 doi:10.20944/preprints202110.0034.v1

Article

An Advax-adjuvanted inactivated cell-culture Japanese encephalitis vaccine induces broadly neutralising anti-flavivirus antibodies and cellular immunity and provides single dose protection

Tomoyoshi Komiya[a], Yoshikazu Honda-Okubo[b,c], Jeremy Baldwin[b] and Nikolai Petrovsky[b,c*]

[a] Hokuriku University, 1-1 Taiyogaoka, Kanazawa, Ishikawa 920-1180, Japan
[b] Vaxine Pty Ltd., 11 Walkley Avenue, Warradale, South Australia 5046, Australia
[c] Flinders University, Bedford Park, South Australia 5042, Australia
* Corresponding Author

ABSTRACT: ccJE+Advax is an inactivated cell culture Japanese encephalitis (JE) vaccine formulated with Advax™, a novel polysaccharide adjuvant based on delta inulin. This vaccine has previously shown promise in murine and equine studies and the current study sought to better understand its mechanism of action and assess the feasibility of single dose vaccine protection. Mice immunised with ccJE-Advax had higher serum neutralisation titres than those immunised with ccJE alone or with alum adjuvant. ccJE+Advax induced extraordinarily broad cross-neutralising antibodies against multiple flaviviruses including West Nile virus (WNV), Murray Valley Encephalitis Virus (MVEV), St Louis Encephalitis virus (SLE) and Dengue-1 and -2 viruses. Notably, the DENV-2 cross-neutralising antibodies from ccJE+Advax immunised mice uniquely had no DENV-2 antibody-dependent infection enhancement (ADIE) activity, by contrast to high ADIE activity seen with DENV-1 cross-reactive antibodies induced by mbJE or ccJE alone or with alum adjuvant. JEV-stimulated splenocytes from ccJE+Advax immunised mice showed increased IL-17 and IFN-γ production, consistent with a mixed Th1 and Th17 response, whereas ccJE-alum was associated with production of mainly Th2 cytokines. There is an ongoing lack of human vaccines against particular flaviviruses, including WNV, SLE and MVEV. Given its ability to provide single-dose JEV protection as well as to induce broadly neutralising antibodies free of ADIE activity, ccJE+Advax vaccine could be highly useful in all situations where rapid protection is desirable but ADE needs to be avoided, e.g. during a local outbreak or for use in travellers or the military requiring rapid travel to JEV endemic regions.

Keywords: Japanese encephalitis; Vaccine; Singles dose protection; Flaviviruses; Antibody-dependent enhancement; Advax; Interferon gamma; culture-grown inactivated JE vaccine

Introduction

Japanese encephalitis virus (JEV) is a flavivirus that is one of the leading cause of viral encephalitis in Asia with over 3 billion estimated at-risk of infection [1]. JEV is transmitted by *Culex* mosquitoes primarily to birds and pigs which act as natural reservoir for the virus, and then secondarily to humans. An estimated 68,000 human cases of Japanese encephalitis (JE) occur annually resulting in about 16,000 deaths [2]. Most of the cases of JE (75%) occur in children 0-14 years old [2]. The virus causes acute inflammation in the central nervous system, and significant portion of survivors (>30%) suffer from permanent neurological, behavioural and cognitive sequelae [3]. A recent study found that 81% cases

 doi:10.20944/preprints202110.0034.v1

occur in areas with well-established or developing JEV vaccination programmes suggesting current JEV vaccines provide inadequate coverage [2].

The first commercially available JE vaccine (JE-VAX) was derived from infected mouse brain tissue (mbJE) and required three-doses for protective immunity in ~ 90% of immunised individuals [4]. However the production and rollout of the vaccine was later halted due to serious side effects including systemic allergic reactions and hypersensitivity [5]. A live attenuated JE vaccine (SA14) was developed in China but still required at least two doses for protection but had issues including the consistency of packaging cell lines and potential carry-over of adventitious agents [6]. In addition, JEV can mutate during passage [7-9] raising concerns of phenotypic reversion of the live attenuated virus. More recently, cell culture-grown inactivated JE vaccines (ccJE) have been developed. The ccJE vaccine has an excellent safety record [10], but requires two or more doses to induce protective immunity. JEV is predominately found in rural areas [11] where access to healthcare infrastructure is not as readily available and therefore a JEV vaccine requiring multiple doses is less feasible logistically and may reduce vaccine uptake and coverage with the population.

An additional vaccine design consideration to take into account is that JEV belongs to a family antigenically-related flaviviruses including, West Nile Virus (WNV), Murray Valley encephalitis virus (MVEV), St Louis Encephalitis (SLE) virus, and Dengue virus (DENV) [12]. This can be both advantageous and disadvantageous as immunity can provide cross protection [13-16] but can also be associated with antibody-mediated disease enhancement (ADE) [15-18]. This is particularly important for JEV and DENV as the two strains are not only genetically and antigenically related, but also continue to co-circulate in the same regions of Southeast Asia [19]. Dengue disease enhancement is thought to be mediated by low titres of non-neutralising antibodies resulting in increased uptake of virus into permissive cells expressing Fcɣ receptors, such as monocytes [20]. Hence achieving an adequate magnitude and quality of neutralising antibody response even against heterologous flaviviruses may be key to preventing disease enhancement.

In addition to a humoral response, generating a strong cellular immune response is critical in mitigating JEV disease pathology. Adoptive transfer of anti-JEV effector T cells was able to clear virus and protect animals from lethal intracerebral JEV challenge [21]. Additionally, a plasmid DNA JEV vaccine conferred significant protection despite absence of detectable antiviral antibodies, suggesting protection was T cell mediated [22]. Furthermore an analysis of JEV endemic areas of Southern India showed a significant negative correlation between interferon gamma (IFN-γ) levels and the severity of post-encephalitic neurological sequelae in patients suggesting IFN-γ acts to reduce clinical pathology of JEV [23]. Altogether, these findings suggest that a Th1-biased cellular response characterised by high IFN-γ production is important in adaptive immune protection against flavivirus.

The level and type of vaccine-induced immune response can be significantly augmented by the choice of adjuvants. The ideal adjuvant for JE vaccines should be (i) well-tolerated, (ii) safe, (iii) induce high serum neutralising antibody titres and memory B cells without increasing the risk of ADE to other related flaviviruses, (iv) induce strong Th1 immunity, and (iv) provide single-dose protection. Advax adjuvants are based on non-reactogenic polysaccharide particles and have shown promise in animal and human vaccines [24]. In a previous study, our group demonstrated that a two dose schedule of ccJE formulated with Advax adjuvant was able to boost neutralising antibodies and provide 100% protection of mice from challenge with the JEV Nakayama strain [25].

The purpose of this current study was to test whether it was possible to fully protect against JEV with a single vaccine dose while also further characterising the effect of Advax

 doi:10.20944/preprints202110.0034.v1

adjuvant alone or combined with a TLR9 agonist CpG oligonucleotide on the ability of the vaccine to cross-neutralise other flaviviruses, in particular WNV, SLE and Dengue virus strains, and any potential effects on the risk of ADE. Given the important role cellular immunity play in mitigating disease pathology of JEV, we also examined the effects of Advax adjuvant on the ccJE vaccine induced cellular immune response. Finally, we tested the ability of the Advax adjuvanted ccJE vaccine to protect against JaTH160 strain, a new and more virulent JEV strain that has higher mortality and more pronounced virus propagation [26], to see whether we could achieve single-dose vaccine protection against this highly virulent strain.

Results

ccJE+Advax vaccine induces broadly cross-neutralising antibody.

Groups of mice were vaccinated with two doses of ccJE with or without Advax or Alum adjuvant. An additional group was immunised with a comparable dose of inactivated mouse brain JE antigen (mbJE). Serum was obtained 3 weeks after the last immunisation, pooled for each group to provide sufficient serum to run all assays and then assayed for its ability to neutralise JEV and the other flaviviruses (WNV, MVEV, SLE and DENV serotypes 1 and 2). mbJE induced the highest titres of neutralising antibodies against the homologous JEV, but induced low or undetectable cross-neutralising antibodies against the other flaviruses **(Table 1 iv)**. ccJE alone induced high neutralising antibodies against homologous JEV but low cross-neutralising antibodies against the other flaviruses **(Table 1 iii)**. The best overall cross-neutralising responses against all flaviviruses were achieved by immunisation with ccJE-Advax which induced detectable neutralising antibody titres against all the tested flaviviruses, namely JEV, WNV, MVEV, SLE, DENV-1 and DENV-2 **(Table 1 i)**. A neutralisation titre of 1:10 is considered seroprotective for flaviviruses [27], indicating that ccJE-Advax was able to induce protective levels of cross-neutralising antibodies against this highly divergent group of flaviviruses, except for SLE, where it induced low but detectable levels of neutralising antibodies. Indeed, for most of flaviviruses, ccJE-Advax induced neutralisation titers almost 10 times higher than the sero-protection cut-off. The order of ranking of neutralisation from highest to lowest in this vaccine group was JEV> DEN2> MVEV> DEN1> WNV> SLE. These result indicate that Advax adjuvant when formulated with ccJE antigen is able to induce broadly cross-reactive neutralising antibodies against a wide range of flaviruses.

Table 1. Advax induces broad cross-neutralising antibodies against Japanese encephalitis virus (JEV) and other Flaviviruses. C57BL/6 mice (n = 10/group) were immunised and boosted after 3 weeks with mbJE alone and ccJE alone or with Advax or Alum. Blood was collected 3 week post last immunisation to assess neutralisation activity. To provide sufficient sera fro all assays all sera for each group was pooled into a single sample. Challenge viruses included JEV, West Nile Virus (WNV), Murray Valley encephalitis virus (MVEV), St Louis Encephalitis (SLE), and Dengue virus 1 and 2 (DENV1/2). Neutralisation titres are presented as log10. Data shown represents pooled sera samples. N.D.: Not detected.

Immunised Mouse Sera	Challenge virus					
	JEV	WNV	MVEV	SLE	DENV1	DENV2
(i) ccJE+Advax	2.67	1.20	1.87	0.37	1.21	1.91
(ii) ccJE+Alum	2.99	0.96	1.45	N.D.	0.89	1.81
(iii) ccJE	2.89	0.87	1.45	N.D.	1.02	1.56
(iv) mbJE	3.37	N.D.	1.19	N.D.	N.D.	0.74

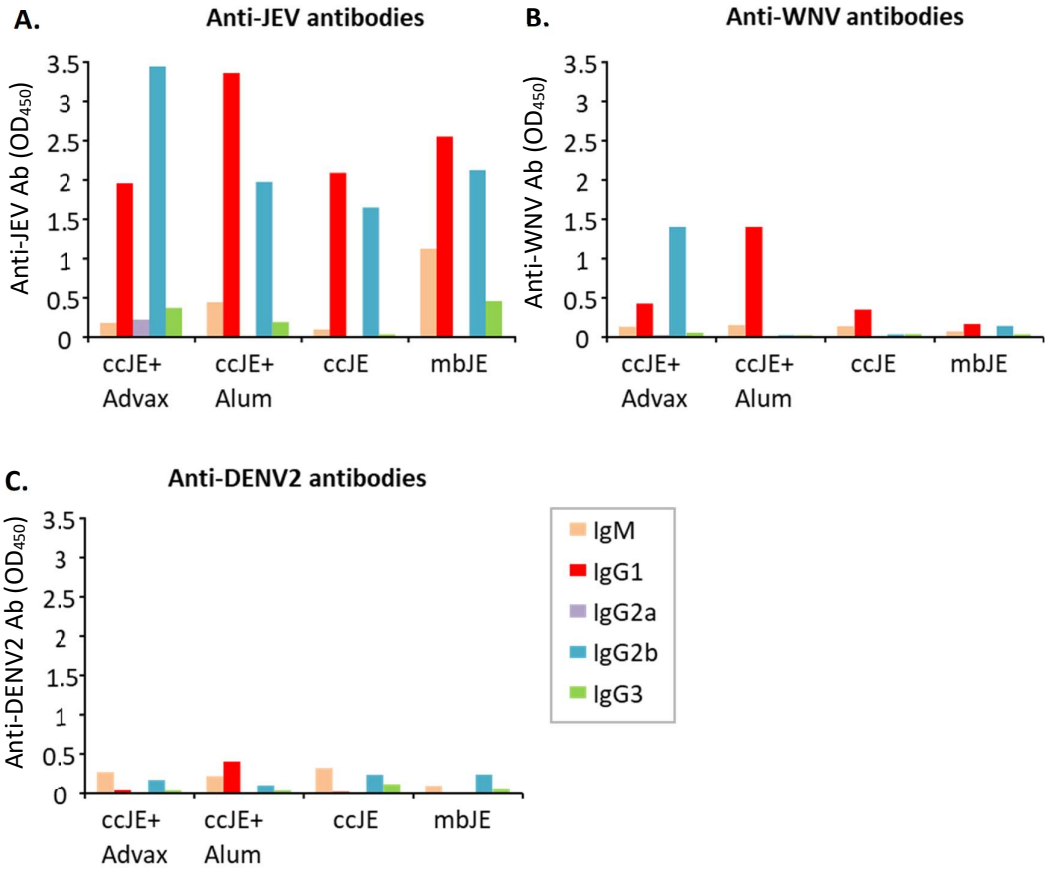
Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 October 2021 doi:10.20944/preprints202110.0034.v1

ccJE+Advax stimulates a balanced Th1/Th2 antibody response

Immunised mouse sera were tested for JEV (Beijing-1 strain) antibody subtype binding by ELISA. ccJE-Advax induced higher production of IgM and IgG subtypes with the exception of IgG1 when compared to immunisation with ccJE alone **(Figure 1A)**. Consistent with the neutralising antibody results, only ccJE-Advax immunised mice showed both WNV-binding IgG1 and IgG2b with low to undetectable levels of anti-WNV antibodies in sera from animals immunised with ccJE or mbJE alone **(Figure 1B)**. DENV-2 binding activity was very low overall for all groups, with IgM the predominant isotype detected **(Figure 1C)**. Overall, ccJE-Advax elicited a balanced but slightly Th1 skewed antibody subtype response, with a higher ratio of IgG2b to IgG1, whereas mbJE and ccJE-alum induced a lower IgG2b to IgG1 ratio, consistent with alum inducing a Th2 biased immune response **(Figure 1Di-iii)**.

To further study the potential role of Advax-specific differences in Th1/Th2 immune bias in induction of broadly neutralising antibodies by the different JEV vaccine formulations, we repeated the JEV immunisations in an IFN-γ knockout mouse model **(Figure 1Div-v)**. IFN-γ has been shown to increase Th1-type antibody isotype production [28] and in B6.129S7-*Ifng*^{tm1Ts}mice this IFN-γ-driven Th1 antibody isotype switching was shown to be abrogated [29]. Analysis of the anti-JEV IgG isotypes again showed no impact of the absence of IFN-γ on the ability of Advax to enhance IgG2b responses to the ccJE antigen, when compared to either ccJE or mbJE alone and did not affect the ability of ccJE-Advax to generate high titres of WNV cross-reactive antibodies. As expected, the Th2 bias of mbJE and ccJE as evidenced by a low IgG2b:IgG1 ratio was maintained in the IFN-γ KO mice. The high IgG2b:IgG1 ratio was preserved in the JE-Advax immunised IFN-γ KO mice was an interesting finding as it indicates that the ability of Advax to induce an IgG2b (Th1-type) isotype switch is not dependent on the Th1 cytokine, IFN-γ.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 October 2021 doi:10.20944/preprints202110.0034.v1



D.

Host mouse	Antigen specific antibodies	Immunised Mouse Sera (IgG2b/IgG1 ratio)			
		ccJE +Advax	ccJE +Alum	ccJE	mbJE
(i) Wild Type	JEV	5.63	0.92	0.94	0.68
(ii) Wild Type	WNV	3.28	0.02	0.10	0.85
(iii) Wild Type	DENV2	0.7	0.227	1.1	1.318
(iv) IFN-γ KO	JEV	1.87	0.36	0.53	0.22
(v) IFN-γ KO	WNV	2.46	0.14	0.26	0.10

Figure 1. Advax induce a Th1-dominant antibody response. C57BL/6 (n = 10/group) and IFN-γ KO (n=5/group) mice were immunised and boosted after 3 weeks with ccJE (50ng) or mbJE (50ng) alone or with Advax (1mg) or Alum (30μg). Blood was collected 3 weeks post last immunisation and antigen-specific antibodies against **(A)** JEV, **(B)** WNV and **(C)** DENV2 were determined by ELISA using isotype/subclass-specific antibodies. Antibody titres on pooled serum for each group are shown as mean OD values at 450nm. **(D)** IgG2b/IgG1 ratios in C57BL/6 and IFN-γ KO mice.

Antibody-dependent Infection Enhancement

Dengue serotype specific antibody-mediated disease enhancement (ADE) has been a major obstacle in the development of dengue vaccines [30] and has also been shown, at least experimentally, for other flaviviruses such as JEV [31]. While initially described as a consequence of previous natural infection, ADE has also been observed after dengue immunisation. As our ccJE-Advax vaccine induced high levels of cross-neutralisation of DENV-1 and -2, we wished to see whether this cross-reactive antibody might be associated with potential antibody-dependent infection enhancement (ADIE) for dengue virus

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 October 2021 doi:10.20944/preprints202110.0034.v1

that could potentially lead to ADE. DENV-2 neutralisation and ADIE were compared using a conventional plaque reduction neutralisation assay using BHK cell or BHK-FcγRIIA cell lines, respectively. Immune sera of mice immunised with ccJE-Advax demonstrated high levels of DENV-2 neutralising activity in both the BHK (PRNT 1.37) and BHK-FcγRIIA (PRNT 1.31) cell lines, but with no evidence of ADIE activity **(Table 2)**. By contrast, all immune sera of mice immunised with ccJE or mbJE alone, or ccJE+alum, demonstrated significant levels of ADIE activity. In particular, the mbJE-immune sera which had no DENV-2 neutralising activity, had the highest ADIE activity, causing over 11-fold increased infection of BHK-FcγRIIA cells. The next highest ADIE activity was seen in sera from the ccJE+alum immunised mice. Altogether, the results indicate that Advax adjuvanted ccJE is able to induce broadly cross-reactive neutralizing antibody against a wide range of flaviviruses but these cross-reactive antibodies, unlike antibodies in mice immunised with vaccines not containing Advax, do not mediate ADIE against DENV2.

Table 2: ccJE with Advax induced neutralising antibody against DENV2 without triggering Fcγ receptor antibody-dependent infection enhancement (ADIE) activity. C57BL/6 (n = 10/group) mice were immunised and boosted after 3 weeks with ccJE (50ng) or mbJE (50ng) alone or with Advax (1mg) or Alum (30μg). Blood was collected 3 weeks post last immunisation. **(A)** Plaque-reduction neutralisation tests (>50% method) ($PRNT_{50}$) with immunised mouse sera using BHK cell or BHK-FcγRIIA cells against DEVN2. $PRINT_{50}$ titers are presented as log10.**(B)** Fold enhancement values. immunised mouse sera using BHK cell or BHK-FcγRIIA cells and DENV2. Underline indicates infection-enhancement activity (see methods section for calculating fold-enhancement, cut of value and infection enhancement activity). N.D.: Not detected.

Immunised Mouse Sera	DENV2			
	(A) Plaque-Reduction Neutralisation Test ($PRNT_{50}$)		(B) Infection enhancement	
	BHK	BHK-FcγRIIA	BHK	BHK-FcγRIIA
(i) ccJE+Advax	1.37	1.31	0.07	0.15
(ii) ccJE+Alum	1.13	N.D.	0.33	3.06
(iii) ccJE	1.51	N.D.	0.20	2.21
(iv) mbJE	N.D.	N.D.	0.66	11.31

Cellular Immune Response

Cellular immunity has been shown to be important for protection against JEV and other flaviviruses. Therefore, cytokine recall responses in pooled splenocytes from each group in response to stimulation with either inactivated or live homologous or heterologous flavivirus antigens was assessed by mouse cytokine multiplex immunoassays (Bio-Plex) and mouse IFN-α ELISA kit **(Figure 2)**. Immunisation with ccJE-Advax markedly increased IL-17 and IFN-γ response to ccJE but not to the other flaviviruses **(Figure 2 D&F)**. Immunisation with ccJE, and to a lesser extent mbJE, increased production of the Th2 cytokines, IL-3, IL-4 and IL-5 after virus stimulation **(Figure 2 A,C&E)**. By contrast, no increase in the IL-3, IL-4 or IL-5 response to any of the flaviviruses was seen in the ccJE-Advax group, consistent with ccJE-Advax biasing towards a Th1 response. The IFN-α response to all flaviviruses was suppressed in the ccJE+alum and to a lesser extent, ccJE alone group **(Figure 2B)**. Cytokine ELISPOT assays showed that addition of CpG oligonucleotide to ccJE +Advax increased the frequency of IFN-γ producing cells when compared to ccJE+Advax alone **(Supplementary Figure 1A)**. IL-17 producing cells were highest in ccJE+Advax or ccJE+AdvaxCpG immunised mice, whilst the number of IL-5 producing cells was highest in ccJE +Alum or ccJE alone immunised mice **(Supplementary Figure 1 B&C)**. This shows that the choice of adjuvant is important in shaping the cytokine profile of antigen-specific immune cells. Given the important role of cytokines,such as IFN-γ in flavivirus protection [32], this suggests Advax or Advax-CpG may be ideally suited for use as adjuvants in flavivirus vaccines.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 October 2021 doi:10.20944/preprints202110.0034.v1

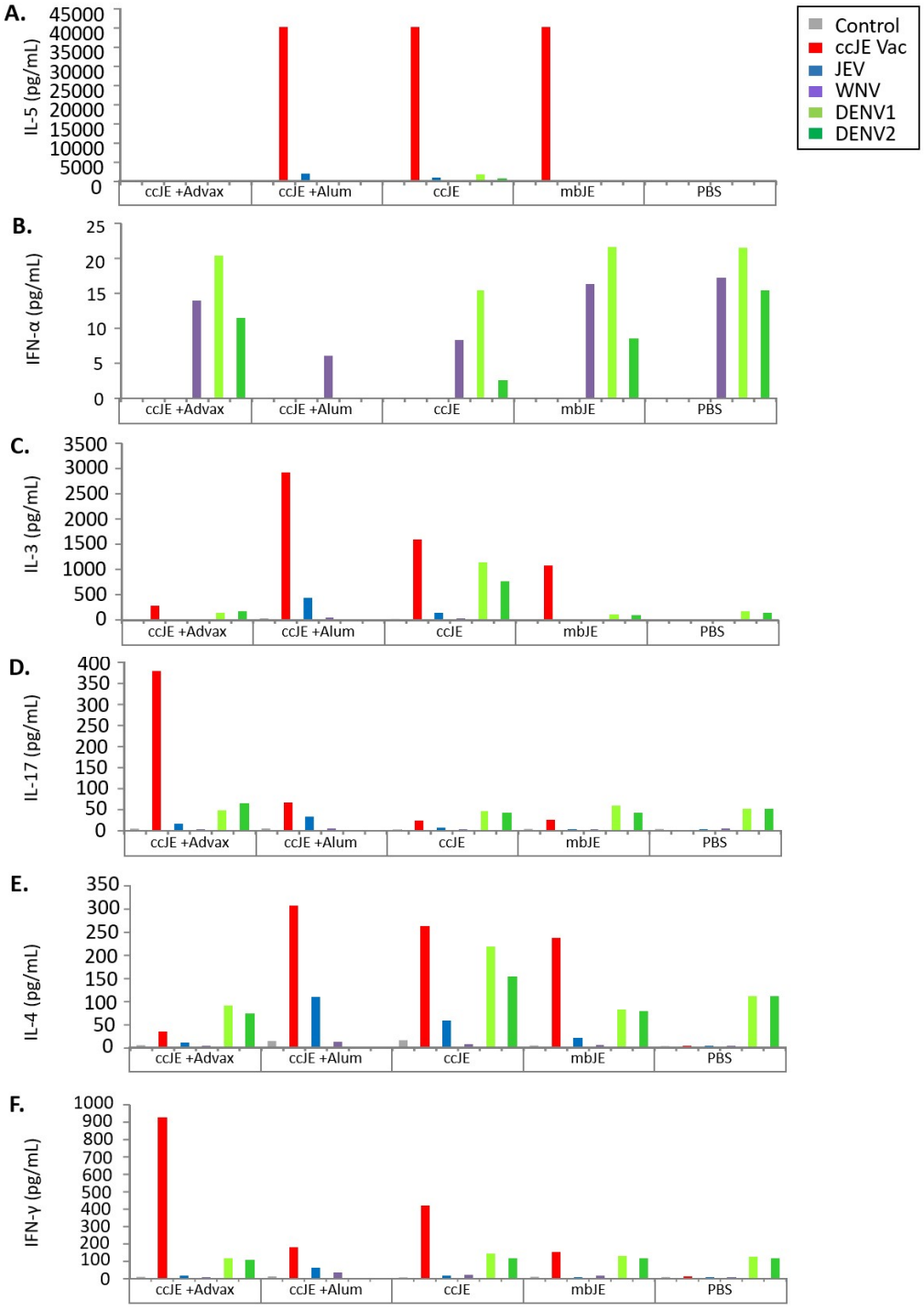


Figure 2: Addition of Advax enhance Th1-associated cytokine production. C57BL/6 mice (n = 10/group) were immunised and boosted after 3 weeks with ccJE (50ng) or mbJE (50ng) alone or formulated with Advax (1mg) or Alum (30μg). Spleens were collected 3 week post last immunisation and pooled for each group. Splenocytes were stimulated with ccJE vaccine (1μg/10^6cells) or flaviruses (JEV, WNV, DENV1 or DENV2) at a MOI of 0.1 for 10^6 cells for 4 days. Cytokines, **(A)** IL-5, **(B)** TNF-α, **(C)** IL-3, **(D)** IL-17, **(E)** IL-4 and **(F)** IFN-γ were measured in culture supernatant using a mouse multiplex immunoassays and a mouse interferon-α ELISA kit.

 doi:10.20944/preprints202110.0034.v1

Advax and Advax-CpG adjuvants enhance broad flavivirus cross-neutralisation

Given the Advax ability to induce a strong cellular response marked by enhanced IFN-γ production and broad cross-neutralizing antibodies against other flaviviruses without enhancement of DENV2 ADIE, we chose to focus on Advax adjuvant for further vaccine development. TLR9 agonist CpG oligodeoxynucleotides have been shown to act synergistically with Advax adjuvant [33-36]. We next tested CpG alone and Advax-CpG combined adjuvant as additional groups. Similar to the Advax group, Advax-CpG induced seroprotective levels of cross-neutralising antibody against all the tested flaviviruses **(Supplementary Table 1)**. By contrast, immunisation with mbJE induced minimal cross-neutralising antibodies against the other flaviviruses, except for MVEV, the closest relative to JEV. ccJE antigen by itself induced moderate cross-neutralisation against the other flaviviruses, not increased by addition of alum adjuvant or CpG. Hence only Advax or Advax-CpG adjuvants were able to induce broad cross-neutralisation responses against all the tested flaviviruses.

ccJE-Advax provides robust JEV protection

To assess whether ccJE-Advax can confer protection against JaTH160, a new and more virulent JEV strain, mice were immunised intramuscularly twice 1 week apart with either inactivated mbJE or ccJE alone or formulated with Advax, CpG or Advax+CpG . One week after the final immunisation, mice were challenged via intraperitoneal route with 3×10^2 PFU JaTH160 strain, equivalent to 20 x LD_{50}. 100% of control PBS-immunised mice succumbed to infection within 2 weeks. Mice that received two doses of ccJE antigen alone or combined with any of the three adjuvant formulations were 100% protected, whereas only partial survival (70%) was observed in the mice that received two doses of mbJE alone **(Figure 3A).**

Next, we tested whether inclusion of Advax adjuvant formulations with ccJE could provide single-dose vaccine protection. Mice at 4 weeks of age were immunised with a single dose of 500ng or 200 ng ccJE antigen formulated with Advax, CpG or Advax-CpG, and challenged 2 weeks later with 3×10^2 PFU JaTH160 strain. After the 500 ng single dose, 100% protection was only seen in the groups immunised with either ccJE+Advax-CpG or with CpG with significantly lower protection for ccJE (60%) or mbJE (50%), alone **(Figure 3B)**. Similar findings were observed for the single 200 ng dose with the highest protection (90%) again seen for ccJE+Advax-CpG, followed by ccJE+CpG (70%), then mbJE (60%) and ccJE (50%) alone **(Figure 3C)**. This shows that Advax-CpG adjuvant enables robust JEV vaccine protection, even after just a single dose.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 October 2021 doi:10.20944/preprints202110.0034.v1

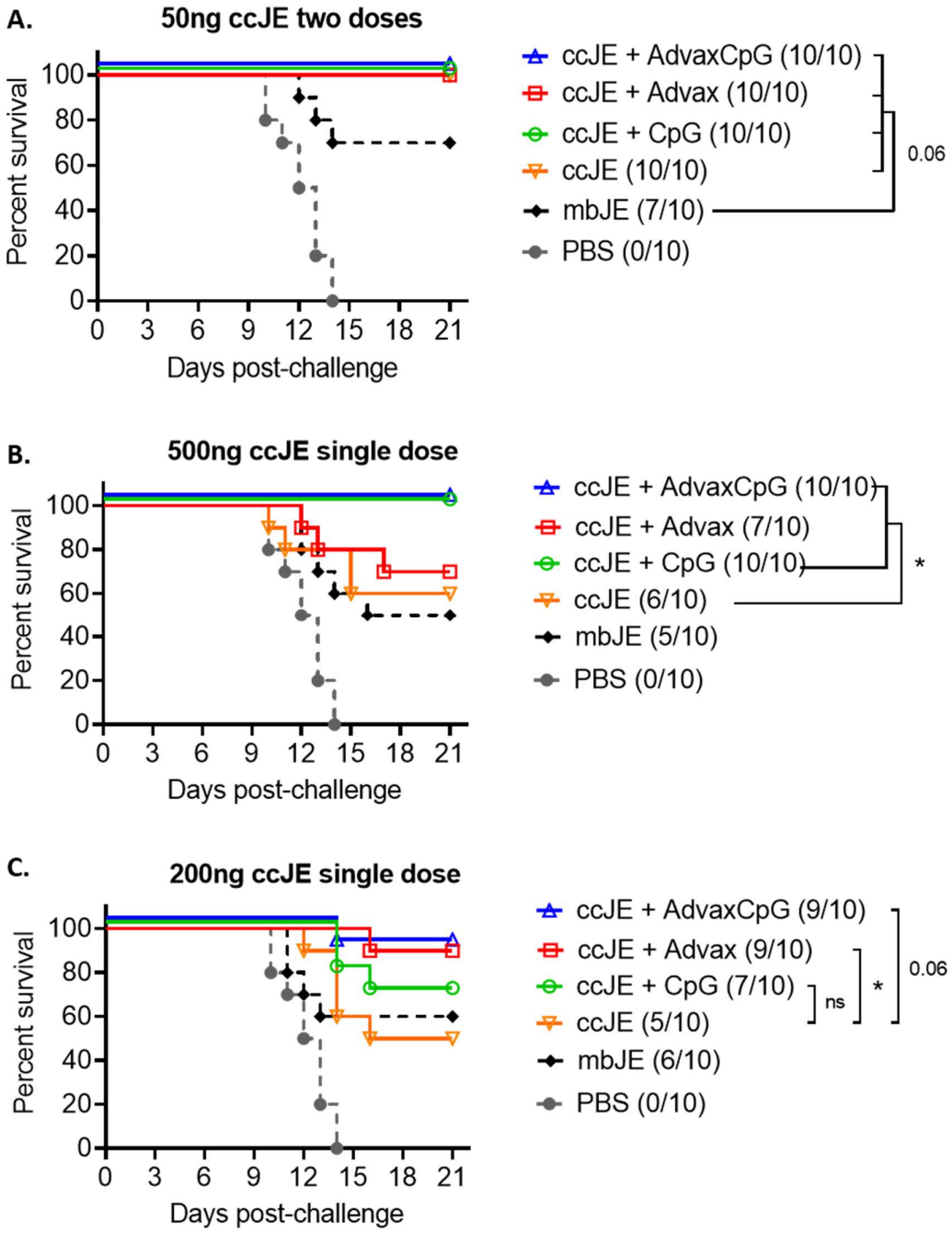


Figure 3: Advax adjuvanted ccJE vaccine provides robust protection against JEV. Four-week-old C57BL/6 strain mice (n = 10/group) were immunised intramuscularly with ccJE alone or with (1mg) or CpG (5μg) or Advax-CpG (1mg/5μg) **(A)** twice 3 weeks apart with a vaccine antigen dose of 50ng or once with a vaccine antigen dose of **(B)** 500ng or **(C)** 200ng. As a control, mice were also immunised mbJE or PBS. One week after last immunisation mice were challenged intraperitoneally with 3 x 10^2 PFU JEV JaTH160 strain. Survival rate (number of survivors / number of challenged mice shown in parenthesis, (*; $p < 0.05$ by Fisher's exact test).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 4 October 2021 doi:10.20944/preprints202110.0034.v1

To assess for a correlation between neutralising antibody titres and protection, sera was collected prior to challenge from mice that received either single (200 ng and 500 ng) or two dose (50 ng) vaccination regimens. Neutralising titres against the Beijing JE strain were highest in mice that received two doses of vaccine. This was followed by the 500ng single dose groups with lowest responses in the 200ng single dose groups **(Table 3)**. There was a correlation between neutralising titre and protection in the twice immunised groups with the mbJE group having the lowest titres and also lowest survival (60%). However, this relationship did not hold true in the 200ng single dose group where the ccJE-Advax group had a relatively modest PRNT of 0.97, just under the accepted seroprotection level of 1, but had the highest survival (90%).Moreover, the mbJE group had the highest PRNT of 1.43, but achieved only 60% survival. Hence the serum neutralising antibody levels in the single immunisation groups showed a poor correlation with the challenge outcome. This suggests that Advax may enhance single dose protection by alternative mechanisms such as via increased numbers of memory B cells, change in functional antibodies or enhanced cellular immunity. In a separate study, we compared the ability of two doses of either ccJE or commercial alum-adjuvanted Ixiaro/Jespect to induce JEV neutralising antibodies (PRNT log10). Again, ccJE-Advax-CpG did best (PRNT 2.5) while the alum-adjuvanted Ixiaro/Jespect vaccine did worst inducing almost 10-fold lower neutralising antibody (PRNT 1.6), than the ccJE+Advax-CpG.

Table 3: Advax and Advax-CpG adjuvants induce strong neutralisation against JEV in either a single or two dose vaccine regimen. Four-week-old C57BL/6 strain mice (n = 10/group) were immunised intramuscularly with ccJE 50ng with Advax(1mg) or CpG (5μg) or Advax-CpG (1mg/5μg) twice 3 weeks apart or once with ccJE 500ng or 200ng with the same adjuvants. Data shown represents pooled sera samples for each group. Neutralisation titres are presented as log10.

Immunised Mouse Sera	JEV		
	Single		Double
	500ng	200ng	50ng
(i) ccJE + Advax	1.972	0.967	2.512
(ii) ccJE + AdvaxCpG	1.730	1.352	2.790
(iii) ccJE + CpG	1.155	0.877	1.972
(iv) ccJE	1.182	0.786	2.098
(v) mbJE	0.966	1.433	1.343

To assess cellular immunity in single and two dose ccJE vaccine schedules, splenocytes were isolated from immunised mice, stimulated with viral antigens, and cytokine production measured Bio-Plex Mouse Cytokine Assay **(Supplementary Figure 2)**. In the two-dose vaccine regime, ccJE+Advax-CpG induced higher levels of all cytokines, except TNF-α when compared to ccJE alone. Unexpectedly, in both single dose immunized groups, IFN-γ wasn't increased in the ccJE+Advax-CpG compared to other groups, hence being different to findings in the two dose schedule. In the 500ng ccJE+Advax-CpG single dose schedule IL-17, MIP-1β, IL-3 and GM-CSF showed a trend to increased levels. The results indicate that other immune parameters, outside of traditional markers such as serum neutralizing antibodies and cellular IFN-γ production, may contribute to protection against JEV after a single vaccine dose.

Discussion

Advax is a novel polysaccharide adjuvant based on microparticles of delta inulin, which potently stimulates vaccine immunogenicity whilst being safe and non-reactogenic [24]. Advax is distinct from typical vaccine adjuvants as it does not appear to work through induction of inflammatory danger signals, but rather potentiates the intrinsic or in-built adjuvant property of co-administered antigens [37]. Vaccines containing Advax adjuvant have been extensively evaluated in human clinical trials, including in hepatitis

 doi:10.20944/preprints202110.0034.v1

B [38], influenza [39-41], insect-sting allergy [42] and SARS-CoV-2 vaccines. Advax adjuvant has previously been shown to enhance ccJE vaccine immunogenicity in mice [25] and horses [43]. The current study explored the mechanisms behind how Advax enhances ccJE vaccine responses including assessing the breadth of cross-reactivity against other flavirus family members, potential for such antibodies to induce ADIE and tested whether Advax adjuvant would allow single-dose vaccine protection against a high virulence JEV strain.

In our study ccJE formulated with traditional alum adjuvant induced predominately IgG1 antibody which conforms with existing literature that alum adjuvant imparts a major Th2 bias in vaccine responses [44]. A strong Th2 bias was also seen after immunisation with ccJE or mbJE alone. On the other hand, ccJE formulated with Advax produced a balanced Th1 and Th2 response as demonstrated by induction of approximately equal amounts of both IgG1 and Ig2b. Splenocytes isolated from mice immunised mice with mbJE or ccJE alone or ccJE+Alum and re-stimulated in vitro produced the high amounts of Th2 cytokines (IL-3, IL-4, IL-5), whereas immunisation with ccJE+Advax-CpG resulted in increased production of both Th1 and Th17 cytokines (IFN-γ and IL-17). This is consistent with Advax adjuvant imparting a significant Th1 bias to the immune response to the ccJE antigen.

An interesting feature not previously reported is the different pattern of antibody responses induced by our new ccJE vaccine formulation when compared to the traditional mbJE vaccine. While the mbJE antigen induced high levels of neutralising antibodies to the homologous JE virus, it failed to induce cross-neutralising antibodies against the other flaviviruses. By contrast, ccJE-Advax induced high levels of cross-neutralising antibodies against a broad range of flaviviruses including WNV, MVEV, DENV-1, DENV-2, and even low neutralisation of SLE. Based a review of current literature, no other flavivirus vaccine approach seems to have demonstrated such extensive flavivirus cross-protection using a single flavivirus antigen. Inactivated JE vaccines JE-VAX [45] or live SA14-14-2 JE vaccine [46] were previously reported to induce undetectable or non-protective levels of neutralising antibody against MVEV and WNV, consistent with our findings for mbJE vaccine. This difference suggests that our ccJE antigen presents to the immune system one or more unique flavivirus neutralising epitopes not presented by the mbJE antigen and recognition of these additional epitopes is enhanced by formulation with Advax adjuvant. The neutralising antibody data is consistent with the IgG binding data that showed the ccJE-Advax vaccine induced detectable WNV and DENV-2 binding IgG just 3 weeks after a single immunisation with ccJE-Advax, whereas such antibodies were not detectable after a single dose of either ccJE or mbJE alone.

Notably, the induction of DENV binding and neutralising antibodies by ccJE+Advax did not induce infection enhancement for DENV2 in the *in vitro* ADIE cellular assays. DENV ADE is a high risk in the presence of sub-neutralising antibodies induced by other DENV sub-strains or related flaviviruses. Only ccJE+Advax showed no signs of DENV-2 induced ADIE in the BHK-RcγRIIA cell assay. Notably, immune sera from all other immunisation groups including ccJE alone, ccJE-Alum and mbJE demonstrated lower neutralisation and increased ADIE activity in the same BHK-RcγRIIA assay. This indicates that not only is Advax adjuvant able to induce high levels of flavivirus cross-neutralisation but that the antibodies that mediate this function uniquely do not cause enhanced infectivity, either because of their potent neutralisation capacity or because they are in some other way functionally different to antibodies induced by alum adjuvant or the antigens by themselves.

At one week post-immunisation in the 2 dose groups, the group that had received ccJE-Advax had significantly higher IL-2, IL-3, IL-6, IL-10, IL-17, Eotaxin, GMCSF, IFN-γ, MIP-1b and Rantes recall responses when compared to the groups that received ccJE or mbJE alone. This suggests that Advax is associated with broad enhancement of T-cell subset responses early after immunisation, whereas at later time points the Advax-induced response matures into a Th1- and to lesser extend Th17-dominant phenotype. This is consistent with findings in other studies of Advax adjuvants where the extent of the T cell

 doi:10.20944/preprints202110.0034.v1

IFN-γ recall response was seen to get progressively stronger out to a year post-immunization (unpublished data). We currently do not know whether this is due to progressive conversion over time of other memory T-cell subsets, e.g. Th0, Th2 and Th-17 cells into IFN-γ producing Th1 cells, continued proliferation and expansion of an IFN-γ producing memory Th1 cell subset, or ongoing activation by long-term antigen depots of naïve T cells into IFN-γ producing Th1 cells.

Due to extensive Th-2 bias with alum adjuvant and potential risk of DENV ADE , we focused on evaluating ccJE with Advax against high virulent JaTH160 JEV strain. In our study the two-dose regime at 50ng was able to confer complete protection against JaTH160. The addition of CpG potentiated the Advax adjuvant and enabled complete protection with a single dose 500ng of ccJE antigen. To our knowledge we are the first group to boost the immunogenicity of ccJE through use of adjuvants (Advax and Advax-CpG) thereby enabling single-dose protection against lethal JEV infection. By comparison a two-dose vaccination schedule in human subjects with the mouse brain derived JE-VAX vaccine failed to generate detectable JE neutralising antibody in ~20% of vaccine recipients [47] and three doses were required to achieve adequate immunity [48]. Similarly, inactivated ccJE vaccines (unadjuvanted) are approved in Japan as three dose pediatric schedules [49] with subsequent periodic boosting presumed to occur due to endemic JE exposure. An alum-adjuvated ccJE traveller vaccine (called Jespect® or Ixaro®) has been licensed as a two-dose vaccination schedule [50].

In the single dose group, analysis of both sera and cytokine-secreting cells showed no increase in neutralization activity or IFN-γ producing cells in response to JEV to explain the enhanced protection seen with the Advax or Advax-CpG adjuvant. In particular, there was a poor correlation between neutralising antibody levels and protection in the single dose group. Future studies are required to determine exact mechanism by which the addition of Advax or Advax-CpG improved JEV protection. Notably, if these results successfully translate to human subjects, a single dose vaccine based on ccJE+Advax could be useful to induce initial JEV immunity in endemic regions as well as for use as a travellers vaccine.

A limitation of the study was that the vaccine formulations were only evaluated in a single preclinical model, namely inbred mice. Despite species-specific differences with humans, mice are one of the most extensively utilized and characterized animal models for JEV and other flavivirues, due to their high degree of susceptibility to flavivirus encephalitis and the similarity in disease presentation and virus tropism between rodents and humans [51,52]. This enables us to directly benchmark our results with the findings of other research groups in the literature. In addition, a previous study [25] published by our lab evaluated ccJE with Advax in horses where the cross-neutralization antibody activity correlated well with observed immunogenicity in our parallel mouse studies. This provides confidence that the single-dose vaccine protection strategy in the current study may be applicable in large animal models and humans.

Methods

Animals.

All studies were performed in accordance with the guidelines specified by the Animal Experimentation and Ethics Committee of the Kitasato Institute for Life Sciences, Kitasato University (Protocols number: 14002). Four-week-old female C57BL/6 mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) and female IFN-ɣ knockout (KO) mice (B6.129S7-*Ifng*^{tm1Ts}) were obtained from Oriental Bioservice, Inc (Kyoto, Japan). Animals were maintained under specific pathogen-free conditions.

Cell and Viruses

Vero cell (JCRB9013) was obtained from Japanese Collection of Research Bioresources Cell Bank (National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan). Working stocks of JEV (Beijing-1, JaTH-160 strain), MVEV (MVE-1-51 strain), Dengue virus 1 (Philippine strain) and 2 (India strain) were 10% suckling mouse

 doi:10.20944/preprints202110.0034.v1

brain homogenates in Hanks' balanced salt solution containing 20 mM HEPES buffer (pH 8.0) and 0.2% bovine serum albumin (HBSS-BSA). West Nile virus (NY-101 strain) and St Louis encephalitis virus were grown Vero cells in MEM containing 2% festal bovine serum. Virus titres were determined by plaque formation on Vero cells, as described [53,54].

Immunisation Schedule

JE-VAX vaccine was obtained from Biken (Osaka, Japan), which contains purified, formalin-inactivated, JEV (mbJE: Nakayama strain). Vero cell culture-grown inactivated JE vaccine (ccJE; Beijing-1 strain) was manufactured by the Kitasato Institute Research Center for Biologicals (Saitama, Japan). Advax delta inulin adjuvant was obtained from Vaxine Pty Ltd (Adelaide, South Australia, Australia). C57BL/6 or IFN-γ KO mice (n = 10/group) were immunised twice 1 week apart with ccJE (50ng) formulation with Advax (1mg) and/or CpG (5μg) or Aluminium hydroxide (30μg). Addition experimental group of mice were immunised with mbJE (50ng) for comparison. Blood and spleens were collected 3 weeks after last immunisation for further analysis.

Antibody Isotypes

Isotypes of JEV, WNV and DENV-2-specific antibodies in serum samples were determined by the ELISA-based Mouse Typer Sub-Isotyping kit (Bio-Rad, Hercules, CA, U.S.A.) according to the supplier's instructions. JEV, WNV or DENV-2-coated ELISA trays were used and serum samples from immunised mice were diluted 100-fold in Blotto/Tween (Novatein Biosciences, Woburn, MA, U.S.A.) and assayed in duplicate. The optical density was measured at 450 nm (OD450). Three naïve control sera were included in each test. To determine relative isotype titres, the mean OD values of test sera were divided by 2 times the corresponding mean OD value of the control sera.

Plaque Reduction Neutralisation Tests

Plaque-reduction neutralisation tests ($PRNT_{50}$) were performed by incubating 200 PFU of JEV (Beijin-1 strain), MVEV (MVE-1-51 stain) or WNV (NY-101 strain) in 110 μl HBSS-BSA with serial 2-fold dilutions of antiserum in the same buffer in a 96-well tray at 37°C for 1 h. Complement was inactivated by heating the sera at 56°C for 0.5 h before use. Duplicate 0.1 ml aliquots were assayed for infective virus by plaque formation on Vero cell monolayers grown in 6-well tissue culture trays. The percentage plaque reduction was calculated relative to virus controls incubated with naïve serum from the same mouse strain. Controls yielded 50 – 100 PFU/well. $PRNT_{50}$ titres are given as the reciprocal of serum dilutions, which resulted in ≥50% reduction of the number of plaques.

Antibody-dependent Infection Enhancement Assay

DENV-2 ADIE activities were determined by conventional plaque reduction neutralization test against DENV-2 using BHK-FcγRIIA cells provided by National Institute of Infectious Disease. Fold enhancement values (FEV) and Positive Infection Enhancement were calculated using the following formulas as previously described [55]:

$$\textbf{FEV} = \frac{\text{Mean Plaque with sera (On BHK-Fc}\gamma\text{RIIA cells)}}{\text{Mean Plaque w/o sera}}$$

$$\textbf{Cut-off value} = \text{Sum of the mean of negative control wells}$$

$$\textbf{Positive Infection Enhancement} = \text{FEV} > (\text{Cut-off value} + 2 \text{ standard deviation})$$

JEV Challenge

C57BL/6 mice (n = 10/group) were immunised intramuscularly with ccJE alone or with Advax (1mg) or CpG (5μg) or AdvaxCpG (1mg/5μg) twice 1 week apart with a vaccine antigen dose of 50ng or once with a vaccine antigen dose of 500ng or 200ng. One (double doses) or 2 (single dose) weeks after the final immunisation, mice were challenged

 doi:10.20944/preprints202110.0034.v1

via intraperitoneal route with lethal dose of 3 x 10^2 PFU JaTH160 strain, corresponding to 20 x LD_{50} and were monitored daily for over 3 weeks.

Cytokine assay

C57BL/6 mice (n = 10/group) were immunised and boosted after 3 weeks with ccJE (50ng) or mbJE (50ng) alone or with Advax (1mg) or Alum (30μg). Spleens were collected 3 week post last immunisation. 1x10^4 splenocytes were stimulated with JE vaccine(50ng), and Cytokine analysis was performed by Bio-Plex Pro™ Mouse Cytokine 23-Plex Immunoassay (Bio-Rad) and VeriKine™ Mouse Interferon Alpha ELISA Kit (PBL Assay Science, Piscataway, NJ, U.S.A.) according to manufacturers' instructions.

Enzyme-linked immunospot (ELISPOT) assay

C57BL/6 mice (n = 10/group) were immunised intramuscularly with mbJE or ccJE alone or with CpG or AdvaxCpG or Alum twice 3 weeks apart with a vaccine antigen dose of 50ng. Spleens were collected 3 weeks after the last immunisation. Antigen-specific IFN-γ and IL-17A ELISPOT assays were conducted with Mouse IFN-γ/IL-17 Dual-Colour ELISPOT Kit (R&D Systems) and Mouse IL-5 ELISpot Kit (R&D Systems) respectively, according to the manufacturer's instructions. Briefly, splenocytes, stimulated by following restimulation with 50ng of ccJE or mbJE vaccine, or with JEV, MVE or WNV (MOI=0.01) were plated at the concentration of 5 x 10^5 cells/well in duplicate for overnight at 37°C. Then plates were washed and incubated with biotinylated anti-IFN-γ, IL-17A or anti-IL-5 antibody overnight at 4ºC. After washing the plates, streptavidin-Alkaline phosphatase was added and incubated for 2 h at room temperature. Finally, the plates were incubated with substrate BCIP/NBT Chromogen at room temperature for colour development. The spots were counted using an immunospot reader system.

Statistics

Differences in survival ratios in mouse challenge experiments were assessed using Fisher's exact test and the Wilcoxon signed-rank test was used to assess differences in antibody titres for significance. Samples with titres below the detection limit of the serological assays were given titres of half that of the detection limit for calculations.

Acknowledgments: This work was supported by Grants No. U01-AI061142 and HHSN272200800039C from the National Institutes of Health, National Institute of Allergy and Infectious Diseases. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health, National Institute of Allergy and Infectious Diseases. Vaxine is the recipient of a grant from DMTC to advance an Advax-adjuvanted JEV vaccine to human clinical trials. The authors thank Hiroko Toriniwa for skilled technical assistance. The authors also thank Dr. Takasaki at the National Institute of Infectious Diseases, Japan, for supplying JaTH 160 strain of JEV and FcR-expressing BHK cells, and Prof. Kariwa at Hokkaido University for supplying NY-101 strain of WNV.

INSTITUTIONAL REVIEW BOARD STATEMENT: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Animal Experimentation and Ethics Committee of the Kitasato Institute for Life Sciences, Kitasato University.

INFORMED CONSENT STATEMENT: Not applicable.

DATA AVAILABILITY STATEMENT: The data presented in this study are available on request from the corresponding author.

POTENTIAL CONFLICTS OF INTEREST: YHO, JB and NP are affiliated with Vaxine Pty Ltd, Adelaide, Australia, which has a commercial interest in Advax adjuvants.

REFERENCES:

1. Erlanger, T.E.; Weiss, S.; Keiser, J.; Utzinger, J.; Wiedenmayer, K. Past, present, and future of Japanese encephalitis. *Emerging infectious diseases* **2009**, *15*, 1.

2. Campbell, G.L.; Hills, S.L.; Fischer, M.; Jacobson, J.A.; Hoke, C.H.; Hombach, J.M.; Marfin, A.A.; Solomon, T.; Tsai, T.F.; Tsu, V.D. Estimated global incidence of Japanese encephalitis: a systematic review. *Bulletin of the World Health Organization* **2011**, *89*, 766-774.
3. Connor, B.; Bunn, W.B. The changing epidemiology of Japanese encephalitis and New data: the implications for New recommendations for Japanese encephalitis vaccine. *Tropical diseases, travel medicine and vaccines* **2017**, *3*, 1-6.
4. Hoke, C.H.; Nisalak, A.; Sangawhipa, N.; Jatanasen, S.; Laorakapongse, T.; Innis, B.L.; Kotchasenee, S.-o.; Gingrich, J.B.; Latendresse, J.; Fukai, K. Protection against Japanese encephalitis by inactivated vaccines. *New England Journal of Medicine* **1988**, *319*, 608-614.
5. Tauber, E.; Dewasthaly, S. Japanese encephalitis vaccines–needs, flaws and achievements. **2008**.
6. Hegde, N.R.; Gore, M.M. Japanese encephalitis vaccines: Immunogenicity, protective efficacy, effectiveness, and impact on the burden of disease. *Human vaccines & immunotherapeutics* **2017**, *13*, 1320-1337.
7. Ni, H.; Chang, G.J.; Xie, H.; Trent, D.W.; Barrett, A.D. Molecular basis of attenuation of neurovirulence of wild-type Japanese encephalitis virus strain SA14. *J Gen Virol* **1995**, *76 (Pt 2)*, 409-413, doi:10.1099/0022-1317-76-2-409.
8. Gromowski, G.D.; Firestone, C.Y.; Bustos-Arriaga, J.; Whitehead, S.S. Genetic and phenotypic properties of vero cell-adapted Japanese encephalitis virus SA14-14-2 vaccine strain variants and a recombinant clone, which demonstrates attenuation and immunogenicity in mice. *Am J Trop Med Hyg* **2015**, *92*, 98-107, doi:10.4269/ajtmh.14-0427.
9. Nitayaphan, S.; Grant, J.A.; Chang, G.J.; Trent, D.W. Nucleotide sequence of the virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA-14-14-2. *Virology* **1990**, *177*, 541-552, doi:10.1016/0042-6822(90)90519-w.
10. Tauber, E.; Kollaritsch, H.; Korinek, M.; Rendi-Wagner, P.; Jilma, B.; Firbas, C.; Schranz, S.; Jong, E.; Klingler, A.; Dewasthaly, S. Safety and immunogenicity of a Vero-cell-derived, inactivated Japanese encephalitis vaccine: a non-inferiority, phase III, randomised controlled trial. *The Lancet* **2007**, *370*, 1847-1853.
11. Le Flohic, G.; Porphyre, V.; Barbazan, P.; Gonzalez, J.-P. Review of climate, landscape, and viral genetics as drivers of the Japanese encephalitis virus ecology. *PLoS neglected tropical diseases* **2013**, *7*, e2208.
12. Rathore, A.P.S.; St John, A.L. Cross-Reactive Immunity Among Flaviviruses. *Front Immunol* **2020**, *11*, 334, doi:10.3389/fimmu.2020.00334.
13. Martina, B.E.; Koraka, P.; van Den Doel, P.; van Amerongen, G.; Rimmelzwaan, G.F.; Osterhaus, A.D. Immunization with West Nile virus envelope domain III protects mice against lethal infection with homologous and heterologous virus. *Vaccine* **2008**, *26*, 153-157.
14. Takasaki, T.; Yabe, S.; Nerome, R.; Ito, M.; Yamada, K.-I.; Kurane, I. Partial protective effect of inactivated Japanese encephalitis vaccine on lethal West Nile virus infection in mice. *Vaccine* **2003**, *21*, 4514-4518.
15. Lobigs, M.; Pavy, M.; Hall, R. Cross-protective and infection-enhancing immunity in mice vaccinated against flaviviruses belonging to the Japanese encephalitis virus serocomplex. *Vaccine* **2003**, *21*, 1572-1579.
16. Lobigs, M.; Larena, M.; Alsharifi, M.; Lee, E.; Pavy, M. Live chimeric and inactivated Japanese encephalitis virus vaccines differ in their cross-protective values against Murray Valley encephalitis virus. *Journal of virology* **2009**, *83*, 2436-2445.
17. Wallace, M.; Smith, D.; Broom, A.; Mackenzie, J.; Hall, R.; Shellam, G.; McMinn, P. Antibody-dependent enhancement of Murray Valley encephalitis virus virulence in mice. *Journal of general virology* **2003**, *84*, 1723-1728.
18. Broom, A.K.; Wallace, M.J.; Mackenzie, J.S.; Smith, D.W.; Hall, R.A. Immunisation with gamma globulin to Murray Valley encephalitis virus and with an inactivated Japanese encephalitis virus vaccine as prophylaxis against Australian encephalitis: evaluation in a mouse model. *Journal of medical virology* **2000**, *61*, 259-265.

19. Singh, K.P.; Mishra, G.; Jain, P.; Pandey, N.; Nagar, R.; Gupta, S.; Prakash, S.; Prakash, O.; Khan, D.N.; Shrivastav, S. Co-positivity of anti-dengue virus and anti-Japanese encephalitis virus IgM in endemic area: co-infection or cross reactivity? *Asian Pacific journal of tropical medicine* **2014**, *7*, 124-129.
20. Wahala, W.M.; De Silva, A.M. The human antibody response to dengue virus infection. *Viruses* **2011**, *3*, 2374-2395.
21. Murali-Krishna, K.; Ravi, V.; Manjunath, R. Protection of adult but not newborn mice against lethal intracerebral challenge with Japanese encephalitis virus by adoptively transferred virus-specific cytotoxic T lymphocytes: requirement for L3T4+ T cells. *J Gen Virol* **1996**, *77 (Pt 4)*, 705-714, doi:10.1099/0022-1317-77-4-705.
22. Ashok, M.S.; Rangarajan, P.N. Immunization with plasmid DNA encoding the envelope glycoprotein of Japanese Encephalitis virus confers significant protection against intracerebral viral challenge without inducing detectable antiviral antibodies. *Vaccine* **1999**, *18*, 68-75, doi:10.1016/s0264-410x(99)00180-2.
23. Kumar, P.; Sulochana, P.; Nirmala, G.; Chandrashekar, R.; Haridattatreya, M.; Satchidanandam, V. Impaired T helper 1 function of nonstructural protein 3-specific T cells in Japanese patients with encephalitis with neurological sequelae. *J Infect Dis* **2004**, *189*, 880-891, doi:10.1086/381768.
24. Petrovsky, N.; Cooper, P.D. Advax™, a novel microcrystalline polysaccharide particle engineered from delta inulin, provides robust adjuvant potency together with tolerability and safety. *Vaccine* **2015**, *33*, 5920-5926.
25. Lobigs, M.; Pavy, M.; Hall, R.A.; Lobigs, P.; Cooper, P.; Komiya, T.; Toriniwa, H.; Petrovsky, N. An inactivated Vero cell-grown Japanese encephalitis vaccine formulated with Advax, a novel inulin-based adjuvant, induces protective neutralizing antibody against homologous and heterologous flaviviruses. *J Gen Virol* **2010**, *91*, 1407-1417, doi:10.1099/vir.0.019190-0.
26. Hayasaka, D.; Shirai, K.; Aoki, K.; Nagata, N.; Simantini, D.S.; Kitaura, K.; Takamatsu, Y.; Gould, E.; Suzuki, R.; Morita, K. TNF-α acts as an immunoregulator in the mouse brain by reducing the incidence of severe disease following Japanese encephalitis virus infection. *PLoS One* **2013**, *8*, e71643, doi:10.1371/journal.pone.0071643.
27. Van Gessel, Y.; Klade, C.S.; Putnak, R.; Formica, A.; Krasaesub, S.; Spruth, M.; Cena, B.; Tungtaeng, A.; Gettayacamin, M.; Dewasthaly, S. Correlation of protection against Japanese encephalitis virus and JE vaccine (IXIARO(®)) induced neutralizing antibody titers. *Vaccine* **2011**, *29*, 5925-5931, doi:10.1016/j.vaccine.2011.06.062.
28. Finkelman, F.; Katona, I.; Mosmann, T.; Coffman, R. IFN-gamma regulates the isotypes of Ig secreted during in vivo humoral immune responses. *The Journal of Immunology* **1988**, *140*, 1022-1027.
29. Dalton, D.K.; Pitts-Meek, S.; Keshav, S.; Figari, I.S.; Bradley, A.; Stewart, T.A. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* **1993**, *259*, 1739-1742.
30. Wilder-Smith, A.; Ooi, E.E.; Horstick, O.; Wills, B. Dengue. *Lancet* **2019**, *393*, 350-363, doi:10.1016/s0140-6736(18)32560-1.
31. Saito, Y.; Moi, M.L.; Takeshita, N.; Lim, C.-K.; Shiba, H.; Hosono, K.; Saijo, M.; Kurane, I.; Takasaki, T. Japanese encephalitis vaccine-facilitated dengue virus infection-enhancement antibody in adults. *BMC Infectious Diseases* **2016**, *16*, 578, doi:10.1186/s12879-016-1873-8.
32. Larena, M.; Regner, M.; Lobigs, M. Cytolytic effector pathways and IFN-γ help protect against J apanese encephalitis. *European journal of immunology* **2013**, *43*, 1789-1798.
33. Layton, R.C.; Petrovsky, N.; Gigliotti, A.P.; Pollock, Z.; Knight, J.; Donart, N.; Pyles, J.; Harrod, K.S.; Gao, P.; Koster, F. Delta inulin polysaccharide adjuvant enhances the ability of split-virion H5N1 vaccine to protect against lethal challenge in ferrets. *Vaccine* **2011**, *29*, 6242-6251, doi:10.1016/j.vaccine.2011.06.078.
34. Honda-Okubo, Y.; Barnard, D.; Ong, C.H.; Peng, B.H.; Tseng, C.T.; Petrovsky, N. Severe acute respiratory syndrome-associated coronavirus vaccines formulated with delta inulin adjuvants provide

enhanced protection while ameliorating lung eosinophilic immunopathology. *J Virol* **2015**, *89*, 2995-3007, doi:10.1128/jvi.02980-14.
35. Honda-Okubo, Y.; Baldwin, J.; Petrovsky, N. Advax-CpG Adjuvant Provides Antigen Dose-Sparing and Enhanced Immunogenicity for Inactivated Poliomyelitis Virus Vaccines. *Pathogens* **2021**, *10*, 500.
36. Counoupas, C.; Pinto, R.; Nagalingam, G.; Britton, W.J.; Petrovsky, N.; Triccas, J.A. Delta inulin-based adjuvants promote the generation of polyfunctional CD4+ T cell responses and protection against Mycobacterium tuberculosis infection. *Scientific reports* **2017**, *7*, 1-11.
37. Hayashi, M.; Aoshi, T.; Haseda, Y.; Kobiyama, K.; Wijaya, E.; Nakatsu, N.; Igarashi, Y.; Standley, D.M.; Yamada, H.; Honda-Okubo, Y. Advax, a delta inulin microparticle, potentiates in-built adjuvant property of co-administered vaccines. *EBioMedicine* **2017**, *15*, 127-136.
38. Gordon, D.; Kelley, P.; Heinzel, S.; Cooper, P.; Petrovsky, N. Immunogenicity and safety of Advax™, a novel polysaccharide adjuvant based on delta inulin, when formulated with hepatitis B surface antigen: a randomized controlled Phase 1 study. *Vaccine* **2014**, *32*, 6469-6477.
39. Gordon, D.L.; Sajkov, D.; Honda-Okubo, Y.; Wilks, S.H.; Aban, M.; Barr, I.G.; Petrovsky, N. Human Phase 1 trial of low-dose inactivated seasonal influenza vaccine formulated with Advax™ delta inulin adjuvant. *Vaccine* **2016**, *34*, 3780-3786.
40. Gordon, D.L.; Sajkov, D.; Woodman, R.J.; Honda-Okubo, Y.; Cox, M.M.; Heinzel, S.; Petrovsky, N. Randomized clinical trial of immunogenicity and safety of a recombinant H1N1/2009 pandemic influenza vaccine containing Advax™ polysaccharide adjuvant. *Vaccine* **2012**, *30*, 5407-5416.
41. Nolan, T.; Richmond, P.C.; Formica, N.T.; Höschler, K.; Skeljo, M.V.; Stoney, T.; McVernon, J.; Hartel, G.; Sawlwin, D.C.; Bennet, J. Safety and immunogenicity of a prototype adjuvanted inactivated split-virus influenza A (H5N1) vaccine in infants and children. *Vaccine* **2008**, *26*, 6383-6391.
42. Heddle, R.; Smith, A.; Woodman, R.; Hissaria, P.; Petrovsky, N. Randomized controlled trial demonstrating the benefits of delta inulin adjuvanted immunotherapy in patients with bee venom allergy. *Journal of Allergy and Clinical Immunology* **2019**, *144*, 504-513. e516.
43. Petrovsky, N.; Larena, M.; Siddharthan, V.; Prow, N.A.; Hall, R.A.; Lobigs, M.; Morrey, J. An inactivated cell culture Japanese encephalitis vaccine (JE-ADVAX) formulated with delta inulin adjuvant provides robust heterologous protection against West Nile encephalitis via cross-protective memory B cells and neutralizing antibody. *Journal of virology* **2013**, *87*, 10324-10333.
44. Kool, M.; Fierens, K.; Lambrecht, B.N. Alum adjuvant: some of the tricks of the oldest adjuvant. *Journal of medical microbiology* **2012**, *61*, 927-934.
45. Kanesa-Thasan, N.; Putnak, J.; Mangiafico, J.; Saluzzo, J.; Ludwig, G. absence of protective neutralizng antibodies to West Nile virus in subjects following vaccination with Japanese encephalitis or dengue vaccines. *The American journal of tropical medicine and hygiene* **2002**, *66*, 115-116.
46. Tang, F.; Zhang, J.-S.; Liu, W.; Zhao, Q.-M.; Zhang, F.; Wu, X.-M.; Yang, H.; Ly, H.; Cao, W.-C. Failure of Japanese encephalitis vaccine and infection in inducing neutralizing antibodies against West Nile virus, People's Republic of China. *The American journal of tropical medicine and hygiene* **2008**, *78*, 999-1001.
47. Poland, J.D.; Bruce Cropp, C.; Craven, R.B.; Monath, T.P. Evaluation of the potency and safety of inactivated Japanese encephalitis vaccine in US inhabitants. *Journal of Infectious Diseases* **1990**, *161*, 878-882.
48. Halstead, S.B.; Jacobson, J.; Dubischar-Kastner, K. Japanese encephalitis vaccines. *Vaccines, 6th ed.; Plotkin, SA, Orenstein, WA, Offit, PA, Eds* **2012**, 312-351.
49. Kuzuhara, S.; Nakamura, H.; Hayashida, K.; Obata, J.; Abe, M.; Sonoda, K.; Nishiyama, K.; Sugawara, K.; Takeda, K.; Honda, T. Non-clinical and phase I clinical trials of a Vero cell-derived inactivated Japanese encephalitis vaccine. *Vaccine* **2003**, *21*, 4519-4526.
50. Jelinek, T.; Cromer, M.A.; Cramer, J.P.; Mills, D.J.; Lessans, K.; Gherardin, A.W.; Barnett, E.D.; Hagmann, S.H.; Askling, H.H.; Kiermayr, S. Safety and immunogenicity of an inactivated Vero

cell_derived Japanese encephalitis vaccine (IXIARO®, JESPECT®) in a pediatric population in JE non-endemic countries: An uncontrolled, open-label phase 3 study. *Travel medicine and infectious disease* **2018**, *22*, 18-24.

51. Clark, D.C.; Brault, A.C.; Hunsperger, E. The contribution of rodent models to the pathological assessment of flaviviral infections of the central nervous system. *Archives of virology* **2012**, *157*, 1423-1440.
52. Kimura, T.; Sasaki, M.; Okumura, M.; Kim, E.; Sawa, H. Flavivirus encephalitis: pathological aspects of mouse and other animal models. *Veterinary pathology* **2010**, *47*, 806-818.
53. Licon Luna, R.M.; Lee, E.; Müllbacher, A.; Blanden, R.V.; Langman, R.; Lobigs, M. Lack of both Fas ligand and perforin protects from flavivirus-mediated encephalitis in mice. *Journal of virology* **2002**, *76*, 3202-3211.
54. Toriniwa, H.; Komiya, T. Long-term stability of Vero cell-derived inactivated Japanese encephalitis vaccine prepared using serum-free medium. *Vaccine* **2008**, *26*, 3680-3689, doi:10.1016/j.vaccine.2008.04.076.
55. Moi, M.L.; Lim, C.-K.; Chua, K.B.; Takasaki, T.; Kurane, I. Dengue virus infection-enhancing activity in serum samples with neutralizing activity as determined by using FcγR-expressing cells. *PLoS neglected tropical diseases* **2012**, *6*, e1536.