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Japanese encephalitis virus produces a CD4$^+$ Th2 response and associated immunoprotection in an adoptive-transfer murine model

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Japanese encephalitis is an acute infection of the central nervous system caused by Japanese encephalitis virus (JEV). The importance of an effective humoral response in preventing JEV infection has already been established, although the contribution of cellular immunity remains unclear. This study used an experimental murine model to understand the protective effects of cell-mediated immunity in JEV infection. Fourteen-day-old mice adoptively transferred with JEV-immune splenocytes were found to be protected from peripheral JEV challenge. The survival rate was reduced when transferred cells were depleted of their CD4$^+$ T-cell population. Correspondingly, increased protection was observed when JEV-primed isolated CD4$^+$ T cells were transferred compared with isolated CD8$^+$ T cells. Mice protected from JEV infection by the adoptive transfer of JEV-immune splenocytes had higher levels of immunomodulatory cytokines and decreased expression of pro-inflammatory cytokines. Concurrent with the increase in Th2 cytokines, JEV-specific IgM and IgG1 antibody titres were found to be elevated in protected mice. Taken together, these data indicate a definite role for CD4$^+$ T cells in protection from lethal JEV infection in naïve 14-day-old mice. Induction of a Th2 cytokine response and IgG1 antibody probably achieves an immunomodulatory effect that results in the enhanced survival of these animals.

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INTRODUCTION

Japanese encephalitis virus (JEV; family *Flaviviridae*) is the leading cause of viral encephalitis in Asia (http://www.cdc.gov/ncidod/dvbid/jencephalitis). There are 35 000–50 000 Japanese encephalitis cases reported annually in the Asian continent, with a high fatality rate of 25–30 % (Koh *et al.*, 2006). Prophylactic vaccination appears to be the only way of controlling JEV outbreaks in endemic areas, and live-attenuated as well as inactivated vaccines are currently in use (Marfin & Gubler, 2005). However, recurring outbreaks in countries like India underline the need for a more effective vaccine (Kabilan *et al.*, 2004; Parida *et al.*, 2006).

Studies carried out in humans and in animal models of the disease have indicated the importance of an effective humoral response in preventing flavivirus infection both in the periphery and within the central nervous system (CNS) (Burke *et al.*, 1985; Roehrig *et al.*, 2001; Ben-Nathan *et al.*, 2003; Diamond *et al.*, 2003).

Cellular immunity is an equally important, although less understood, mechanism of protection against flavivirus infections. Kunjin, Murray Valley encephalitis and West Nile virus-specific CD8$^+$ cytotoxic T lymphocytes have been shown to be important for the control of infection in experimental murine models (Parrish *et al.*, 1991; Licon Luna *et al.*, 2002; Wang *et al.*, 2003; Shrestha & Diamond, 2004). JEV-specific CD8$^+$ T cells are recruited during infection with either live virus or recombinant vaccine and are capable of recognizing epitopes on structural or non-structural (NS) viral proteins (Takada *et al.*, 2000; Konishi *et al.*, 2003). The contribution of CD4$^+$ cells to the control of infection has been reported for JEV and other flaviviruses (Mathews *et al.*, 1991; Aihara *et al.*, 1998; Liu & Chambers, 2001; Kumar *et al.*, 2004; Sitati & Diamond, 2006).

The adoptive transfer of CD4$^+$ or CD8$^+$ T cells can confer protective immunity in many viral infections (Offit & Dudzik, 1990; Tishon *et al.*, 2006). An important role for cellular immunity in JEV infection was demonstrated when the adoptive transfer of JEV-immune T cells protected mice from subsequent virus challenge (Mathur *et al.*, 1983; Murali-Krishna *et al.*, 1996). Virus infection in these studies, however, was by the intracerebral (i.c.) route, which may not adequately involve peripheral immune responses in the host.

The objective of this study was to analyse the protective effects of cell-mediated immunity in JEV infection by the adoptive transfer of JEV-immune splenocytes into naïve mice before virus challenge. Fourteen-day-old mice were used as recipients as they are susceptible to peripheral

infection with JEV. Thus, the role played by the peripheral immune response in preventing virus entry and spread into the brain could be studied. In addition, the contribution of CD4$^+$ and CD8$^+$ T-cell subsets to the control of JEV infection was also examined, as this aspect has not previously been clearly elucidated.

METHODS

Virus and mice. An Indian isolate of JEV (733913) was used for all experiments (Ghosh *et al.*, 1989). A 10 % suspension of infected mouse brain in 0.75 % BSA in PBS was prepared. Virus titre in the suspension was determined by inoculation via the intraperitoneal (i.p.) route in 14-day-old mice. The 50 % lethal dose (LD_{50}) was derived using the method of Reed & Muench (1938).

BALB/c mice were obtained from the animal housing facility of the National Institute of Virology, Pune, India, and maintained according to the Committee for Protection, Supervision and Control of Experiments on Animals guidelines. Adult BALB/c mice were immunized with three doses of 100 µl (10^4 p.f.u.) of the live virus by the i.p. route on days 0, 14 and 28. Mice were bled at appropriate time points to estimate the level of JEV neutralizing antibodies. The mean JEV neutralizing antibody titre after three immunization doses was estimated to be approximately 850.

Cell separation. JEV-immune or naïve mice were sacrificed by CO_2 asphyxiation on day 7 after the last immunization. Blood was drained by cardiac puncture and spleens were harvested. A single-cell suspension was prepared and red blood cells (RBCs) were lysed with RBC lysis buffer (eBioscience). Splenocytes were incubated with CD4$^+$ or CD8$^+$ T cell-specific antibodies conjugated to magnetic beads (Miltenyi Biotec). Magnetic cell separations were carried out using either isolation or depletion columns. The purity of the population was confirmed by flow cytometric analysis using phycoerythrin (PE)-conjugated rat anti-mouse CD4$^+$ and CD8$^+$ antibodies (BD Biosciences). The percentage purity of depletion was found to be >98 % (see Fig. 2c), whilst the percentage purity of isolation was found to be >94 % (see Fig. 2d).

Lymphocyte proliferation assay. Splenocytes from naïve mice were plated at a concentration of 2×10^5 cells per well in a 96-well flat-bottomed microtitre plate (Nunc). After incubation for 2 h at 37 °C to allow the cells to adhere, the plates were washed with Dulbecco's modified Eagle's medium to remove non-adherent cells. The remaining cells were then pulsed overnight with live or inactivated JEV antigen. The inactivated antigen used was a sucrose density gradient-purified virus, inactivated using β-propiolactone (BPL). Concanavalin A (1 µg ml^{-1}) served as a positive mitogenic control.

CD4$^+$ or CD8$^+$ T cells (2×10^5) from immunized mice spleens were added to the stimulated antigen-presenting cells. After 3 days, the microtitre plates were pulsed with 1 µCi (37 kBq) [^{3}H]thymidine (BRIT) per well for 18 h. The cells were then harvested onto GF/C (Whatman) filter discs and the cell-incorporated radioactivity was measured by liquid scintillation as counts min^{-1} (c.p.m.) in a β-counter (LKB).

Adoptive transfer. In total, 1×10^7 splenocytes from immunized mice spleen were transferred into naïve 14-day-old BALB/c mice by the intravenous (i.v.) route. Other batches of mice received either 1×10^7 CD4$^+$- or CD8$^+$-depleted splenocytes or 1×10^7 isolated CD4$^+$ or CD8$^+$ T cells. Control mice were transferred with splenocytes from naïve, non-immunized animals. This was followed 24 h later by i.p. infection with 50 LD_{50} live JEV. Mice were monitored for 25 days for survival. Mice that were bled every alternate day for cytokine analysis and antibody-isotyping studies were found to succumb to JEV infection faster than those that were not, and were not included in the survival analysis.

Quantification of virus in the organs of animals. Mice receiving splenocytes from either naïve or immunized animals were sacrificed every alternate day post-infection (p.i.) for comparison of viral titres in various organs by real-time PCR. Mice were euthanized by CO_2 asphyxiation and their blood was drained by cardiac puncture. Fresh blood was immediately processed for RNA extraction. Spleens and brains were excised and weighed and 20 % suspensions were made in 0.75 % BSA in PBS using a tissue homogenizer.

Viral RNA from supernatants of tissue homogenates was isolated by using a QIAamp viral RNA kit (Qiagen) and virus titres were quantified by using a Geno Sen real-time RT-PCR kit for JEV (Genome Diagnostics). Analysis was performed on an ABI 7300 real-time PCR system (Applied Biosystems). Primers were specific for a 130 bp region of the JEV envelope gene. The probe was labelled with the reporter dye FAM at the 5′ end and the quencher dye TAMRA at the 3′ end. Virus copy numbers in the samples were calculated from a standard graph generated by using pre-quantified JEV-specific RNA standards with known copy numbers, provided with the kit.

Antibody isotyping. JEV-specific immunoglobulin subtypes from the sera of mice receiving either naïve or immune splenocytes were measured using a Monoclonal Antibody Isotyping kit (Pierce) following the manufacturer's protocol. Wells were coated with sucrose density gradient-purified JEV. Sera from uninfected mice were used as negative controls. The ratios of the absorbance values (405 nm) of IgG1 and IgG2a were determined for control and experimental mice. IgM, IgG1 and IgG2a titres were determined using twofold serial dilutions of test sera and were expressed as the reciprocal of the highest dilution of sera that gave an absorbance two times higher than that of the negative control.

Cytokine profiling. Sera from mice receiving either naïve or immune splenocytes were analysed for Th1 and Th2 cytokines using a mouse Th1/Th2 Cytometric Bead Array (CBA) kit (BD Biosciences) according to the manufacturer's instructions. Following data acquisition on a FACSCalibur (Becton Dickinson), cytokine concentrations in the samples were determined using BD CBA software using standards provided with the kit. Levels of gamma interferon (IFN-γ), tumour necrosis factor (TNF)-α and interleukins (IL) 2, 4 and 5 were determined for each time point p.i., and cytokine levels from normal mouse sera were used to set the threshold.

In addition, total cellular RNA was isolated from the brains of infected mice by using an RNeasy kit (Qiagen). RNA (1 µg) from infected and control mice was reverse-transcribed at 50 °C for 1 h using a Thermoscript RT-PCR System (Invitrogen) with 0.5 µg random primers. A sample of the cDNA (2 µl) was then used for PCR amplification using gene-specific primers for TNF-α, IFN-γ, IL-10 and the housekeeping gene β-actin using PCR reagents from Invitrogen.

Statistical analysis. Kaplan–Meier survival curves and a log-rank test were carried out for adoptive-transfer studies. An independent samples *t*-test was used for other experiments. All tests were analysed with SPSS version 11 software.

RESULTS

CD4$^+$ T cells show increased proliferation in response to JEV *in vitro*

The capacity of CD4$^+$ and CD8$^+$ T-cell splenocyte subsets from immunized mice to proliferate in response to live and

inactivated JEV antigen was analysed in an *in vitro* lymphocyte-proliferation assay. Splenocytes from JEV-immunized mice were found to proliferate in response to both live and inactivated JEV antigen (8108 ± 534 and 8331 ± 741 c.p.m., respectively; data not shown). JEV-primed $CD4^+$ T cells proliferated to a significantly greater extent in response to an m.o.i. of 10 and 5 of live JEV compared with $CD8^+$ T cells (P=0.001 and P=0.019, respectively; Fig. 1a). In addition, $CD4^+$ T cells showed increased proliferation with 20 and 10 μg inactivated JEV antigen ml^{-1} (10664 ± 212 and 9316 ± 116 c.p.m., respectively; Fig. 1b), compared with $CD8^+$ T cells (8580 ± 548 and 6925 ± 116 c.p.m., respectively). $CD4^+$ and $CD8^+$ T cells from control animals showed comparatively less proliferation in response to both live and inactivated JEV antigen (Fig. 1a, b).

Adoptive transfer of immune $CD4^+$ T cells protect mice from lethal JEV challenge

The adoptive transfer of 1×10^7 JEV-immune splenocytes by the i.v. route into 14-day-old recipient mice resulted in increased resistance to lethal challenge with JEV. Control mice, transferred with splenocytes from non-immunized animals, began to succumb to JEV infection by day 7 p.i., resulting in a survival rate of 5 % and a mean survival time of 11 days, whilst 95 % of mice transferred with JEV-immune splenocytes were protected from lethal challenge with JEV (P=0.000; mean survival time, 24 days). To determine the role of $CD4^+$ and $CD8^+$ T-cell subsets in protection from JEV infection, 1×10^7 JEV-immune spleen cells, depleted of either the $CD4^+$ or $CD8^+$ population, were transferred into naïve recipients (Fig. 2a). Mice receiving $CD4^+$-depleted spleen cells had reduced survival (34.62 %) upon lethal challenge with JEV compared with mice receiving $CD8^+$-depleted spleen cells (64.52 %, P=0.04).

Further studies were carried out using isolated $CD4^+$ and $CD8^+$ cells from JEV-immune splenocytes (Fig. 2b). Mice that were subjected to adoptive transfer with $CD4^+$ isolated T cells had an increased survival percentage over mice injected with isolated $CD8^+$ T cells (53.85 and 28.57 % survival, respectively), although the difference was not statistically significant. Thus, $CD4^+$ T cells appear to play an important role in the protection of JEV-challenged mice.

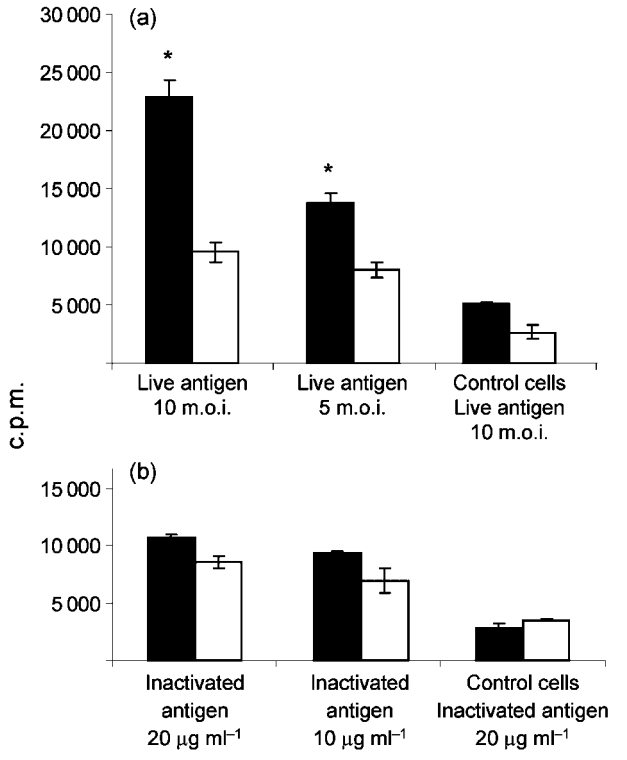

Fig. 1. *In vitro* proliferation assay of $CD4^+$ (filled columns) and $CD8^+$ (empty columns) T cells from immunized mice in response to different doses of live (a) and inactivated (b) JEV antigen. $CD4^+$ and $CD8^+$ T cells from unimmunized mice were used as controls. Significant differences (*) were seen between proliferation of $CD4^+$ and $CD8^+$ cells in response to an m.o.i. of 10 and 5 of live JEV, respectively. Data represent the means ± SEM of three experiments.

Virus titres in organs of mice receiving immune and non-immune splenocytes

Virus titres were determined by real-time PCR to verify which tissues are important in supporting peripheral virus growth and whether the magnitude of virus production in these tissues is a decisive factor in determining the incidence of CNS involvement. JEV was detected on days 2 and 4 p.i. in the blood of mice receiving non-immune splenocytes (Fig. 3a). In the spleen, peak levels of virus replication were observed on days 4 and 6 p.i. (Fig. 3b). Virus levels in the brains of mice receiving non-immune splenocytes increased exponentially, reaching peak levels on days 6 and 8 p.i. (Fig. 3c). Viral loads in mice receiving non-immune splenocytes were thus seen to peak earlier in peripheral organs. Once productive infection had been established in the CNS, virus titres reached extremely high levels, with a corresponding decline in virus replication in peripheral organs.

In mice that were subjected to adoptive transfer with immune spleen cells before infection, virus was detected in only a few animals (Fig. 3a–c). In those mice that had detectable virus titres, the course of infection did not parallel that observed in mice receiving non-immune splenocytes. Mice receiving immune splenocytes had much lower viral titres in the brain than mice receiving non-immune splenocytes.

Increased production of pro-inflammatory cytokines in mice receiving non-immune splenocytes

The levels of Th1 and Th2 cytokines in the sera of mice receiving either immune or non-immune splenocytes were

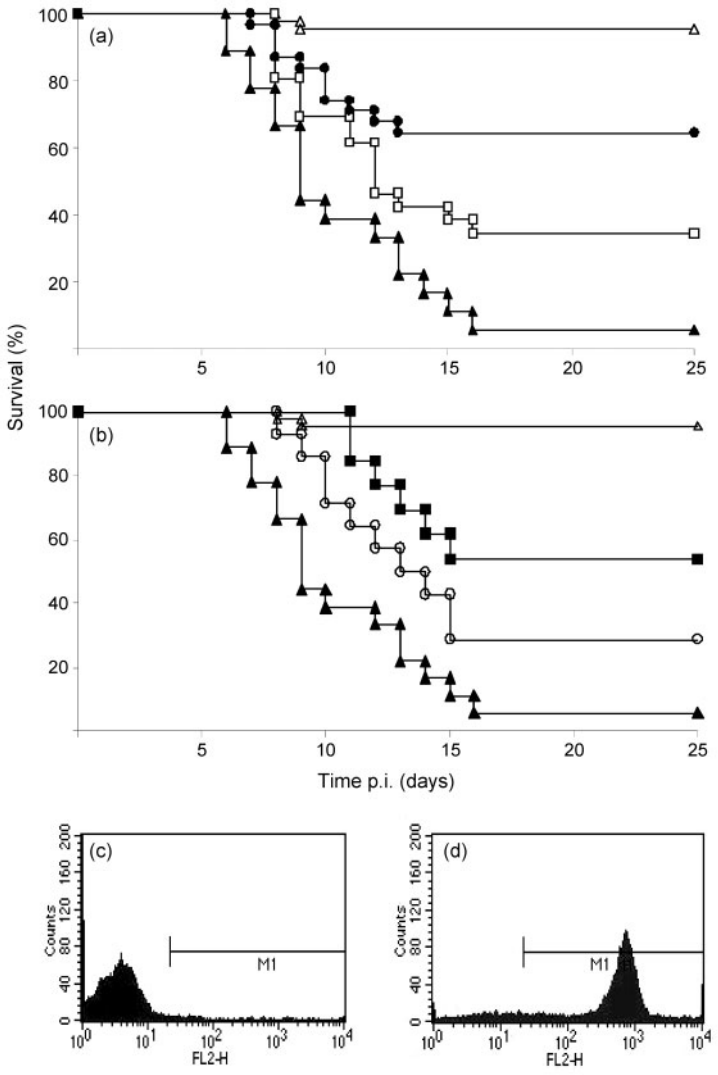

Fig. 2. (a) Survival curves of mice transferred with JEV-immune splenocytes (△, n=47), CD4$^+$ T cell-depleted immune splenocytes (□, n=26), CD8$^+$ T cell-depleted immune splenocytes (●, n=31) and control splenocytes (▲, n=18) following challenge with 50 LD_{50} JEV 733913. Kaplan–Meier survival curves were plotted and a log-rank test was performed to determine significance. The significance of groups receiving CD4$^+$- and CD8$^+$-depleted cells was P=0.017 and P=0.00, respectively, compared with controls. (b) Survival curves of mice transferred with JEV-immune splenocytes (△, n=47), CD4$^+$ T-cell isolated immune cells (■, n=13), CD8$^+$ T-cell isolated immune cells (○, n=14) and control splenocytes (▲, n=18), following challenge with 50 LD_{50} JEV 733913. The significance of groups receiving isolated CD4$^+$ and CD8$^+$ T cells was P=0.0009 and P=0.035, respectively, compared with controls. (c, d) Representative histogram plot of splenocytes depleted of (c), or isolated for (d), a specific subset of T cells (CD4$^+$ or CD8$^+$) following magnetic separation through a depletion column. Cells were stained using PE-conjugated CD4$^+$ or CD8$^+$ anti-mouse antibodies.

determined by CBA. Mice that were susceptible to lethal JEV infection had higher serum levels of the pro-inflammatory cytokines TNF-α and IFN-γ (Fig. 4a, b) compared with mice that were protected from infection by immune cell transfer. Expression of these cytokines seemed to parallel the progression of disease in infected mice, with peak expression levels corresponding to sickness and death of the animal. Cytokine mRNA detection of TNF-α and IFN-γ by RT-PCR showed the same pattern of expression in the brains of infected animals as observed in the sera by CBA analysis (Fig. 5).

Expression of IL-2 and the Th2 cytokines IL-4 and IL-5 was seen to decrease in susceptible mice as infection progressed compared with mice that were protected from infection, which had sustained levels of IL-4 and IL-5 at all time points p.i. (Fig. 4c–e). In the brain, mRNA expression of the anti-inflammatory cytokine IL-10 was observed on day 8 p.i. (Fig. 5).

Increased production of anti-JEV IgG1 antibodies in mice receiving immune splenocytes

Induction of the antibody response following JEV infection in mice receiving either non-immune or immune splenocytes was determined. Mice receiving non-immune cells had increased IgM antibody expression from day 4 p.i., reaching titres of 400 by day 8 p.i. (Fig. 6a). Mice receiving immune splenocytes (Fig. 6b) had elevated IgM antibody levels as early as day 2 p.i., with titres that were higher overall (560) than in mice receiving non-immune splenocytes.

Analysis of IgG subtypes in mice receiving either non-immune or immune splenocytes revealed a significant increase in IgG1 production in the sera of animals that were protected from infection (Fig. 6b). In these mice, IgG1 levels rose significantly by day 6 p.i. (P=0.01), reaching titres of 400 by day 8 p.i. (P=0.007). IgG1 levels in mice receiving non-immune splenocytes were barely

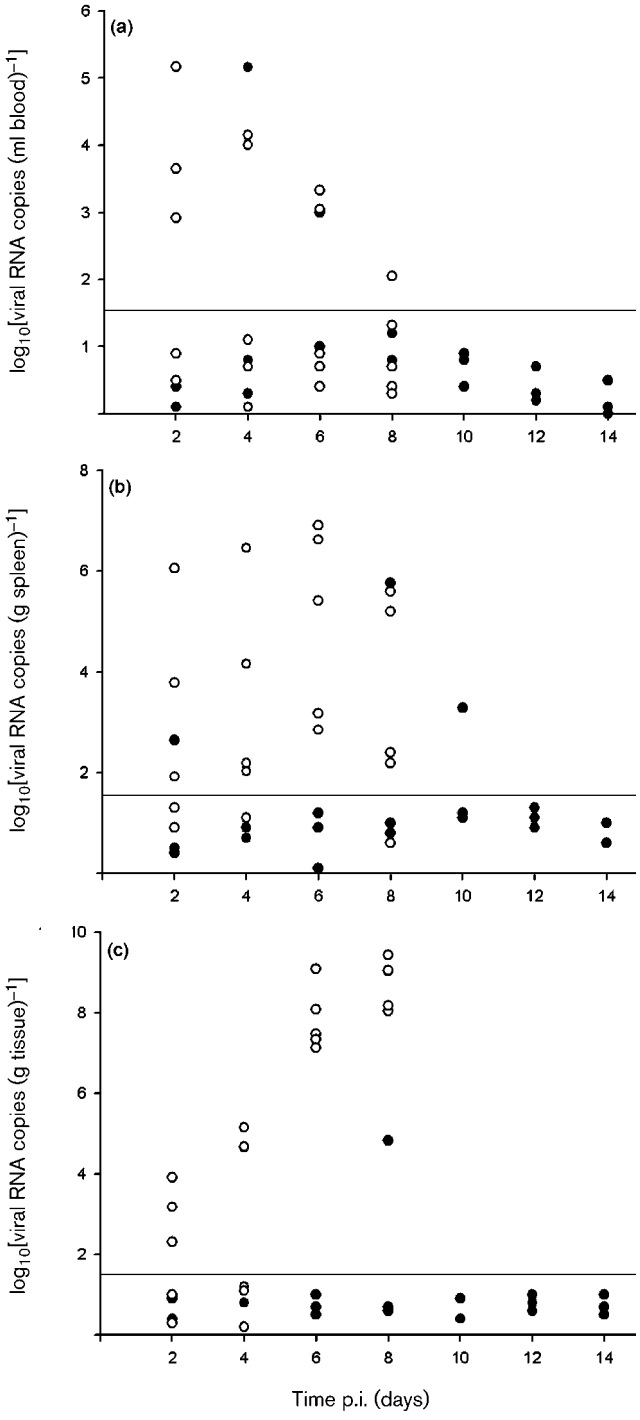

Fig. 3. Scatter plots showing viral RNA copies in the blood (a), spleen (b) and brain (c) of mice receiving either JEV-non-immune splenocytes (○) or immune splenocytes (●), taken at various days p.i. The limit of sensitivity of the JEV real-time PCR assay is represented by the horizontal line.

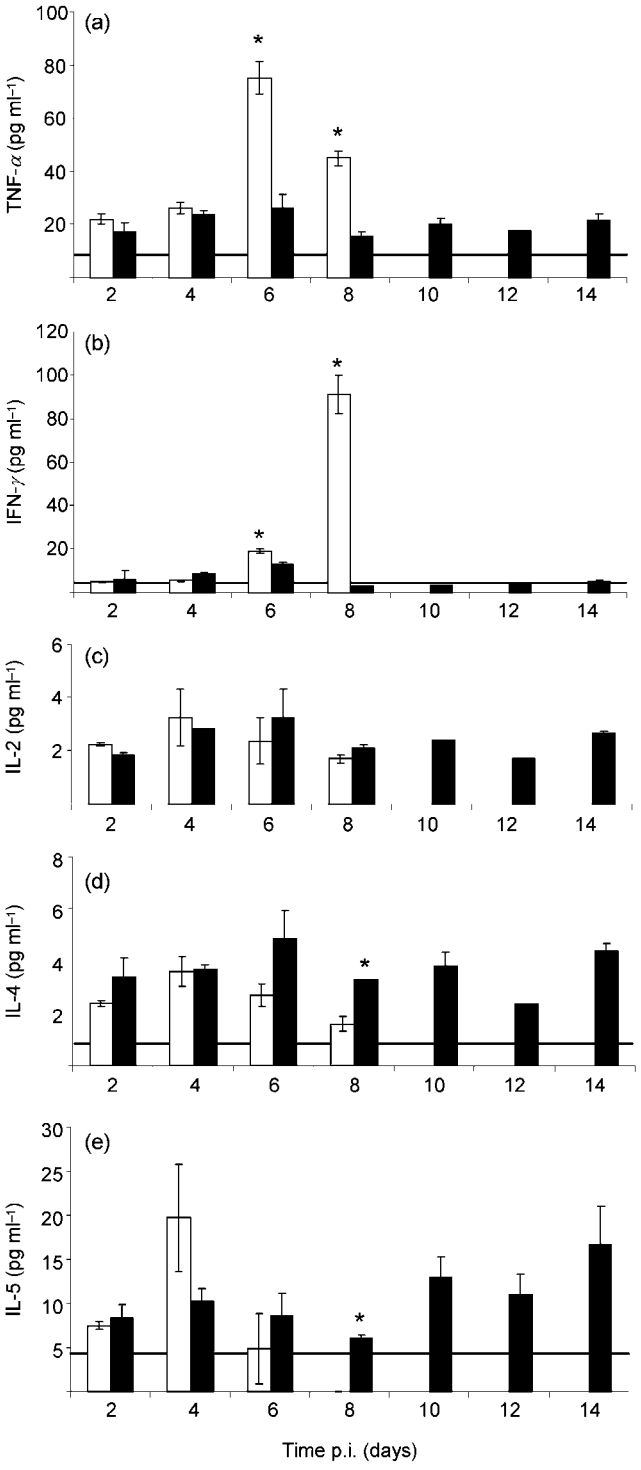

Fig. 4. Induction of the pro- and anti-inflammatory cytokines TNF-α (a), IFN-γ (b), IL-2 (c), IL-4 (d) and IL-5 (e) in the sera of mice receiving either non-immune (empty columns) or immune (filled columns) splenocytes. Cytokine levels from normal mouse sera were used to set the threshold (horizontal lines). The data presented here are means ± SD of three datasets. Each dataset represents sera from a pool of eight mice. Significant differences between groups are denoted by asterisks.

detectable, showing a constant titre of 40 (Fig. 6a). Sera from mice receiving immune splenocytes showed an increased IgG1 : IgG2a ratio by day 6 p.i., indicating a switch towards a Th2 response (Fig. 6c).

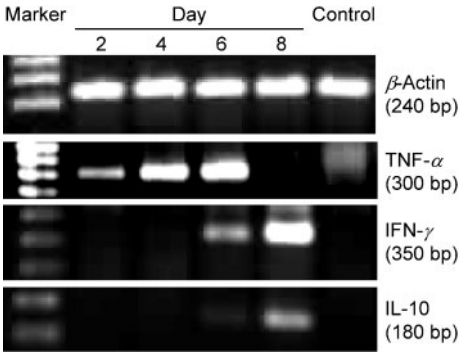

Fig. 5. RT-PCR of cytokines from brains of mice lethally infected with JEV, harvested at various days p.i. Total RNA from brains of infected and control animals was reverse-transcribed and amplified with cytokine-specific primers. Lanes (from left to right): DNA marker; samples from infected mice on the indicated days; control mouse.

DISCUSSION

The effective clearance of virus infection requires an appropriate antiviral response to be generated without causing significant injury and loss to the host. The host response to JEV infection has not been fully characterized. The importance of an early antibody response in mediating recovery from JEV infection has been documented, but in many instances antibody titres did not correlate with protection (Burke *et al.*, 1985). This indicates that a major role might be played by cell-mediated immunity, especially once infection has been established. The T-lymphocyte subsets involved in protective immunity, however, have not been studied in detail.

In this study, an important role for cellular immunity in protection against JEV was demonstrated through the adoptive transfer of splenocytes from JEV-immune mice. The contribution of CD4$^+$ and CD8$^+$ T-cell subsets to protection against lethal virus infection and the T-helper responses (Th1 or Th2) induced in mice that either succumbed to or were protected from JEV infection was compared.

Young mice are more susceptible to peripheral JEV infection than adult mice, which have a well-developed immune response (Grossberg & Scherer, 1966; Smith *et al.*, 2001). The adoptive transfer of JEV-immune splenocytes into naïve 14-day-old recipient mice resulted in the protection of 95% of the recipients from peripheral JEV challenge. Although earlier studies using an adoptive-transfer model in JEV infection have been reported, these were carried out in adult mice using the i.c. route of challenge, which allows the accumulation of high virus titres in the brain with little involvement of extraneural cells and tissues (Mathur *et al.*, 1983; Murali-Krishna *et al.*, 1996). The peripheral route of virus challenge used in our study mimics the natural route of disease progression into the brain and allowed us to analyse each component of the host immune system involved in protection.

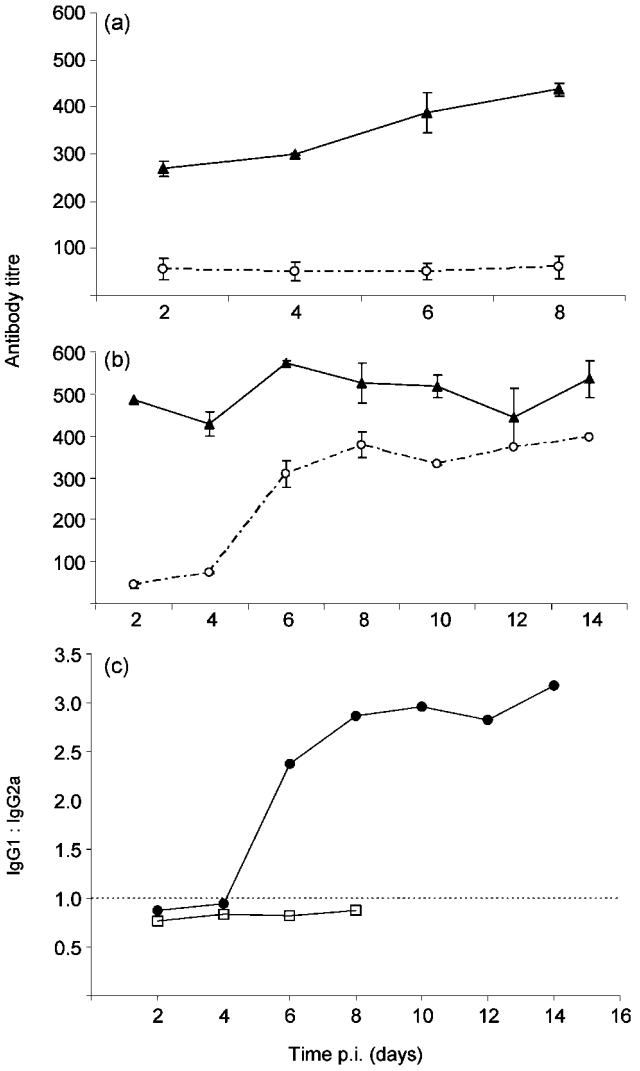

Fig. 6. (a, b) IgM (▲) and IgG1 (○) titres from the pooled sera of mice receiving JEV-non-immune splenocytes (a) and mice receiving JEV-immune splenocytes (b), collected at different days p.i. Titres were obtained by using serial twofold dilutions of sera. Values shown represent means ± SD. (c) IgG1 : IgG2a ratios from pooled sera of mice receiving either JEV-non-immune splenocytes (□) or immune splenocytes (●). For each group, a least-squares regression line was fitted and comparison of slopes, using univariate general linear model, showed a significantly higher trend for mice that received immune splenocytes ($P<0.05$) compared with mice that succumbed to infection. Each dataset represents pooled sera from eight mice, and the ratios represented are a mean of three independent datasets.

In a study using sublethally irradiated mice, splenocytes from live JEV-immunized donors conferred only a low level of protection to JEV challenge, suggesting that JEV infection might stimulate faster kinetics of the host immune response with the help of transferred JEV-specific T cells (Pan *et al.*, 2001). However, adoptive transfer experiments into athymic nude mice carried out by us have

shown a considerable level of protection against lethal JEV challenge, demonstrating that transferred immune T cells are capable of functional activities in the recipient animal (unpublished observations).

Earlier studies have shown both $CD4^+$ and $CD8^+$ T-cell subsets to be involved in protection against JEV. The *in vitro* proliferation of both $CD4^+$ and $CD8^+$ T cells in response to live and inactivated JEV antigen has been observed in Japanese encephalitis patients and vaccinees (Konishi *et al.*, 1995). We found that primed $CD4^+$ T cells from JEV-immunized animals showed faster kinetics of restimulation to JEV *in vitro*. The increased proliferation of $CD4^+$ T cells in response to live virus compared with inactivated antigen might be because of T-cell responses elicited towards the non-structural proteins of live JEV. Kumar *et al.* (2003) identified the NS3 protein of JEV as a dominant $CD4^+$ as well as $CD8^+$ T-cell-eliciting antigen in a healthy JEV-endemic cohort, indicating that a Th1 immune response to the NS3 protein may be a critical determinant of immune control of JEV infection.

Virus clearance from mouse brains acutely infected with JEV has been reported by virus-specific $CD8^+$ T cells adoptively transferred into the CNS; however, $CD4^+$ T cells were also required (Murali-Krishna *et al.*, 1996). Our results indicate a major role for $CD4^+$ T cells in protection from JEV infection. Depletion of $CD4^+$ T cells before adoptive transfer resulted in a reduced level of protection against JEV infection (36 % survival) compared with mice that received $CD8^+$ T cell-depleted immune splenocytes (65 % survival). Correspondingly, increased protection was observed when primed $CD4^+$ isolated T cells were transferred compared with $CD8^+$ isolated T cells.

Generally, $CD4^+$ T cells are believed to control virus infection through several mechanisms. These cells are known to prime B-cell responses following infection by measles virus and lymphocytic choriomeningitis virus and are also involved in class switching of B-cell immunoglobulins to the IgG isotype (Homann *et al.*, 1998; Kennedy *et al.*, 2003; Tishon *et al.*, 2006). An important priming role of $CD4^+$ T cells for memory $CD8^+$ T cells was observed in a yellow fever virus challenge model (Liu & Chambers, 2001). In addition, an independent role of $CD4^+$ T cells in controlling infection, primarily by the production of IFN-γ, has been observed in gammaherpesvirus and mouse hepatitis virus infections (Sparks-Thissen *et al.*, 2005; Stohlman *et al.*, 2008).

A comparison of the cytokine profiles in mice that were either susceptible to or protected from JEV infection revealed a progressive increase in levels of the pro-inflammatory cytokines IFN-γ and TNF-α in mice acutely infected with JEV. In contrast, mice that received JEV-immune splenocytes had depressed levels of IFN-γ and TNF-α in the sera. Increased levels of pro-inflammatory cytokines have been associated with a poor prognosis in many reports of JEV infection (Ravi *et al.*, 1997; Winter *et al.*, 2004; Babu *et al.*, 2006). Although mice deficient in TNF-α, IFN-γ or their receptors were seen to have enhanced mortality following flavivirus infection, other studies suggest that flavivirus encephalitis is immunopathological in nature, with IFN-γ playing a crucial role (King *et al.*, 2003).

A corresponding decline in the production of the Th2 cytokines IL-4 and IL-5 was observed in the sera of mice receiving JEV-non-immune splenocytes. In contrast, mice protected from JEV infection by the adoptive transfer of JEV-immune splenocytes showed sustained expression of IL-4 and IL-5 at all time points p.i. Expression of IL-10 was also detected by RT-PCR in the brains of acutely infected mice late during infection, probably in response to inflammation in the CNS. IL-10 is critical during most major CNS diseases and promotes the survival of neurons and glia (Liu & Chambers, 2001; Strle *et al.*, 2002). Recent differential expression studies carried out by us revealed a considerable increase in the expression of immunomodulatory cytokines such as IL-4, IL-10 and transforming growth factor-β in the brains of mice that were protected from JEV infection, whereas the converse was observed in mice that were lethally infected with JEV (S. M. Biswas *et al.*, unpublished observations), thus lending support to our findings.

Concurrent with the increase in Th2 cytokines, titres of JEV-specific IgM and IgG1 antibodies were seen to be elevated in the sera of mice that were adoptively transferred with JEV-immune splenocytes. Similarly, the IgG1 : IgG2a ratio was increased in these animals, signifying a Th2 antibody response, whilst it remained constant in mice that received JEV-non-immune splenocytes. It has been reported previously that levels of IgM and IgG in the plasma and CSF tend to be higher in survivors of JEV infection (Winter *et al.*, 2004).

In conclusion, protection from lethal JEV infection in naïve 14-day-old mice involved a $CD4^+$ T-cell-mediated, Th2 immune response. Analysis of virus titres in the organs of mice that were susceptible to or protected from lethal infection showed similar kinetics of virus replication at peripheral sites such as blood and spleen, whilst very high levels of virus were observed in the brains of acutely infected mice. In mice receiving JEV-immune splenocytes, faster kinetics of Th2 antibody production resulted in higher levels of JEV-specific antibody in the sera, which probably helped to reduce viral load in the CNS. Reduced levels of pro-inflammatory cytokines such as IFN-γ and TNF-α in the sera of protected mice, combined with an increase in Th2 cytokines such as IL-4 and IL-5, probably achieved an immunomodulatory effect that resulted in the enhanced survival of these animals.

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