

Research Article

Japanese Encephalitis Vaccine Generates Cross-Reactive Memory T Cell Responses to Zika Virus in Humans

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Received 21 May 2022; Revised 22 October 2022; Accepted 2 November 2022; Published 19 November 2022

Academic Editor: Maoshui Wang

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Objective. Zika virus (ZIKV) and Japanese encephalitis virus (JEV) are mosquito-borne flaviviruses with sequence homology. ZIKV circulates in some regions where JEV also circulates, or where JE vaccination is used. Cross-immunity between flaviviruses exists, but the precise mechanisms remain unclear. We previously demonstrated that T cell immunity induced by the live-attenuated Japanese encephalitis (JE) SA14-14-2 vaccine conferred protective immunity against ZIKV infection in mice, which could even bypass antibody-dependent enhancement. However, the role of T cell immune, especially memory T cell subsets, in cross-reactive immune responses between JE vaccine and ZIKV in humans has not been reported. **Methods.** We examined central and effector memory CD4⁺ and CD8⁺ T cell (T_{CM} and T_{EM}) responses (including degranulation, cytokines, and chemokines) in the presence of JEV and ZIKV, respectively, by using qualified peripheral blood mononuclear cell samples from 18 children who had recently received a two-dose course of JE vaccine SA14-14-2 as well as seven children without JE vaccination. **Results.** Cross-reactive CD8⁺ T_{CM} in response to ZIKV was characterized by secretion of IFN- γ , whereas CD8⁺ T_{EM} did not show significant upregulation of functional factors. In the presence of ZIKV, IFN- γ and TNF- α expression was upregulated by CD4⁺ T_{EM}, and the expression signature of CD4⁺ T_{CM} is more cytotoxic potential. **Conclusions.** We profiled the cross-reactive memory T cell responses to ZIKV in JE vaccine recipients. These data will provide evidence for the mechanism of cross-reactive memory T cell immune responses between JEV and ZIKV and a more refined view of bivalent vaccine design strategy.

1. Introduction

Zika virus (ZIKV) and Japanese encephalitis virus (JEV) belong to the mosquito-borne flaviviruses [1]. Although ZIKV infection is mainly asymptomatic or mildly symptomatic, it can cause Guillain-Barre syndrome and other

neurological syndromes in adults and serious fetal defects such as microcephaly [2, 3]. The epidemic caused by ZIKV has been declining since 2016, but ongoing transmission remains with the attendant risk of severe disease [4]. Reduced case numbers mean that there is still no licensed and available vaccine against ZIKV [5]. JEV is a flavivirus sharing

some biological characteristics with ZIKV, which mostly causes asymptomatic infection or mild disease. However, JEV infection can also progress to acute Japanese encephalitis (JE), with a fatality rate of 20–30%, and 30–50% of recovered patients have sequelae [6]. Compared with the homology between DENV and ZIKV, JEV is more closely related to ZIKV [7, 8]. On average, JEV shares a 56.1% protein sequence identity with ZIKV. JEV is widely distributed in the Asia-Pacific region, in many countries in East, South, and Southeast Asia [9]. Among them, China was once the most affected country by JEV and one of the earliest countries to initiate JE-vaccination program [10].

Cross-reactive T cell responses induced by prior flavivirus exposure or vaccination to heterogeneous flaviviruses remains are widely reported between flaviviruses [11]. Unlike antibodies that can both prevent and enhance the subsequent infection with heterogeneous flaviviruses, the effect of cross-reactive T cell responses may be more inclined to protect against secondary infection [12–15]. With the ZIKV pandemic, the role of immunodominant protection of cross-reactive CD4⁺ and CD8⁺ T cells induced by primary DENV infection in secondary ZIKV infection has been described [13, 16, 17]. However, the degree of cross-reactivity and protective potential is influenced by factors such as the degree of homology, the sequence of infection, and the interval between primary and secondary infection. JEV has a much wider geographic range than DENV, previously for disease and now for vaccination, in China [18]. Since the implementation of the national Expanded Program on Immunization (EPI) in China in 2008, almost all Chinese people have been immunized against JE [19, 20], which prompted researchers to focus on the cross-reactive immune response between JEV and ZIKV. We and other groups have previously characterized the cross-reactive immune response between the two viruses. These studies suggest that the cross-protection is mainly conferred by the JEV-induced T cell response [15, 21]. These conclusions are mostly drawn from experiments in mice. ZIKV-specific cytotoxic CD8⁺ T cells can effectively suppress ZIKV infection [22]. In a model of CD8⁺ T cell adoptive transfer in mice, JE SA14-14-2-vaccination-induced CD8⁺ T cells can bypass or resist the ADE-mediated by cross-reactive antibodies, biasing the pathogenesis protection balance in global ZIKV infection in favor of protection [21]. The CD4⁺ T cells evoked by the JE-vaccination do not serve as the most dominant protective components but trigger Th1/Th2 cytokine generation through recognizing conserved epitopes [23]. Amplified cross-reactive clones boosted subsequent ZIKV vaccine responses, resulting in a higher degree of virus clearance [23]. When mapping the DENV-ZIKV cross-reactive CD4⁺ T cell response, it was observed that Th1-type cytokines played a more prominent role in inhibiting ZIKV replication [13].

The live-attenuated vaccine SA14-14-2 and the inactivated vaccine (JE-VC/IXIARO) and chimeric vaccine (ChimeriVax-JE) derived from SA14-14-2 strain are widely used and studied worldwide [24, 25]. Given the potential for T cells to mediate a cross-protective response, coupled with the fact that ZIKV is still circulating or potentially at risk of

spreading [26], understanding the cross-reactive T cell response in children vaccinated with SA14-14-2 against ZIKV will be critical in assessing its potential to protect against ZIKV infection. Such data will also provide important clues for the development of a bivalent vaccine against JEV/ZIKV aimed at inducing a robust T cell response, with good safety profile (avoiding ADE responses) and an effective T cell response. Therefore, in this study, we used peripheral blood mononuclear cell (PBMC) samples collected from children who were vaccinated with two doses of JE vaccine SA14-14-2 to detect cross-reactive central memory (T_{CM}) and effector memory (T_{EM}) to ZIKV among CD4⁺ and CD8⁺ T cell memory T lymphocyte responses including cytokine secretion and degranulation upon ZIKV antigen stimulation, respectively [27–29].

2. Materials and Methods

2.1. Ethical Approval. Written informed consent in Chinese was obtained from all guardians of vaccinated children before enrollment, and the ethical approval was given by Beijing Children's Hospital, Capital Medical University (approval number: 2020-k-85). All procedures performed were in accordance with the Declaration of Helsinki. The study was explained in detail, and a section of the consent granted the investigators' permission for possible future use of the serum and PBMC samples.

2.2. Study Cohort. In total, we took peripheral venous blood samples and separated sera and PBMCs from 18 apparently healthy children (2 years old) who had previously received a prime and boost vaccination with live-attenuated JE SA14-14-2 vaccine for less than half a year from Jan through Feb 2022. Seven unvaccinated children's (6 months old) PBMCs were used as system controls. None of the subjects had visited an area where ZIKV was endemic and had no history of seeking medical attention for symptomatic ZIKV infection. The sex and age of study individuals are shown in Table S1. Before we analyzed the induction of JEV-specific and ZIKV cross-reactive CD4⁺ or CD8⁺ memory T cells among JE vaccinated individuals, hemogram parameters were analyzed in an automatic analyzer (Lifotronic Technology Co., Ltd.) within one hour after the blood samples were taken. There were no individuals with elevated C-reactive protein above the threshold. Seroconversion was confirmed by both enzyme-linked-immune-sorbent assay (ELISA) and plaque reduction neutralization test (PRNT).

2.3. ELISA. The presence of JEV-specific IgG antibodies was measured by using an indirect ELISA kit (Shanghai B&C Biological Technology, China) according to the manufacturers' instructions, and it was previously described [20, 30]. Briefly, serum samples were diluted at 1:41 dilution with buffer that goes with the kit. The diluted test sera and control samples (100 μL/well) were added to each well and incubated at 37°C for half an hour, followed by five washes. Then, 100 μL of horseradish peroxidase-conjugated mouse anti-human IgG monoclonal antibody was added to each well at

37°C for half an hour. The initiation of the peroxidase reaction occurred after incubation at 37°C for 15 min in the dark. The reaction was then halted by the addition of 50 μ L of 2 M sulfuric acid per well. The result was represented as optical density, which was read at 450 nm using an ELISA plate reader (Thermo, USA). The cut-off value was calculated based on the manufacturers' instructions. The optical density of recipients' sera greater than the cut-off value was considered positive.

2.4. Cell and Lines Viruses. C6/36 cells and Vero cells were used for virus propagation and PRNT, respectively. Vero cells were cultured in the MEM medium containing 5% fetal bovine serum; C6/36 cells were cultured in the RPMI-1640 medium containing 10% fetal bovine serum. JEV (Beijing-1 strain) and ZIKV (SMGC_1 strain) were propagated in C6/36 cells and stored in a -80°C freezer. The virus was inactivated by UV irradiation for 1 h. Inactivated viral particles were harvested from the culture supernatant of C6/36 cells that had been infected by JEV or ZIKV, concentrated by 8% polyethylene glycol precipitation and then purified from clarified extracts by ultracentrifugation at $100,000 \times g$ for 3 h at 4°C .

2.5. PRNT. The PRNT is considered as gold standard for detecting neutralizing antibodies (nAbs) against flaviviruses after vaccination or natural infection [20]. Seroconversion of nAbs is an indicator that flavivirus vaccine-induced immune protection has been successfully established. Heat inactivated sera were two-fold serially diluted from 1 : 10 to 1 : 160. The diluted serum was mixed with an equal volume of 100 plaque forming units (PFU) of JEV and incubated for 1 h at 37°C . The mixture was incubated with Vero cells for 1 h. Cells with removal of the inoculum were cultured under the MEM overlay medium and visualized by crystal violet staining. PRNT₅₀ was defined as the reciprocal of the highest serum dilution that produced a 50% reduction in mean plaque number serum compared to control wells containing virus alone. With reference to the guideline, PRNT₅₀ titers $\geq 1 : 10$ are considered positive [31].

2.6. Ex Vivo Intracellular Staining (ICS). Venous blood samples were collected in EDTA-K₂-anticoagulated tubes. PBMCs were isolated by using lymphocyte separation medium density gradients (Dakewe Biotech Co., Ltd., China) [28]. ICS was performed as described previously [29, 32, 33]. Briefly, a total of 3×10^6 freshly extracted PBMCs were divided into three equal parts with 1×10^6 cells, and the two parts were stimulated with concentrated inactivated JEV or ZIKV particles at a final concentration of 1×10^5 PFU/mL in a final volume of 500 μ L (MOI = 0.1) for 16 h at 37°C in the presence of 1 μ g/mL monoclonal antibodies CD28 (clone: CD28.2) and CD49d (clone: 9F10), GolgiPlug, monensin, and surface stained with BV605-anti-CD107a (clone: H4A3). Dead cells were labeled using Zombie NIR™ Fixable Viability Kit. Surface markers, including BV650-anti-CD3 (clone: SK7), BUV395-anti-CD4 (clone: SK3), BV421-

anti-CD8 (clone: SK1), BUV737-anti-CD27 (clone: L128), and BV480-anti-CD45RO (clone: UCHL1) were stained. Cells were then washed, fixed with Cytofix/Cytoperm™ Fixation/Permeabilization Solution (BD Biosciences, USA), and stained with FITC-anti-IFN- γ (clone: 4S.B3), PE-anti-TNF- α (clone: MAb11), BV785-anti-IL-2 (clone: MQ1-17H12), and APC-anti-MIP-1 α (clone: W16009B). The remaining part of PBMCs as negative controls without concentrated virus particles stimulation but combined with CD28 and CD49d were run for each sample. All reagents were from BioLegend (USA) unless otherwise stated. All samples were acquired on a BD FACSymphony™ (BD Biosciences, USA) flow cytometer and analyzed using FlowJo version 10 software (TreeStar, USA). Cytokine responses were background subtracted individually before further analysis.

2.7. Statistical Analysis. Statistical analysis was performed with SPSS Statistics version 17.0 (SPSS Software Inc., USA), and the figures were made with GraphPad Prism version 6 (GraphPad Software Inc., USA). The Mann-Whitney *U* test or Kruskal-Wallis test (for multiple comparisons) were used to compare variables between two groups. The chi-square test was used to assess differences in the composition ratio of pluripotent T_{PF} between stimuli. Statistical significance was set at **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. All of the tests were two tailed.

3. Results

3.1. Complete Blood Count, IgG, and nAb Results. After preliminary testing of blood and isolated serum samples, complete blood count results of the 16 vaccinated children were all within the reference interval. Both IgG binding and nAb antibody measurement against JEV showed seroconversion, following two doses of SA14-14-2 vaccine among these children (Table 1).

3.2. Memory CD8⁺ T Cells. To characterize and compare the functional response profiles of JEV-specific and cross-reactive CD8⁺ T cells to ZIKV in vaccinated children, we assessed the frequency, function, and the memory phenotype of memory CD8⁺ T cells by multicolor flow cytometry. The gating strategy is shown in Figure S1. For this analysis, we defined central memory CD8⁺ T cells as CD27⁺ CD45RO⁺ and effector memory CD8⁺ T cells as CD27⁻ CD45RO⁺, respectively [34]. We found that a large fraction of CD8⁺ T_{CM} cells in vaccinated children expressed CD107a ($1.53\% \pm 0.54\%$ vs. $1.03\% \pm 0.53\%$, *P* < 0.01) and IFN- γ ($1.28\% \pm 0.53\%$ vs. $0.35\% \pm 0.19\%$, *P* < 0.001) under JEV stimulation compared with controls, and higher expression of TNF- α ($0.47\% \pm 0.30\%$ vs. $0.30\% \pm 0.19\%$, *P* < 0.05) and IL-2 ($1.20\% \pm 0.85\%$ vs. $0.47\% \pm 0.23\%$, *P* < 0.01) were also detected, whereas MIP-1 α did not show a significant increase ($0.28\% \pm 0.32\%$ vs. $0.11\% \pm 0.15\%$, *P* > 0.05, Figure 1). The frequencies of CD107a, IFN- γ , TNF- α , and IL-2 positive cells were higher after JEV stimulation than after ZIKV stimulation (*P* < 0.05). When pulsed with ZIKV, the responses of cross-reactive CD8⁺ T_{CM} in vaccinated children

TABLE 1: Results of complete blood count and JEV antibody testing/titration of subjects in this study.

No.	Parameter												
	CRP (mg/L)	WBC count ($\times 10^9/L$)	RBC count ($\times 10^{12}/L$)	Hgb (g/L)	Plt count ($\times 10^9/L$)	NEUT count ($\times 10^9/L$)	LYMPH count ($\times 10^9/L$)	MONO count ($\times 10^9/L$)	EO count ($\times 10^9/L$)	BASO count ($\times 10^9/L$)	JEV IgG	JEV nAb	
Reference interval (2 y-<6 y)		4.4-11.9	4.0-5.5	112-149	188-472	1.2-7	1.8-6.3	0.12-0.93	0-0.68	0-0.1			
VAC 1	<8	5.94	4.21	120	274	1.49	3.92	0.38	0.13	0.02	+	1:80	
VAC 2	<8	7.13	4.13	116	372	3.78	2.64	0.53	0.13	0.05	+	1:160	
VAC 3	<8	9.84	4.46	119	348	6.93	2.41	0.49	0	0.01	+	1:20	
VAC 4	<8	7.97	4.57	126	351	2.52	4.79	0.57	0.06	0.03	+	>1:160	
VAC 5	<8	5.62	4.3	122	312	1.68	3.33	0.38	0.17	0.06	+	1:80	
VAC 6	<8	6.87	4.7	126	400	1.62	4.54	0.55	0.13	0.03	+	1:40	
VAC 7	<8	6.53	4.38	123	253	1.48	4.21	0.73	0.1	0.01	+	1:80	
VAC 8	<8	5.29	4.15	120	275	1.64	3.1	0.38	0.14	0.03	+	1:80	
VAC 9	<8	6.65	4.5	131	338	2.09	3.67	0.74	0.12	0.03	+	1:80	
VAC 10	<8	8.53	4.9	129	371	3.63	4.32	0.43	0.11	0.04	+	1:80	
VAC 11	<8	7.87	4.97	134	359	3.15	3.99	0.55	0.15	0.03	+	1:160	
VAC 12	<8	5.39	4.67	123	226	2.78	2.2	0.33	0.06	0.02	+	1:40	
VAC 13	<8	6.08	4.9	129	304	1.51	3.77	0.47	0.3	0.03	+	1:80	
VAC 14	<8	7.09	4.14	113	361	3.24	3.09	0.55	0.18	0.03	+	>1:160	
VAC 15	<8	8.49	5.01	141	281	2.14	5.73	0.42	0.16	0.04	+	1:20	
VAC 16	<8	8.46	4.41	116	326	3.66	4.25	0.53	0.01	0.01	+	1:80	
VAC 17	<8	8.6	4.85	130	271	1.77	6.22	0.51	0.06	0.04	+	1:160	
VAC 18	<8	10.88	5.05	134	313	5.36	5.6	0.66	0.17	0.09	+	>1:160	
Mean \pm standard	—	7.4 \pm 1.55	4.57 \pm 0.32	125.11 \pm 7.36	324.17 \pm 60.26	2.8 \pm 1.49	3.99 \pm 1.12	0.51 \pm 0.12	0.12 \pm 0.07	0.03 \pm 0.02			
Reference interval (28 d-<6 m)		4.3-14.2	3.3-5.2	97-183	183-614	0.6-7.5	2.4-9.5	0.15-1.56	0.07-1.02	0-0.1			
UNV 1	<8	9.87	3.54	99	292	4.79	3.9	1.02	1.02	0.07	-	<1:10	
UNV 2	<8	8.62	4.65	138	257	6.92	3.97	0.72	0.08	0.01	-	<1:10	
UNV 3	<8	11.5	5.1	169	377	5.62	5.81	1.23	0.38	0.03	-	<1:10	
UNV 4	<8	9.47	3.65	125	405	2.6	4	1.08	0.77	0.02	-	<1:10	
UNV 5	<8	10.95	4.92	172	302	4.7	3.96	1.01	0.36	0.04	-	<1:10	
UNV 6	<8	6.4	3.57	109	258	0.67	3.86	0.6	0.26	0.01	-	<1:10	
UNV 7	<8	5.87	3.88	112	247	1.50	3.61	0.56	0.08	0.02	-	<1:10	
Mean \pm standard	—	8.95 \pm 2.15	4.19 \pm 0.68	132 \pm 29.07	305.43 \pm 62.23	3.83 \pm 2.29	4.16 \pm 0.74	0.89 \pm 0.26	0.42 \pm 0.35	0.03 \pm 0.02			

BASO: basophil, CRP: C-reaction protein, EO: eosinophil, Hgb: hemoglobin, LYMPH: lymphocyte, MONO: monocyte, NEUT: neutrophil, Plt: platelet, RBC: red blood cell, UNV: unvaccinated individuals, VAC: vaccinated individuals, and WBC: white blood cell.

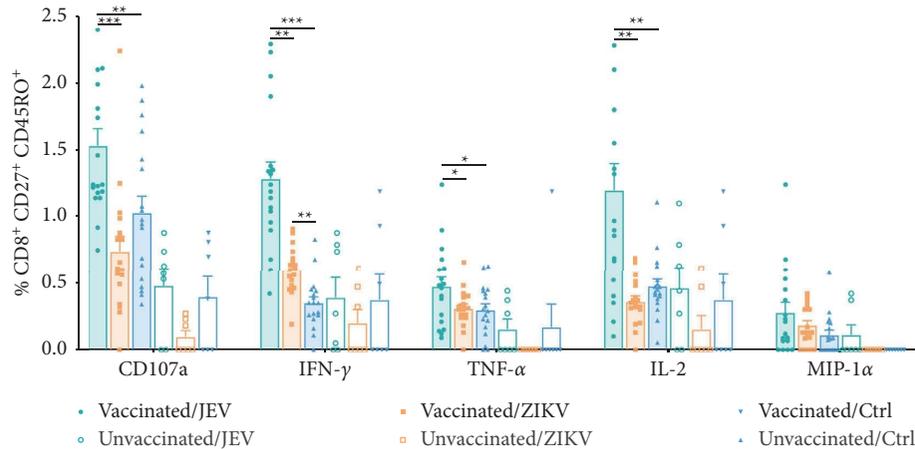


FIGURE 1: Multifunctional characterization of $CD8^+$, $CD27^+$ $CD45RO^+$ central memory T cell (T_{CM}) responses to JEV or ZIKV. $CD8^+$ T_{CM} subpopulations were gated on cells expressing at least one of the five T cell functions analyzed, and the frequency of cells expressing any of the five cell functions was assessed. Vaccinated, $n=18$; unvaccinated, $n=7$. Results are expressed as mean \pm SD. Differences between unmatched groups were compared using an unpaired t -test, the Mann–Whitney U test, or the Kruskal–Wallis rank-sum test with Dunn’s post hoc test for multiple comparisons. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

were significantly different from that of the control group in IFN- γ ($0.60\% \pm 0.17\%$ vs. $0.35\% \pm 0.19\%$, $P < 0.01$) but not in other indicators. T_{EM} cells are thought to exert antiviral effects directly upon restimulation with JEV. JEV-specific IFN- γ ($1.23\% \pm 0.64\%$ vs. $0.37\% \pm 0.30\%$, $P < 0.001$) positive cells were of dominant type responding to JEV, compared with those of the control group (Figure 2). Also, IL-2 ($0.95\% \pm 0.95\%$ vs. $0.23\% \pm 0.19\%$, $P < 0.01$) positive cells were detected with higher frequency after JEV stimulation. However, significantly elevated CD107a ($1.35\% \pm 1.35\%$ vs. $0.96\% \pm 1.45\%$, $P > 0.05$), TNF- α ($0.47\% \pm 0.46\%$ vs. $0.23\% \pm 0.32\%$, $P > 0.05$), and MIP-1 α ($0.17\% \pm 0.36\%$ vs. $0.05\% \pm 0.10\%$, $P > 0.05$) were not detected. Responding cells expressing CD107a ($0.75\% \pm 0.66\%$ vs. $0.96\% \pm 1.45\%$, $P > 0.05$), IFN- γ ($0.51\% \pm 0.27\%$ vs. $0.37\% \pm 0.30\%$, $P > 0.05$), TNF- α ($0.20\% \pm 0.22\%$ vs. $0.23\% \pm 0.32\%$, $P > 0.05$), and IL-2 ($0.24\% \pm 0.23\%$ vs. $0.23\% \pm 0.19\%$, $P > 0.05$) were not significantly elevated after ZIKV stimulation. The level of IFN- γ in ZIKV cross-reactive T_{EM} was lower than in the JEV group ($0.51\% \pm 0.27\%$ vs. $0.37\% \pm 0.30\%$, $P < 0.01$). The commonality is that neither JEV nor ZIKV can stimulate $CD8^+$ T_{EM} to secrete MIP-1 α . These results suggest that only cross-reactive $CD8^+$ T_{CM} is activated by ZIKV.

3.3. Memory $CD4^+$ T Cells. The role of $CD4^+$ T cells in against flavivirus infection is also important. The simultaneous activation of memory $CD4^+$ T cells and $CD8^+$ T cells is the ideal cellular immune response. Therefore, we analyzed whether memory $CD4^+$ T cell function could be cross-reactively evoked by ZIKV. Similar to $CD8^+$ T_{CM} , the proportion of IFN- γ^+ and CD107a $^+$ cells among the five functional subsets were higher in the presence of JEV (Figure 3). Compared with the control group, JEV antigen successfully induced higher levels of CD107a ($0.78\% \pm 0.42\%$ vs. $0.22\% \pm 0.18\%$, $P < 0.001$), IFN- γ ($1.09\% \pm 0.49\%$ vs. $0.22\% \pm 0.10\%$, $P < 0.001$), TNF- α ($0.41\% \pm 0.17\%$ vs. $0.14\% \pm 0.07\%$, $P < 0.001$), and IL-2

($0.51\% \pm 0.39\%$ vs. $0.17\% \pm 0.11\%$, $P < 0.001$). Surprisingly, ZIKV stimulation appeared to trigger the expression of the CD107a, IFN- γ , and TNF- α and productions of CD107a ($0.48\% \pm 0.20\%$ vs. $0.22\% \pm 0.18\%$, $P < 0.01$), IFN- γ ($0.61\% \pm 0.15\%$ vs. $0.22\% \pm 0.10\%$, $P < 0.01$), and TNF- α ($0.32\% \pm 0.14\%$ vs. $0.14\% \pm 0.07\%$, $P < 0.01$). No significant increase in MIP-1 α was detected in neither in JEV-specific nor in ZIKV cross-reactive $CD4^+$ T_{CM} compared to the control group. It can be seen that $CD4^+$ T_{CM} produced a broader cross-reactive cytokine profile after ZIKV stimulation than $CD8^+$ T_{CM} . $CD4^+$ T_{EM} cells in the JEV group expressed high levels of CD107a ($1.09\% \pm 0.94\%$ vs. $0.41\% \pm 0.35\%$, $P < 0.01$), IFN- γ ($1.25\% \pm 0.53\%$ vs. $0.27\% \pm 0.14\%$, $P < 0.001$), TNF- α ($0.42\% \pm 0.36\%$ vs. $0.16\% \pm 0.12\%$, $P < 0.01$), and IL-2 ($0.57\% \pm 0.31\%$ vs. $0.17\% \pm 0.10\%$, $P < 0.01$) than those in the control group (Figure 4). As was the case for $CD8^+$ T_{EM} cells, MIP-1 α was also not expressed following stimulation with either virus. Notably, IFN- γ ($0.64\% \pm 0.14\%$ vs. $0.27\% \pm 0.14\%$, $P < 0.001$), TNF- α ($0.35\% \pm 0.28\%$ vs. $0.16\% \pm 0.12\%$, $P < 0.05$), and IL-2 ($0.33\% \pm 0.18\%$ vs. $0.17\% \pm 0.10\%$, $P < 0.05$) in $CD4^+$ T_{EM} were detected after stimulation with ZIKV, when compared with control, although the frequencies of IFN- γ and IL-2 in the ZIKV group were still lower than that in the JEV group ($P < 0.05$). MIP-1 α was not significantly increased upon stimulation with either JEV or ZIKV ($P > 0.05$).

4. Discussion

The role of T cell-mediated adaptive immune system in controlling viral infection should be of interest [35]. In addition to nAbs, T cells play an important role in host defense against viruses. As well as helping antibody responses, $CD4^+$ T cells also aid in the initiation of cytotoxic T cells, the generation and maintenance of memory $CD8^+$ T cells, as well as direct killing of target cells. $CD8^+$ T cells can clear viruses from infected tissues by killing infected cells. For the optimal vaccine design, simultaneous activation of

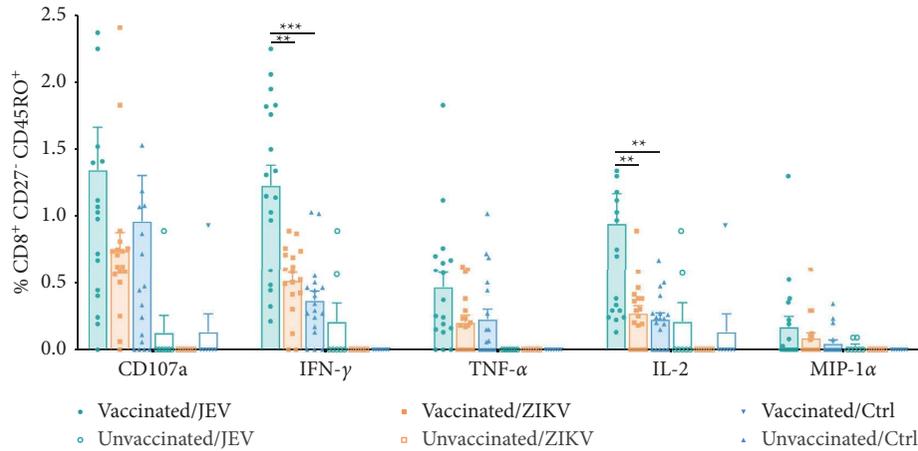


FIGURE 2: Multifunctional characterization of $CD8^+$ $CD27^-$ $CD45RO^+$ effector memory T cell (T_{EM}) responses to JEV or ZIKV. $CD8^+$ T_{EM} subpopulations were gated on cells expressing at least one of the five T cell functions analyzed, and the frequency of cells expressing any of the five cell functions was assessed. Vaccinated, $n = 18$; unvaccinated, $n = 7$. Results are expressed as mean \pm SD. Differences between unmatched groups were compared using an unpaired t -test, the Mann–Whitney U test, or the Kruskal–Wallis rank-sum test with Dunn’s post hoc test for multiple comparisons. ** $P < 0.01$; *** $P < 0.001$.

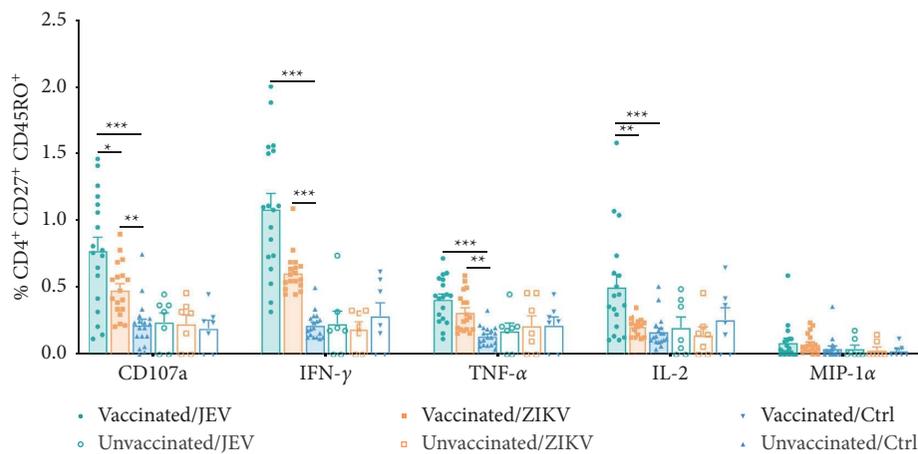


FIGURE 3: Multifunctional characterization of $CD4^+$ $CD27^+$ $CD45RO^+$ central memory T cell (T_{CM}) responses to JEV or ZIKV. $CD4^+$ T_{CM} subpopulations were gated on cells expressing at least one of the five T cell functions analyzed, and the frequency of cells expressing any of the five cell functions was assessed. Vaccinated, $n = 18$; unvaccinated, $n = 7$. Results are expressed as mean \pm SD. Differences between unmatched groups were compared using an unpaired t -test, the Mann–Whitney U test, or the Kruskal–Wallis rank-sum test with Dunn’s post hoc test for multiple comparisons. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

$CD4^+$ and $CD8^+$ T cells is an ideal strategy for vaccine-induced cellular immunity.

An earlier study reported that DENV-specific $CD8^+$ and $CD4^+$ T cells could produce IFN- γ upon flavivirus stimulation and lyse infected target cells [36]. Indeed, evidence accumulated from our group and other group’s studies in mouse models suggests that T cells are actually protective against the flavivirus infection in both infection and vaccination settings, both in specific and cross-reactive responses [15, 32, 37, 38]. The immunization of immunodominant $CD8^+$ T cell epitopes of DENV can improve viral clearance and protection during primary DENV infection [39]. $CD8^+$ T cells can even confer protection from ADE-mediated infection with DENV and ZIKV in mice [21, 40, 41]. The protective effect of $CD4^+$ T cells against

flaviviruses has been clearly demonstrated in a mouse model [42]. Protective and long-lived immunity is closely related to the production of $CD4^+$ T cells [43], which includes cytokine production, recruitment and activation of innate immune cells, enhancement of $CD8^+$ T cell responses, promotion of immune memory, and direct cytotoxicity to infected cells [44]. Although some studies indicate that $CD4^+$ T cells are not required for the control of primary DENV infection, their induction by epitope immunization nevertheless contributes to virus clearance and reduces tissue viral burden [38].

In humans, the exact role of JEV-induced T cells in preventing ZIKV infection and pathogenesis is unclear. We found that JEV–ZIKV cross-reactive T cells were detected in PBMC samples from children vaccinated with JEV, similar

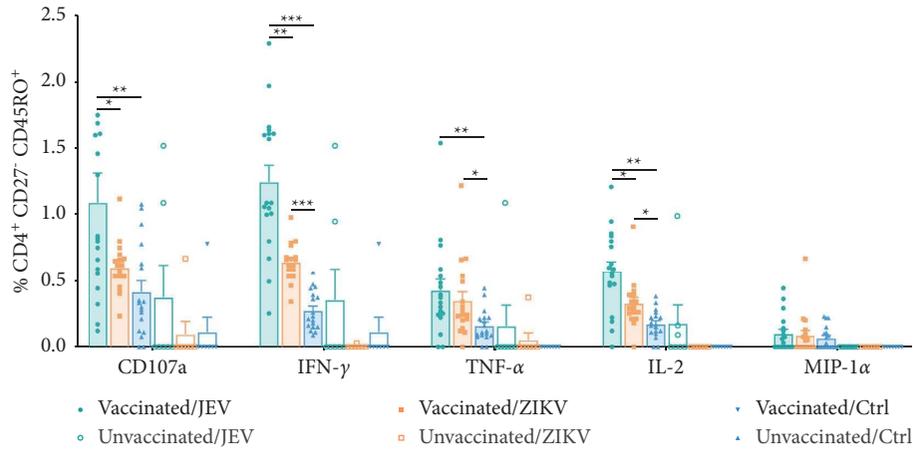


FIGURE 4: Multifunctional characterization of CD4⁺ CD27⁻ CD45RO⁺ effector memory T cell (T_{EM}) responses to JEV or ZIKV. CD4⁺ T_{EM} subpopulations were gated on cells expressing at least one of the five T cell functions analyzed, and the frequency of cells expressing any of the five cell functions was assessed. Vaccinated, $n = 18$; unvaccinated, $n = 7$. Results are expressed as mean \pm SD. Differences between unmatched groups were compared using an unpaired t -test, the Mann–Whitney U test, or the Kruskal–Wallis rank-sum test with Dunn’s post hoc test for multiple comparisons. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

to our results in mice, and reported that these cells responded after restimulation *in vitro*. JEV–ZIKV cross-reactive CD8⁺ T_{CM} is only IFN- γ -producing upon ZIKV stimulation, but this cytokine appears to be critical in cross-protection in the mouse model. ZIKV cross-reactive CD8⁺ T_{EM} did not have detectable potential for cytotoxic and chemotactic activity. Here, in terms of CD4 memory T cells, we showed that among those who received two doses of SA14-14-2 vaccine, peripheral CD4⁺ T_{CM} and T_{EM} cells were characterized by the expression of three markers following ZIKV stimulation. Normally, T_{CM} are highly sensitive to antigenic stimulation, while the dependence on costimulatory signals is reduced. After homing to the T cell area of secondary lymphoid organs, T_{CM} cells present reactive memory and proliferate rapidly. They have almost no effector function but can proliferate stably and differentiate into effector T cells in the presence of antigen [45]. T_{CM} cells mainly produce IL-2, and a small amount of IFN- γ and perforin through T cell receptor signaling [46]. In this study, we observed a similar polyfunctional feature in cross-reactive CD4⁺ T_{CM} to that in the JEV group itself, with a high level of CD107a, IFN- γ , and TNF- α , indicating an important role for CD4⁺ T_{CM} in cross-reactive T cell responses. Cross-reactive CD4⁺ T_{EM} mainly expresses the markers IFN- γ , TNF- α , and IL-2. Studies have shown that immunity generated by flaviviruses sharing the CD4⁺ T cell epitope promotes protection during subsequent heterologous infection [12], which is speculated to be mediated by the NS3 protein [47, 48].

T cells express two or more of the above five markers, namely, polyfunctional T cells (T_{PF}). We measured the coexpression of more than two markers; however, we found that the frequency of induction of ZIKV cross-reactive T_{PF} by JEV-vaccination was low. We detected two or more cytokine repertoires only in CD4⁺ T_{CM} and T_{EM} but not in CD8⁺ T_{CM} and T_{EM}. In the CD4⁺ T_{CM} of JEV-vaccinated individuals, the frequency of the IFN⁺ TNF⁺ population was

0.02%, 0.01%, and 0.01% in the JEV-specific, ZIKV cross-reactive, and unstimulated groups, respectively, without differences across groups; in CD4⁺ T_{EM}, the frequency of IFN⁺ TNF⁺ population was 0.04%, 0.01%, and 0% in these three groups, respectively.

It should be noted that existing anti-JEV antibody tests cannot completely rule out isolated ZIKV infection, as the available kits do have partial cross-reactivity to ZIKV in specificity, albeit at a very low level. In addition, the cases of latent infection with ZIKV have been detected in the population of Guangxi Province, a border province in southern China [26], bringing some uncertainty to the immune background of the study individuals in this study. However, we took into account the following three points: (1) the existing reported local cases of ZIKV infection were in border provinces in southern China but not yet prevalent in northern China, and these subjects did not travel outside of China in those ZIKV endemic areas; (2) they did not travel to the Chinese provinces (Yunnan and Guizhou) where ZIKV was detected in wild mosquitoes but no domestic ZIKV cases were reported; and (3) theoretically, it is unlikely that the level of T cell immune response caused by primary infection with ZIKV is lower than that of the cross-reaction elicited by JEV vaccination. Moreover, the COVID-19 pandemic poses considerable difficulties in the availability of larger sample sizes; thus, larger sample sizes would be beneficial to the firmness of the aforementioned conclusions.

Two issues to be considered in future investigations are explained. (1) To ensure that immune responses restricted by different HLA alleles and different species of JEV vaccines are adequately represented in JEV–ZIKV cross-reactive T cell response studies, the subjects of this study were all individuals vaccinated with the live-attenuated SA14-14-2 vaccine, but did not include individuals vaccinated with inactivated vaccines or recombinant chimeric vaccines, whose immune characteristics were different [49]. (2) The breadth of the immunodominant T cell epitope repertoire

needs to be investigated, which has implications for the vaccine design, cross-reactivity, and immune escape by cross-reactive immune response. T cell epitopes of flaviviruses are generally conserved [43], and there are very few instances of T cell epitopes causing acute infections in viral escape (such as those caused by flaviviruses). In contrast, viruses that drive the progression of chronic viral infection evade T cell epitope recognition, which is due to a fundamental difference in selection pressure [50]. Given the importance of T cells in cross-reactive immune responses, boosting T cell responses to improve vaccine efficacy is desirable. This can be achieved by generating broad flavivirus cross-reactive T cell responses by sequential immunization against flaviviruses that share T cell epitopes.

5. Conclusions

In conclusion, we enrich our current understanding of how T cells induced in JEV-vaccinated children cross-react with ZIKV through experiments, and we put forward that the role of JEV-specific and ZIKV cross-reactive T cells in the infection control may be the strategy for the development of bivalent vaccines that induce dual protection with safety and efficacy. Further expansion of these findings will significantly improve our understanding of T cell function and highlight the potential clinical benefit of incorporating JEV-ZIKV cross-reactive T cell epitopes into experimental vaccine formulations to improve cellular immune responses.

Data Availability

The data used to support the findings are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ran Wang and Meng Zhang contributed equally to this study.

Acknowledgments

RW was supported by Beijing Natural Science Foundation, China (No. 7222059), National Natural Science Foundation of China (No. 82002130), and the Open Research Fund Program of the State Key Laboratory of Virology of China (No. 2021IOV002). XZ was supported by the CAMS Innovation Fund for Medical Sciences (No. 2019-I2M-5-026). The authors are grateful to Dr. Lance Turtle (University of Liverpool) for helping with analyzing the data and revising the manuscript.

Supplementary Materials

Table S1: the sex and age of study individuals. Figure S1: gating strategy. (*Supplementary Materials*)

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