JE-ADVAX™ Vaccine Protection against Japanese Encephalitis
Mediated by Memory B Cells in the Absence of CD8⁺ T Cells and Pre-exposure Neutralizing Antibody

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Running Title: Mechanism of vaccine protection against JEV

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JE-ADVAX™ is a new, delta inulin-adjuvanted, Japanese encephalitis (JE) candidate vaccine with strong safety profile and potent immunogenicity that confers efficient immune protection not only against JE virus but also related neurotropic flaviviruses such as West Nile virus. In this study we investigated the immunological mechanism of protection by JE-ADVAX™ vaccine using knockout mice deficient in B cells or CD8+ T cells and poor persistence of neutralizing antibody, or by adoptive transfer of immune splenocyte subpopulations. We showed that memory B cells induced by JE-ADVAX™ provided long-lived protection against JE even in the absence of detectable pre-exposure serum neutralizing antibodies, and without the requirement of CD8+ T cells. Upon virus encounter, these vaccine-induced memory B cells were rapidly triggered to produce neutralizing antibodies that then protected immunized mice from morbidity and mortality. The findings suggest that the extent of the B-cell memory compartment might be a better immunological correlate for clinical efficacy of JE vaccines than the currently recommended measure of serum neutralizing antibody. This may explain the paradox where JE protection is observed in some subjects even in the absence of detectable serum neutralizing antibody. Our investigation also established the suitability of a novel flavivirus challenge model (β2-microglobulin knockout mice) for studies of the role of B-cell memory responses in vaccine protection.
INTRODUCTION

Japanese encephalitis virus (JEV) is a neurotropic flavivirus that can cause severe central nervous system disease in humans and animals (reviewed in (1, 2)). It is a mosquito-borne pathogen that is prevalent in south and south-east Asia, China and the Asia-Pacific region, where it is responsible for approximately 50,000 annual Japanese encephalitis (JE) clinical presentations with 20-30% resulting in death and 30-50% in irreversible neurologic damage among survivors (3, 4). JE is primarily a disease of children since most adults in endemic regions show natural immunity, but it is also a health risk to travellers to endemic regions. Vaccination is the most important control measure against JE, and has been highly successful in countries that have implemented national immunization programs since availability of the first JE vaccine in the late 1960s. Nevertheless, vaccination has failed to halt the spread of JEV in Asia and the Asia-Pacific region (5), and transmission of JEV is likely to continue to increase in low-income countries (4).

The first licensed JE vaccine was a mouse brain-derived formalin-inactivated antigen (JE-VAX™) supplied from Japan for decades for internal and international use (reviewed in (6)). In recent years, JE-VAX™ has been superseded by second-generation formalin-inactivated vaccines produced from cell culture-grown JEV, or by live attenuated vaccines (reviewed in (7)). However, JE-VAX™ remains the gold-standard for immunogenicity and safety comparisons of new generation vaccines against JE (8). Using JE-VAX™ as a comparator, we showed that JE-ADVAX™, a Vero cell culture-grown inactivated JEV antigen (ccJE) (9) combined with Advax™, a novel polysaccharide adjuvant derived from delta inulin (10), provided greatly superior immunogenicity to JE-VAX™ in mice and horses (11). In the same study,
we also found that JE-ADVAX™ elicited levels of neutralizing antibody against serologically related flaviviruses of medical significance (West Nile and Murray Valley encephalitis viruses) that were indicative of cross-protective immunity, because they exceeded the titers against the homologous virus (JEV) generated by immunization with the gold-standard JE-VAX™ (11). The possible feasibility of cross-protective vaccination against multiple flaviviruses belonging to the JE serocomplex using a single antigen had previously only been proposed for live attenuated JE vaccines (12, 13) (reviewed in (14)).

In view of the excellent immunogenic properties of JE-ADVAX™, it was of interest to delineate the immunological correlates underlying vaccine protection (reviewed in (15)). In studies with knockout mice lacking B cells or CD8⁺ T cells or with poor persistence of neutralizing antibody, or by passive transfer of immune effector cells from immunized donor to naïve recipient mice, we show that JE-ADVAX™ mediates durable, protective immunity by induction of a long-lived memory B-cell population that affords protection against JEV without the need for CD8⁺ T cells or pre-exposure neutralizing antibody.
MATERIALS AND METHODS

Viruses and cells. Vero cells (African green monkey kidney) were obtained from the American Type Culture Collection, and were grown at 37°C in a 5% CO₂ atmosphere in Eagle’s minimal essential medium plus nonessential amino acids (MEM; Invitrogen) supplemented with 5% fetal bovine serum (FBS). Working stocks of JEV (strain Nakayama) were prepared as infected Vero cell culture supernatants (2x10⁸ PFU/ml) and stored in single-use aliquots at -80°C. Virus titration was by plaque assay on Vero cell monolayers, as previously described (16).

Mice. C57Bl/6 (B6), congenic B cell-deficient (µMT-/-) (17), and β₂-microglobulin-deficient (β₂m-/-) (18) 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 (ANU), Canberra. Female mice were used in all experiments. All animal experiments were approved by and conducted in accordance with the ANU Animal Ethics Committee.

Vaccines and adjuvant. Vaccines were formulated and administered to mice as previously described (11). JE-ADVAX™ was a formulation of Vero cell culture-grown, inactivated JE vaccine (ccJE; Beijing-1 strain) (9) together with Advax™ adjuvant (Vaxine Pty Ltd, Adelaide, Australia) comprising delta inulin by itself or in a formulation further containing a CpG oligonucleotide (Advax™-2). ChimeriVax-JE (19) was supplied by Acambis Inc (Cambridge, MA, USA), amplified for one passage on Vero cells, and titrated by Vero cell plaque assay. Vaccines were diluted in PBS to the required dose and injected subcutaneously (s.c.) in a volume of 0.1 ml.

Real-time RT-PCR. For determination of viral burden in mouse serum and spleen samples, total RNA in 50 μl splenic homogenates (10% [wt/vol]) and 50 μl
serum was extracted using Trizol (Invitrogen) as described previously (20), and virion RNA content, expressed in genome equivalents, was determined by quantitative RT-PCR. JEV RNA extracted from a Vero cell-grown virus stock and quantitated by spectrophotometry was used for a genome copy standard. RT was performed at 43°C for 90 min in a 10 μl mixture containing 2 μl sample RNA, Expand reverse transcriptase (Roche), RNase inhibitor (Invitrogen), 10 mM deoxynucleoside triphosphate, 10 pmol downstream primer (5'-TTGACCGTTTACTGCAAGGC-3'), 10 mM dithiothreitol, and the manufacturer’s recommended buffer conditions. Real-time PCR was performed using IQSybr qPCR mixture (Bio-Rad) and 0.2 nM downstream and upstream primers (5'-GCTGGATTCAACGAAAGCCACA-3') under cycling conditions of 95°C for 3 min for 1 cycle and 95°C for 30 sec, 63°C for 30 sec and 72°C for 60 sec for 40 cycles. Each sample was tested in duplicate, and genome copy numbers were determined by extrapolation from a standard curve generated within each experiment. The detection limit of the assay was 4x10^3 RNA copies/ml.

Adoptive transfer experiments. Donor B6 or β2m-/- mice were immunized with a defined dose of vaccine, and were sacrificed at an indicated time point for aseptic removal of spleens. Single-cell splenocyte suspensions were prepared by pressing the spleen tissue gently through a fine metal-mesh tissue sieve. Erythrocyte lysis was by suspension of the splenocyte pellet in 4.5 ml distilled water followed immediately by the addition of 0.5 ml of 10x PBS. Lysed cells were discarded after centrifugation at 400 g for 5 min. B and CD4+ T cell purification was performed as previously described (21). For B cell enrichment, isolated splenocytes were incubated with 1:3 dilutions of anti-CD4 (RL172) plus anti-CD8 (31M) supernatants in MEM plus 5% FBS for 30 min at 4°C, followed by incubation with rabbit serum.
complement (Cedarlane Laboratories) for 30 min at 37°C. Cells were washed twice
with PBS before transfer into recipient mice (5x10^6 cells/mouse). The efficiency of
depletion of CD^4^+ and CD^8^+ cells was >95% as assessed by flow-cytometry. For
CD^4^+ T cell enrichment, B cells were depleted by magnetic bead separation (Miltenyi
Biotec), as previously described (21). Efficiency of B cell depletion was >99% as
assessed by FACS analysis. B cell-depleted splenocytes were next incubated with a
1:3 dilution of anti-CD8 (31M) supernatant in MEM plus 5% FBS for 30 min at 4°C,
followed by incubation with rabbit serum complement (Cedarlane Laboratories) for
30 min at 37°C. Cells were washed twice with PBS before transfer into recipient mice.
The efficiency of depletion of CD^8^+ cells was 95% as assessed by FACS. Splenocytes
(5x10^6 cells) were resuspended in 100 μl PBS and injected through the lateral tail vein
of 8-week-old B6 recipient mice. Recipient mice were challenged a day later with
1x10^3 PFU JEV via footpad injection.

**Serological tests.** For titration of JEV specific antibody isotypes in mouse
serum, ELISAs were performed with HRP-conjugated rabbit anti-mouse Ig and the
peroxidase substrate, 2,29-azino-di(3-ethyl-benzthiasoline sulfonate). The JEV
Nakayama strain was used for ELISA antigen production as described (22). For
determination of ELISA end-point titers, absorbance cut-off values were established
as the mean absorbance of eight negative-control wells containing sera of naive mice
plus 3 SD. Absorbance values of test sera were considered positive if they were equal
to or greater than the absorbance cut-off, and end-point titers (log_{10}) calculated as the
reciprocal of the last dilution giving a positive absorbance value. Neutralization titers,
measured in a 50% plaque reduction neutralization test (PRNT_{50}), were determined as
previously described (11).
**Statistical analyses.** Mortality data were plotted into Kaplan-Meier curves and assessed for significance by the log-rank test. The Student’s t-test was applied to assess differences between data gathered from two experimental groups. A $P$ value of $\leq 0.05$ was considered significant.
RESULTS

Essential requirement of B cells but not CD8\(^+\) T cells for vaccine-mediated protection against JE. To better define the immune parameters of protection against JE afforded by JE-ADVAX\(^\text{TM}\) vaccination, we first addressed the absolute requirement of B cells and CD8\(^+\) T cells for resistance of immunized mice to lethal JEV challenge. Mice genetically deficient in mature B cells and antibody (µMT-/- mice) or CD8\(^+\) T cells (β2m-/- mice) were immunized with the JE-ADVAX\(^\text{TM}\) candidate vaccine previously shown to be superior to a traditional licensed reference vaccine (JE-VAX\(^\text{®}\)), or in the case of µMT-/- mice also with a live recombinant vaccine (ChimeriVax-JE). ChimeriVax-JE contains the non-structural genes of yellow fever vaccine and the premembrane and envelope genes of JEV, and induces both humoral and cell-mediated immunity. Mice were challenged four weeks later with a low-dose JEV inoculum (10\(^3\) PFU) deposited s.c. in the footpad (Table 1).

Vaccination of µMT-/- mice with either vaccine conferred no survival advantage or extension of mean survival time (MST) relative to a naïve control group. By contrast, immunization of β2m-/- mice with JE-ADVAX\(^\text{TM}\) efficiently protected against clinical disease. While naïve β2m-/- mice showed high susceptibility to JEV infection (mortality: 13/15, 87%), virtually all β2m-/- mice vaccinated with ccJE antigen adjuvanted with either of two Advax\(^\text{™}\) formulations survived the challenge, even when only very low doses of antigen (0.05 µg) were used in a two-dose schedule. In the absence of adjuvant, ccJE also provided some protection of β2m-/- mice against JEV challenge, although the protective value of low doses of ccJE was significantly enhanced by formulation with the adjuvant.

The result confirms that humoral immunity is required for vaccine-mediated protection of mice against lethal JEV challenge and that this protection is maintained...
in the absence of CD8\(^{+}\) T cells. It also confirms a >10-fold dose-sparing effect of Advax\(^{TM}\) adjuvant for ccJE antigen, as was shown previously (11, 23).

Immunized β2m-/- mice are protected against JEV in the absence of detectable pre-challenge neutralizing antibody. The widely accepted correlate of clinical efficacy for JE vaccines is a neutralizing antibody titer of \(\geq 10\) determined, in vitro, in the PRNT\(_{50}\) assay (24-26). However, β2m-/- mice in addition to their lack of CD8\(^{+}\) T cells are unable to produce long-lived IgG antibodies after viral or model antigen exposure (18, 27-29). The latter is the result of a deficiency of the neonatal Fc receptor (FcRn) in these mice. FcRn has a major histocompatibility complex (MHC) class I-like molecular structure (30) and in association with β2m protects plasma IgG from catabolism (reviewed in (31, 32). Accordingly, we predicted that the anti-JEV IgG and PRNT\(_{50}\) responses in vaccinated β2m-/- mice would rapidly wane.

To address this question, β2m-/- and wt B6 control mice were immunized twice, at a two-week interval, with low doses of ccJE (0.05µg) in the presence or absence of Advax\(^{TM}\) adjuvant, and anti-JEV IgG1 and IgG2b titers determined over a period of 28 days post-vaccination (Table 2). Wild-type mice immunized with ccJE plus Advax\(^{TM}\) had detectable anti-JEV IgG1 and IgG2b titers on day 8 post-vaccination, that increased by ~100-fold by days 18 and 28 post-immunization. Non-adjuvanted ccJE gave barely detectable IgG responses in wt B6 mice. IgG1 and IgG2b titers were at or below the detection threshold of the ELISA assay in immunized β2m-/- mice over the course of the experiment, regardless of whether the vaccine was adjuvanted or not (Table 2).

The abnormal IgG response in JE vaccine recipient β2m-/- mice was reflected in an absence of detectable neutralizing antibody at 4 weeks post-immunization.
(Table 3). This was in striking contrast to the anti-JEV PRNT\textsubscript{50} titers in wt B6 mice immunized with JE-ADVAX\textsuperscript{TM}. As reported previously (11), the low-dose ccJE vaccine regimen elicited a very poor neutralizing antibody response even in wt B6 mice when delivered without the adjuvant.

The observation of protection against JEV challenge despite the absence of detectable neutralizing antibody in JE-ADVAX\textsuperscript{TM} immunized β\textsuperscript{2m-/-} mice (Table 1) was contrary to the notion that pre-challenge neutralizing antibody titers are a critical correlate of protection against JE. We therefore hypothesized that despite the rapid catabolism of IgG antibodies in β\textsuperscript{2m-/-} mice leading to the appearance of a minimal almost non-existent vaccine response, JE-ADVAX\textsuperscript{TM} immunization generated a memory B-cell population that when stimulated by the actual live JEV challenge was able to produce neutralizing anti-JEV antibody with sufficient speed and quantity to neutralize the virus and attenuate clinical disease. Post-challenge PRNT\textsubscript{50} titers were therefore compared in immunized and saline-treated β\textsuperscript{2m-/-} mice: at 5 days post-challenge with JEV, all β\textsuperscript{2m-/-} mice vaccinated with JE-ADVAX\textsuperscript{TM} had PRNT\textsubscript{50} titers \(\geq 10\) that by day 10 post-challenge rose to a level comparable to those in vaccinated wt B6 mice. By contrast, saline-treated mice showed markedly lower post-challenge neutralizing antibody titers than mice immunized with JE-ADVAX\textsuperscript{TM} (Table 3). The post-challenge PRNT\textsubscript{50} titers in wt and β\textsuperscript{2m-/-} mice vaccinated with ccJE without adjuvant were intermediate relative to those in saline-treated and JE-ADVAX\textsuperscript{TM} immunized mice.

Together, the data suggest that JE-ADVAX\textsuperscript{TM}-mediated protective immunity against JEV in β\textsuperscript{2m-/-} mice may correlate better with the presence of memory B cells rather than neutralizing antibody present at the time of challenge.
Critical role of B cells and subsidiary contribution of CD4⁺ T cells in vaccine-mediated protection of β2m⁻/⁻ mice against JE. While a role of CD8⁺ T cells in protection of JE-ADVAX™-immunized β2m⁻/⁻ mice could be excluded, it was unclear whether B cells with or without CD4⁺ T cells were required for vaccine protection against JE. Therefore, adoptive transfer of purified B or CD4⁺ T cells from JE-ADVAX™-immunized β2m⁻/⁻ donor mice into naïve β2m⁻/⁻ recipients was performed, and recipient mice challenged with JEV (Fig. 1). Another group of β2m⁻/⁻ mice that received naive splenocytes served as a negative control. Immune CD4⁺ T-cell recipient mice had a reduced mortality rate relative to the control group (89% vs. 67%, respectively), although this did not reach statistical significance with the group sizes used. The mean survival time of mice that succumbed to the JEV challenge in the CD4⁺ T-cell recipient group was also longer than for the control group (13.3±2.0 vs. 11.9±1.4 days, respectively), consistent with some protective value of immune CD4⁺ T cells, though this also did not reach statistical significance. On the other hand, immune B-cell transfer significantly reduced mortality relative to the control group (mortality: 10% vs. 89%, respectively). The almost complete protection against JE provided by immune B cells correlated with a rapid appearance of anti-JEV plasma antibodies after JEV challenge (Table 4).

JE-ADVAX™ induces long-lived memory B cells that mediate durable protection against JE. Next, we examined the durability of protection against JE provided by immune B cells induced by JE-ADVAX™ immunization. First, β2m⁻/⁻ mice were challenged with JEV 6 months after completion of a prime-boost immunization schedule with low doses (0.05 µg) of JE-ADVAX™: all immunized mice survived the challenge, while the mortality rate in a saline-treated control group...
was 60% (Fig. 2). Second, immune B cells were isolated from JE-ADVAX™ immunized wt B6 donor mice at 3 and 6 months post-immunization and adoptively transferred to 8-week-old B6 recipients that were then challenged with JEV. Mice that received immune B cells harvested from donors at 3 months post-immunization had significantly improved survival relative to control mice that received naïve B cells (mortality: 20% vs. 80%, respectively; Fig 3A). Even immune B cells isolated from mice at 6 months post-immunization with JE-ADVAX™ conferred significant protection of recipient mice when compared to controls (mortality: 40% vs. 90%, respectively; Fig. 3B). Measurement of anti-JEV PRNT50 titers in serum of mice on day 5 post-challenge showed that these were 8-fold (98, range: 10-320) and 4-fold (84, range: 20-160) higher in recipients of 3 and 6 months post JE-ADVAX™ immunization B cells, respectively, than those in the naïve B-cell recipient control group (16, range: <10-40).

β2m-/- mice are more susceptible to primary infection with JEV than congenic wt B6 mice. The results from this investigation suggested that β2m-/- mice are suitable for use as a challenge model for JE vaccine efficacy testing because (i) protection from challenge in this model is a measure of the immunization-induced antiviral memory B-cell response, the most critical parameter in vaccination against JEV, and (ii) adult (12-week-old) β2m-/- knockout mice can be lethally challenged by low-dose s.c. infection with JEV (≥90% mortality rate), which contrasts to an age-dependent resistance to JEV infection found in wt strains (reviewed in (33)). To better characterize this β2m-/- JEV mouse model, the susceptibility and immune responses to primary JEV infection in β2m-/- mice was compared to that in wt B6 mice. Using a JEV infection model of s.c. deposition of a low dose inoculum (10^3 PFU) into the
footpad that results in ~60% mortality in 8- to 12-week-old wt B6 mice (21), we found that β2m-/- mice were significant more susceptible to JEV infection (mortality rate ~95%; Fig. 4A, B).

To test whether this difference in resistance against JEV could be attributed to defective control of virus growth in peripheral and CNS tissues in β2m-/- mice, groups of wt B6 and β2m-/- mice were inoculated with JEV, and tissues and serum collected at 48 h time intervals. Viral load in spleen and serum was determined by real-time RT-PCR, and that in brain and spinal cord by plaque assay. While viral titers in serum and spleen were marginally higher in β2m-/- than wt mice, the difference was not significant, and the kinetics of virus growth was similar in both strains (Fig. 4C, D). However, viral load in brain and dissemination into the spinal cord were significantly increased in β2m-/- relative to wt mice (Fig. 4E, F).

As earlier described, β2m-/- mice display a short-lived IgG isotype antibody response. To test whether this humoral immune defect could account for the increased susceptibility of the knockout mice to JEV, the magnitude and kinetics of IgM, IgG and neutralizing antibody responses against a primary JEV infection in B6 wt and β2m-/- mice were measured (Fig. 4G, H, I). No difference in anti-JEV IgM and neutralizing antibody responses was found; however, the IgG1 and IgG2b titers were significantly lower in β2m-/- than wt mice at day 10 pi.
DISCUSSION

The important role of neutralizing antibody in vaccine protection against JE is widely accepted, because in previously described animal models protection correlated with the presence of neutralizing antibody (26, 34), and passive transfer of neutralizing antibody conferred resistance to challenge with JEV (35, 36). Moreover, immunization approaches in mice that preferentially elicited neutralizing antibody against E protein vs. CD8+ T cell immunity against other regions of the viral polyprotein showed that the former afforded complete protection against lethal challenge, while virus-specific CD8+ memory T cells were at best only partially protective (37, 38). Therefore, neutralizing antibody in serum, measured by the PRNT50 assay, is the accepted surrogate immunological read-out of clinical efficacy of JEV vaccines (24, 25). However, applying this surrogate can result in a disparity in apparent vaccine efficacy and clinical protection, reflected in resistance to JEV in vaccine recipients with low or undetectable PRNT50 titers (39). It was previously hypothesized that this might be due to viral exposure resulting in a rapidly induced anamnestic antibody response in immunized individuals devoid of pre-exposure neutralizing antibodies (40), although no experimental evidence was advanced in support of this proposition.

In this study two approaches were used to confirm that memory B cells elicited with an adjuvanted inactivated JEV candidate vaccine are the key factor, in the absence of neutralizing antibody and CD8+ T cells, underlying vaccine-mediated protection against lethal JEV challenge. First, complete vaccine protection against JEV was achieved in β2m-/- mice, despite absence of CD8+ T cells and undetectable pre-exposure neutralizing antibody. Moreover, immunization of β2m-/- mice
produced a durable B-cell memory compartment that was rapidly triggered to secrete neutralizing antibody upon virus encounter. Second, adoptive immune B-cell transfer confirmed that this immune cell population was able to confer resistance to JEV when B cells from either JE-ADVAX™-immunized wt or β2m-/- mice were transferred to naïve recipient mice, without a requirement for help from primed antiviral CD4⁺ or CD8⁺ T cells. These findings were complemented with the demonstration of an absolute requirement of B cells, but not CD8⁺ T cells, in vaccine protection against JE, since immunization with live or inactivated vaccines of mice lacking mature B cells (μMT-/- mice) did not provide protection. Overall, the results of this study underscore the cardinal contribution of memory B cells to vaccine protection against JE. In effect, high serum neutralising antibody titers correlate with JEV protection because they are a surrogate for the presence of a memory B-cell population, whereas negative serum titers are not predictive of susceptibility because they do not exclude the presence of an underlying memory B-cell population. Nevertheless, it is highly probable that memory T-cell responses further contribute to the strength of vaccine-mediated protection. Notably, the pleiotropic role of CD4⁺ T cells in orchestrating B and T cell immunity against JEV, and supporting antiviral CD8⁺ T-cell effector functions, requires further investigation (reviewed in (41)).

Persistent antibody responses that may in some instances last a lifetime have previously been thought to be the exclusive hallmark of live virus vaccines, and are thought to depend on activation of dendritic cells via stimulation of multiple Toll-like receptors (42). A surprising finding in this study was that robust JEV protection persisting for at least six months post-immunization could similarly be conferred by JE-ADVAX™, which is an adjuvanted inactivated vaccine. The extent to which this protection might be a unique property of the particular adjuvant used in this...
investigation awaits further comparative studies, but is consistent with other studies showing that the addition of Advax™ adjuvant significantly enhanced the efficiency and duration of vaccine-mediated protection against seasonal or pandemic influenza with enhancement of anti-influenza neutralizing antibody, B-cell, and CD4+ and CD8+ T-cell memory responses (23).

Antibody responses develop along two distinct pathways, the extra-follicular pathway that rapidly generates short-lived antibody-secreting cells, and the germinal center pathway that leads to production of memory B cells and long-lived plasma cells that sequester to the bone marrow and other secondary lymphoid tissues where they secrete high-affinity antibodies (reviewed in (43)). Memory B cells produce antibody only upon re-stimulation with specific antigen, and their ability to confer protection from disease following re-infection in the absence of pre-existing serum antibody titers has been questioned (44). Our study showed that JE-ADVAX™ efficiently stimulated the germinal center pathway of memory B-cell formation in wt B6 mice, but the vaccine’s ability, in particular, to induce robust protection in β2m−/− mice suggested that memory B cells alone are sufficient for vaccine protection against a neurotropic viral infection. However, this conclusion comes with the caveat that in our s.c. challenge model the window for development of a protective immune response was a number of days before JEV spread from the periphery into the CNS, and this may have been a critical time to allow memory B cells to produce antibody that, in turn, prevented virus entry into the brain. In other viral diseases with a shorter incubation window, memory B cells may have less opportunity to serve a protective role. That said, our JEV s.c infection model arguably reflects the normal JEV mosquito-bite infection route and hence suggests that in natural infection an adequate duration may similarly exist for memory B-cell mediated protection.
Our study also investigated the pathogenesis of JEV in β2m-deficient mice, and underscored their suitability for vaccine studies that focus on memory B-cell immune responses. In comparison to wt B6 mice, primary JEV infection in β2m-/− mice resulted in higher mortality and virus burden in the CNS, although no significant difference in the kinetics and magnitude of extraneural virus growth and in the neutralizing antibody response was observed. The latter is most likely a reflection of the dominant role of IgM in antibody-mediated control of primary flavivirus infection (45), which is unaffected by β2m deficiency. Moreover, we previously established that CD8+ T cells are mostly dispensable for recovery in the mouse model of JE (21). Accordingly, the two prime antiviral immune pathways that are defective in β2m-/− mice, the humoral and CD8+ T cell responses, cannot fully account for the poorer clinical outcome of β2m-deficient mice relative to wt in our model of JE, and also that of others using a different JEV strain and younger mice (46). An additional immune deficiency in β2m-/− mice that has been associated with their enhanced susceptibility to viral infection is a suboptimal IFN-γ response (47). Given our recent finding that IFN-γ contributes to disease resolution by helping to clear JEV infection from the CNS (M Larena, M Regner and M Lobigs, manuscript submitted), a deficiency in IFN-γ production may contribute to the more severe disease in primary infections of β2m-/− mice with JEV. While the precise mechanism for the increased mortality rate of β2m-/− mice following JEV challenge is not fully understood, it is an important parameter of the knockout strain that defines its suitability as a stringent challenge model for pre-clinical evaluation of vaccines against JE. The positive outcomes obtained with JE-ADVAX™ in this stringent challenge model attest to the effectiveness of this particular vaccine that is currently advancing into human clinical trials.
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Financial and competing interests disclosure

NP is affiliated with Vaxine Pty Ltd, a company with commercial interests in Advax™ and JE-ADVAX™.
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Figure 1 Immune B cells mediate protection of JE-ADVAX™ vaccine against JEV challenge. Eight-week-old β2m⁻/⁻ donor mice were immunized with two doses of 0.5 μg of ccJE + 1 mg Advax™, delivered 2 weeks apart, B cells and CD4⁺ T cells purified at 1 month after completion of the vaccination schedule, and 5x10⁶ cells adoptively transferred into 8-week-old β2m⁻/⁻ recipient mice. A control group of β2m⁻/⁻ mice received naive total splenocytes. One day post-transfer, mice were challenged s.c. with 10³ PFU of JEV. Mice were monitored twice daily for morbidity and mortality over a 21-day observation period. Asterisks denote statistical significance (***, P < 0.001).

Figure 2 Low-dose JE-ADVAX™ vaccine exerts long-term protection against JEV challenge. Six-week-old β2m⁻/⁻ mice were immunized with two doses of ccJE (0.05 μg/dose) + Advax™ (1 mg/dose) given two weeks apart, and a negative control group was injected with PBS. Six months after the last dose, mice were challenged s.c. with 10³ PFU of JEV. Mice were monitored twice daily for morbidity and mortality over a 28-day-observation period. Asterisks denote statistical significance (*, P < 0.05).

Figure 3 JE-ADVAX™ vaccine induces a durable, protective B-cell response in B6 mice. Eight-week-old B6 wt donor mice were immunized with two doses of ccJE (0.5 μg/dose) + Advax™ adjuvant, delivered two weeks apart. B cells were purified from these mice at three (A) or six months (B) post-immunization, and adoptively transferred into 8-week-old naïve B6 recipients. A control group of B6...
mice received naive B cells purified from unvaccinated mice. One day after transfer, mice were challenged s.c. with $10^3$ PFU of JEV. Mice were monitored twice daily for morbidity and mortality over a 28-day observation period. Asterisks denote statistical significance (*, $P < 0.05$).

**Figure 4** Pathogenesis and humoral immunity in primary JEV infections of $\beta_2m^{-/-}$ mice. Survival data of (A) 8- and (B) 12-week-old $\beta_2m^{-/-}$ and wt B6 control mice infected s.c. with $10^3$ PFU of JEV. Morbidity and mortality were recorded daily, and surviving mice were monitored for 28 days. The data shown were constructed from three independent experiments. Viral burden in serum (C), spleen (D), brain (E) and spinal cord (F) following s.c. infection of 8-week-old mice with $10^3$ PFU of JEV was measured by qRT-PCR (for serum and spleen samples) or plaque titration on Vero cells (for CNS samples). Lower limits of detection are denoted by the dotted line, and geometric mean titers by a horizontal line. The data shown were constructed from two independent experiments. Anti-JEV IgM (G) and IgG (H) isotype antibody titers were determined by ELISA, and neutralizing antibody titers (I) by PRNT$_{50}$ assay. The data presented are mean titers representative of 4 or 5 mice per time point with the SEM indicated by error bars. Asterisks denote statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
Table 1 Essential role of B cells and dispensable contribution of CD8<sup>+</sup> T cells in vaccine protection against Japanese encephalitis

<table>
<thead>
<tr>
<th>Mouse strain and immunogen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mortality (no. of deaths/total)</th>
<th>MST ± SD, days</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>µMT-/- mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>100% (4/4)</td>
<td>11.8 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ccJE (1 µg) + Advax™</td>
<td>100% (5/5)</td>
<td>11.2 ± 1.1</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>ChimeriVax-JE (10&lt;sup&gt;5&lt;/sup&gt; PFU)</td>
<td>100% (4/4)</td>
<td>11.0 ± 0</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>β&lt;sup&gt;2&lt;/sup&gt;m&lt;sup&gt;-/-&lt;/sup&gt; mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>87% (13/15)</td>
<td>16.2 ± 4.9</td>
<td>0.0029</td>
<td></td>
</tr>
<tr>
<td>ccJE (0.5 µg)</td>
<td>6% (1/16)</td>
<td>16</td>
<td></td>
<td>0.0029</td>
</tr>
<tr>
<td>ccJE (0.5 µg) + Advax™-2</td>
<td>0% (0/10)</td>
<td>-</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>ccJE (0.05 µg)</td>
<td>53% (8/15)</td>
<td>17.9 ± 5.8</td>
<td>0.0004</td>
<td>0.007</td>
</tr>
<tr>
<td>ccJE (0.05 µg) + Advax™-2</td>
<td>0% (0/10)</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>0.04</td>
</tr>
<tr>
<td>ccJE (0.05 µg) + Advax™</td>
<td>10% (1/10)</td>
<td>13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Eight-week-old mice were immunized s.c. with 2 doses of ccJE with or without Advax™ adjuvant (1 mg/dose) given 2 weeks apart. One group of mice received a single dose of the live ChimeriVax-JE vaccine and negative control mice were treated with saline. One month after completion of the vaccination schedule, mice were challenged with 10<sup>3</sup> PFU of JEV injected into the footpad.

<sup>b</sup> Statistical significance in survival was computed in comparison to the saline-treated control groups.

<sup>c</sup> Statistical significance was computed in comparison to the non-adjuvanted ccJE (0.05 µg) group.
Table 2  JEV-specific IgG responses in wt and β2m⁻/⁻ mice after vaccination.

<table>
<thead>
<tr>
<th>Mouse strain and immunogen</th>
<th>IgG1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IgG2b&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D8</td>
<td>D18</td>
</tr>
<tr>
<td>B6 wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ccJE 0.05 µg</td>
<td>&lt;2.0 (&lt;2.0-2.0)</td>
<td>2.5 (2.0-2.9)</td>
</tr>
<tr>
<td>ccJE 0.05 µg + Advax™</td>
<td>2.4 (2.0-2.6)</td>
<td>&gt;4.1 (&gt;4.1)</td>
</tr>
<tr>
<td>β2m⁻/⁻</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ccJE 0.05 µg</td>
<td>&lt;2.0 (&lt;2.0-2.3)</td>
<td>2.1 (&lt;2.0-2.3)</td>
</tr>
<tr>
<td>ccJE 0.05 µg + Advax™</td>
<td>&lt;2.0 (&lt;2.0-2.0)</td>
<td>2.2 (&lt;2.0-2.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Groups of 8-week-old B6 wt or β2m⁻/⁻ mice were immunized with two doses, given two weeks apart, of ccJE formulated with or without Advax™ adjuvant. Sera were collected on days 8, 18, and 28 post-immunization for anti-JEV IgG1 and IgG2b antibody determination.

<sup>b</sup> ELISA endpoint titers of individual test sera were determined as described in the Materials and Methods section; mean log<sub>10</sub> titers and range are given.
Table 3 Post-immunization and post-challenge JEV-specific neutralizing antibody response in wt and β2m-/- mice.

<table>
<thead>
<tr>
<th>Mouse strain and immunogen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day post-immunization&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Day post-challenge&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D8</td>
<td>D28</td>
</tr>
<tr>
<td>B6 wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ccJE 0.05 μg</td>
<td>&lt;10 (&lt;10-10)</td>
<td>&lt;10 (&lt;10-10)</td>
</tr>
<tr>
<td>ccJE 0.05 μg + Advax&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>32 (10-80)</td>
<td>116 (10-320)</td>
</tr>
<tr>
<td>β2m-/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ccJE 0.05 μg</td>
<td>&lt;10 (&lt;10-10)</td>
<td>&lt;10 (&lt;10)</td>
</tr>
<tr>
<td>ccJE 0.05 μg + Advax&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>16 (&lt;10-40)</td>
<td>&lt;10 (&lt;10-10)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Groups of 8-week-old B6 wt or β2m-/- mice were immunized as indicated using a two-dose immunization schedule. Serum was collected on days 8 and 28 post-immunization, and on days 5 and 10 post-challenge with 10<sup>3</sup> PFU of JEV.

<sup>b</sup> Plaque reduction neutralization was determined as described in the Materials and Methods section; mean titers and range are given.
Table 4 Post-challenge serum antibody and neutralization titers.

<table>
<thead>
<tr>
<th>Transferred cells</th>
<th>Mean Ab titer [log₁₀] (range)</th>
<th>Mean PRNT₅₀ titer (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D5</td>
<td>D10</td>
</tr>
<tr>
<td>Naive</td>
<td>2.4 (2.0-2.3)</td>
<td>2.5 (2.0-2.9)</td>
</tr>
<tr>
<td>B cells</td>
<td>&gt;4.1 (3.8-&gt;4.1)</td>
<td>&gt;4.1(3.2-&gt;4.1)</td>
</tr>
<tr>
<td>CD₄⁺ T cells</td>
<td>2.8 (2.0-3.2)</td>
<td>3.8(3.2-&gt;4.1)</td>
</tr>
</tbody>
</table>

*a Groups of 10- to 12-week-old β2m⁻⁻ recipients of immune B or CD₄⁺ T cells isolated from β2m⁻⁻ donor mice that had been vaccinated twice with ccJE (0.5 μg/dose) + Advax™ (1 mg/dose). Naive control mice received naive total splenocytes.