), nor did viral titers in brain and spinal cord on day 10 pi (Fig. 6E and F).

Discussion

Here we showed that mice with a combined defect in granule exocytosis and Fas–Fas ligand pathways displayed increased susceptibility to infection with JEV. Deficiency in cytotoxicity resulted in increased viral burden in the CNS late in infection relative to wt mice, suggesting that the contact-dependent cytotoxic effector functions of the cell-mediated immune response acted in the CNS to reduce viral replication and disease severity. It is not entirely clear if the disease-ameliorating effect of immune-mediated cytotoxicity was limited to suppression of virus growth in the CNS; while extraneural virus titers did not differ significantly between wt and Fas$^{-/-}$xGzmAxB$^{-/-}$ mice, loss of cytotoxic effector function may nevertheless have contributed to increased virus spread into the CNS. The relatively moderate improvement in disease outcome in mice with functional Fas–Fas ligand and granule exocytosis pathways relative to animals deficient in both pathways was consistent with a previously described subsidiary role of CD8$^+$ T cells in protection against JE [1], and may reflect that the antiviral activity of the cytolytic pathways was predominantly apparent in the CNS where the benefit of cytolytic virus clearance is constrained by the limited capacity of neurons to regenerate.

The uncontrolled growth of JEV in the CNS of Fas$^{-/-}$xGzmAxB$^{-/-}$ mice resembles the increased pathogenesis of mouse hepatitis virus in mice lacking both Fas–Fas ligand and granule exocytosis-mediated effector functions, but not in animals with single deficiencies in either Fas or Perf [34, 35]. Immune defense in both models of neurotropic viral disease is characterized by a redundancy of the contact-dependent cytolytic mechanisms in control of lethal CNS infection. Intriguingly, in the JEV model, the dispensability of either Fas–Fas ligand or granule exocytosis pathways in disease outcome — as measured by mortality rate — contrasted with the markedly increased viral

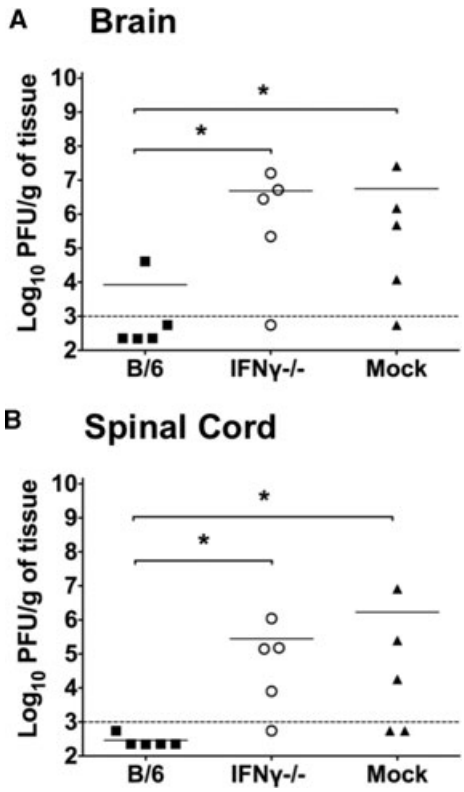

Figure 5. JEV burden in CNS after adoptive transfer of immune wt or IFN-$\gamma^{-/-}$ splenocytes. (A, B) The JEV burden in (A) brain and (B) spinal cord samples from B6 mice infected with JEV (10^3 PFU, s.c.) one day after transfer of naive total splenocytes from wt (mock), or JEV-immune T cells from wt or IFN-$\gamma^{-/-}$ donor mice is shown. At 10 days pi, mice were sacrificed, and viral content in tissues measured by plaque assay. The lower limit of virus detection is indicated by the dotted line. Each symbol represents an individual mouse and geometric mean titers are indicated by horizontal lines. Data shown were pooled from two independent experiments performed. *$p < 0.05$; two-tailed Mann–Whitney test.

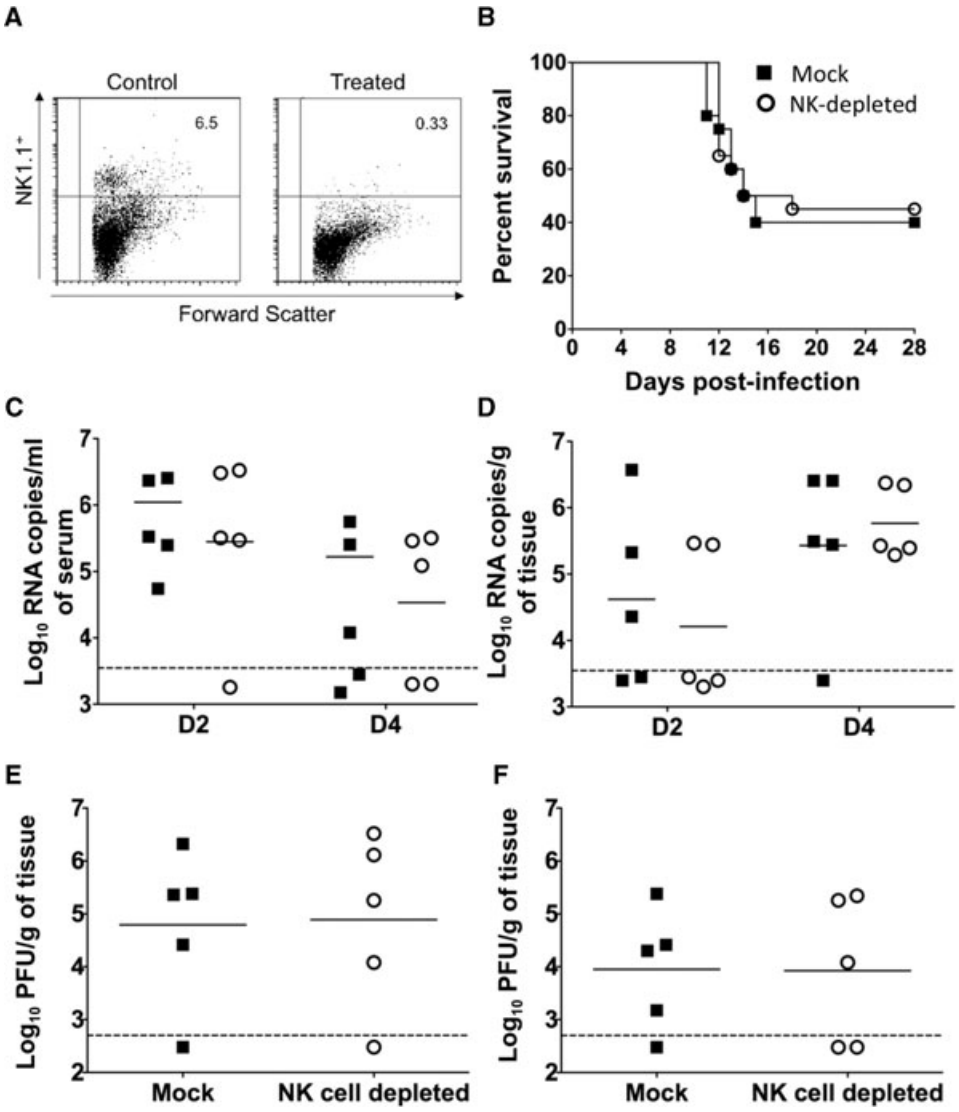

Figure 6. Susceptibility to JEV after in vivo NK-cell depletion. Groups of 12-week-old B6 mice were depleted of NK cells with anti-NK1.1 Ab, or mock-treated, and infected s.c. with 10^3 PFU of JEV. (A) Representative flow cytometry analysis on a serum sample collected at day 7 pi showing 95% depletion of NK cells. (B) Morbidity and mortality in groups of NK-cell-depleted ($n = 20$) and mock-treated mice ($n = 20$). Data shown were pooled from two independent experiments performed; difference between groups was not significant ($p > 0.05$) with log-rank test. (C and D) The JEV burden in (C) serum and (D) spleen samples collected at 2 and 4 days pi measured by real-time RT-PCR is shown. (E and F) The viral burden in (E) brain and (F) spinal cord samples collected at 10 days pi was measured by plaque assay. The lower limit of virus detection is indicated by the dotted line. Each symbol represents an individual mouse and horizontal lines indicate geometric mean titers.

burden in the CNS in single KO mice defective in either Fas, Perf, or Gzm [1]. The latter finding, previously explained by a counteracting balance of protection versus immunopathology mediated by cytolytic leukocytes in the CNS, casts doubt on an interpretation that posits solely absence of cytolytic clearance of JEV as the reason for the observed mortality rate increase in the double-deficient mice, because viral burden in brain increased by a similar level and with a similar kinetics in mice lacking one or both contact-dependent cytolytic pathways. Therefore, noncytolytic functions of Fas and Gzms may contribute to recovery from JE and account for the discrepancy. Accumulating data suggest a physiological role of Fas in neuronal development, growth, differentiation, and regeneration (reviewed in [36]). Intriguingly, Fas is strongly upregulated in brains of mice infected with JEV and WNV [37, 38] such that it is tempting to speculate that disruption of the putative neuroprotective role of Fas may in part explain the increased susceptibility of $Fas^{-/-}$xGzmAxB$^{-/-}$ mice to JEV infection. Noncytolytic activities mediated by Gzms include diverse biological effects such as (i) stimulation of proinflammatory cytokines, (ii) stimulation of type I IFN responses by deregulating the activity of the three-prime repair exonuclease, TREX1, and (iii) proteolytic cleavage and inactivation of viral or host factors essential for viral replication (reviewed in [11, 39]). Regarding the latter, it is of interest that GzmB-deficient but not Perf KO mice showed increased susceptibility to WNV (Sarafend strain) [40], suggesting a beneficial noncytotoxic effect of the molecule against flavivirus infection, which was also observed in growth experiments using fibroblasts ectopically expressing GzmB [41]. The mechanism of noncytotoxic, antiflaviviral, activity of GzmB has not been elucidated, but may involve cleavage by the serine protease of viral or host proteins required for replication.

The second major finding of this study was the substantial — predominantly CNS T-cell-mediated — contribution of IFN-γ to protection against fatal JE. Mechanistically, the protective role of IFN-γ against JE was distinct from that against WNV, given that in

the mouse model of West Nile encephalitis, the cytokine was crucial in suppressing early virus replication in peripheral lymphoid tissues, thereby preventing virus dissemination into the CNS [20], while in the JE model IFN-γ deficiency resulted in significantly increased viral burden in the CNS but not in extraneural tissues. The finding further underscores disparities in immune protection between the closely related flaviviruses [1, 13, 31]. Overall, our results were consistent with a model of noncytolytic virus clearance from the CNS by IFN-γ secreted from infiltrating T cells (reviewed in [42]), and place JEV in a heterogeneous group of neurotropic viruses that are controlled by IFN-γ in the CNS, including Sindbis [43], mouse hepatitis [44], Theiler's murine encephalomyelitis [45], Borna disease [46], dengue [47] and measles viruses [48]. While our finding in mice was consistent with a reported protective role of IFN-γ in human JE [27], it would be of considerable interest if noncytolytic virus clearance mediated by the cytokine also contributed to recovery from human neurotropic virus infection. Mechanistically, IFN-γ-mediated suppression of virus replication in the CNS relies upon Jak/STAT signaling [48], and may involve generation of NO, an effector molecule with activity against JEV [49].

Finally, this investigation resolved the question of whether the cytolytic and IFN-γ-mediated effector functions of NK cells played a role in host resistance against JE. We showed that while infection with JEV resulted in early activation of NK cells, the response did not significantly contribute to host survival. It is unlikely that NK and $CD8^+$ T cells were redundant in recovery from disease, given that depletion of $CD8^+$ T cells resulted in increased viral burden in the CNS [1], while a similar effect was not seen in NK-cell-depleted mice in this study. Our data are consistent with the absence of a protective value of NK cells against WNV [33], and supports a previously proposed model of flavivirus immune escape from NK-cell attack involving upregulation of MHC-I on infected cells (reviewed in [50]). MHC-I can engage with NK cell inhibitory receptors and thereby downregulate the NK-cell response, which may explain why despite apparent NK-cell activation, this immune effector arm did not promote a survival advantage against flaviviral encephalitis.

Materials and methods

Virus

Working stocks of JEV (strain Nakayama) were infected Vero cell culture supernatants (2×10^8 PFU/mL), and were titrated by plaque-assay on Vero cells as previously described [16].

Mice

C57BL/6 (B6) and congenic Fas receptor-deficient ($Fas^{-/-}$; [51]), Perf- and GzmA and B-deficient ($Perf^{-/-}xGzmAxB^{-/-}$; [28]), Fas- and GzmA and B-deficient ($Fas^{-/-}xGzmAxB^{-/-}$; [52]) and IFN-γ-deficient (IFN-$\gamma^{-/-}$; [53]) mice were bred under specific-pathogen-free conditions, and supplied by the Animal Breeding Facility at the John Curtin School of Medical Research, The Australian National University, Canberra. Female 8- to 12-week-old mice were used. Mice were infected s.c. via the footpad as described [1], or by an alternative route as indicated in the figure legends. All animal experiments were approved by and conducted in accordance with the Australian National University Animal Ethics Committee.

Quantitation of viral burden in mouse tissues

Viral burden in serum and spleen was determined by real-time RT-PCR as previously described [1]. Quantitation of viral load in brain and spinal cord was by plaque-assay on Vero cells [16].

Cell surface and intracellular cytokine staining

The surface marker staining utilized the following Ab (all from Becton Dickinson): allophycocyanin-conjugated anti-CD8, PE-conjugated anti-NK1.1, FITC-conjugated anti-CD4: 10^5 events were acquired for each sample on a four-color FACSort flow-cytometer. Results were analyzed using Cell Quest Pro Software. For intracellular cytokine staining, 1×10^6 splenocytes were stimulated with an $H\text{-}2D^b$-binding JEV NS4B protein-derived peptide, SAVWNSTTA [1], in the presence of 1 μL/mL brefeldin A (eBioscience), surface-stained with anti-CD8–allophycocyanin Ab, followed by paraformaldehyde fixation and permeabilization with saponin (Biosource), before intracellular staining with anti-IFN-γ–FITC (BioLegend) and/or anti-TNF-α–PE Ab (Invitrogen) as described [1].

In vivo depletion of $NK1.1^+$ cells

$NK1.1^+$ cells in B6 mice were depleted with rat anti-mouse $NK1.1^+$ mAb (100 μg in 0.5 mL PBS; BioXcell) injected ip one day before, on the day and one day after virus infection, and every 4 days thereafter. Control mice were injected with a corresponding volume of PBS.

Transfer experiments

B6 and IFN-$\gamma^{-/-}$ mice were iv infected with 1×10^3 PFU of JEV, and sacrificed a week later for aseptic removal of spleens. Preparation of single-cell splenocyte suspensions and B-cell depletion by magnetic bead separation (Miltenyi Biotec) was performed as described [1]. Efficiency of B-cell depletion was >99% as assessed by FACS analysis. Splenocytes were resuspended in 100 μL PBS and injected through the lateral tail vein of 8-week-old B6 mice that were challenged a day later with JEV.

Serological tests

For titration of JEV-specific Ab in serum, ELISA was performed and end-point titers calculated as described [1]. Neutralizing Ab titers were measured in a 50% plaque reduction neutralization test as described [54].

Acknowledgments: This work was funded by the Australian National University, Australia.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **Gzm**: granzyme · **JE**: Japanese encephalitis · **JEV**: Japanese encephalitis virus · **MHC-I**: MHC class I · **Perf**: perforin · **pi**: postinfection · **WNV**: West Nile virus

Full correspondence: Dr. Mario Lobigs, Australian Infectious Diseases Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, 4072, QLD, Australia
Fax: +61-7-33654620
e-mail: m.lobigs@uq.edu.au

Current address: Maximilian Larena, Institute of Biomedicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Received: 14/11/2012
Revised: 5/2/2013
Accepted: 3/4/2013
Accepted article online: 9/4/2013

