Evaluation of porcine T lymphocytes following West Nile virus vaccination

by

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Abstract

Japanese encephalitis virus (JEV) is a foreign zoonotic flavivirus of significant concern to the United States (U.S.) swine industry. Currently, there are no approved veterinary vaccines or treatments for JEV in the United States. In contrast, West Nile virus (WNV), a related flavivirus and zoonotic pathogen of concern, does have approved commercial equine vaccines available. Within the *Flaviviridae* family, some cross-reactivity exists between these viruses, as JEV and WNV share similar structural proteins that often serve as vaccine targets. Cross-neutralizing antibodies between flaviviruses have been documented in both humans and animals following vaccination or natural infection. However, the immune response against JEV infection in swine remains poorly understood, and the mechanisms of protective immunity following vaccination have yet to be investigated. To evaluate potential cross-reactive T-cell immunity, we analyzed CD4+, CD8+, and CD4+CD8+ double-positive T lymphocyte populations within peripheral blood mononuclear cells (PBMCs) isolated from swine vaccinated with commercial equine WNV vaccines. PBMCs were stimulated *in vitro* with WNV and JEV, and T cell activation was assessed through phenotypic characterization of naïve, central memory, and effector memory subsets. We then assessed the action of these memory T cells by measuring IFNγ expression. An observed increase in IFNγ expression in response to JEV stimulation suggests that these T cell populations could be capable to contributing to protective immunity against JEV through the IFNγ upregulation of defensive immune mechanisms. The generation of cross-reactive immunity from WNV vaccination could enable leveraging available commercial WNV vaccines to provide cross-protection against JEV in the event of JEV introduction into U.S. swine populations.

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Chapter 1 - An Introduction to Orthoflaviviruses and Cellular Immune Response

Orthoflaviviruses

Orthoflaviviruses are a genus of enveloped positive single-stranded RNA viruses within the family *Flaviviridae*. Viruses in this genus are transmitted primarily by arthropod vectors, such as mosquitoes. Orthoflaviviruses infect a range of vertebrate hosts, including humans, birds, horses, pigs, rodents, and bats. This broad host range makes them zoonotic pathogens of public health concern. Many orthoflaviviruses involve animal reservoirs in their transmission cycles; mosquitoes feed on these animals and can infect humans during subsequent blood meals (Marino et al., 2025; Monath, 1988). The genus includes several disease-causing arboviruses, such as West Nile virus (WNV), Japanese encephalitis virus (JEV), dengue virus (DENV), yellow fever virus, and Zika virus (ZIKV). Clinical outcomes range from asymptomatic infection to neurological or hemorrhagic disease (Gaunt et al., 2001). Orthoflavivirus infections are increasing, with an estimated 60 million cases reported in 124 countries and territories in 2021, a 1.12-fold rise from 2011 (Liang & Dai, 2024). This underscores the importance of a One Health approach in preventing infections in both humans and animals through vaccination strategies.

Genomic structure

The orthoflavivirus genome encodes three structural proteins: the capsid, the pre-membrane, and the envelope (E). It also encodes seven non-structural proteins (NS): NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Dey et al., 2021). The Japanese encephalitis virus serocomplex includes flaviviruses with morphological heterogeneity and are grouped based on cross reactive antibodies. This cross-reactivity can affect innate and adaptive immunity. JEV and

WNV are a part of this serogroup. The gene encoding the E protein is one of the most conserved regions across Orthoflaviviruses (Khare & Kuhn, 2022). This region, along with the non-structural proteins, could be targeted to develop cross-reactive antibodies and T cells.

West Nile virus

WNV was first identified in Uganda in 1937 (Lanciotti et al., 1999). It has since emerged as a major public health threat. The virus was first introduced into North America in 1999 (Lanciotti et al., 1999). It has contributed to sporadic severe outbreaks and is now the leading cause of arboviral neuroinvasive disease in North America (Brinton, 2002). The virus is transmitted within an enzootic cycle between *Culex* species mosquitoes and viremic birds. Humans and horses are incidental dead-end hosts. They can develop disease, but cannot pass the virus back to mosquitos (Petersen et al., 2013). WNV can cause a range of symptoms in humans. These include high fever, chills, malaise, headache, joint pain, nausea, vomiting, diarrhea, cough, and sore throat (Gould & Solomon, 2008). Typically, individuals remain asymptomatic. However, at-risk populations such as the elderly and children are more susceptible to developing symptoms or severe infections. Around 1% of infected individuals will develop neurological symptoms. These can progress to encephalitis, meningitis, and West Nile poliomyelitis (Sejvar, 2016). The fatality rate of patients experiencing neurological symptoms is estimated to be 5 to 10% (Gould & Solomon, 2008).

WNV is also an important veterinary pathogen for horses. Affected horses may exhibit fever, incoordination, stumbling, weakness, muscle twitching, seizures, head drooping, grinding teeth, and abnormal sensitivity to touch or sound. In severe cases, neurological involvement

manifests as limb ataxia and recumbency. Reported mortality rates for naïve horses diagnosed with WNV range from 22 to 44% (Estell, 2025).

Japanese encephalitis virus

JEV was first isolated in 1935 from the brain of an individual who succumbed to encephalitis in Japan (Webster 1938). Since then, JEV has been endemic to Asia and the Western Pacific. The virus primarily transmits within an enzootic cycle involving birds and swine as amplifying hosts and mosquito vectors such as *Culex* species (Lord et al., 2015). Japanese Encephalitis (JE) caused by JEV infection is the leading cause of viral encephalitis in Asia (Campbell et al., 2011). In 2011, an estimated 68,000 JE cases occurred annually in 24 JE endemic countries (Campbell et al., 2011). Historically the virus remained geographically confined to eastern, southeastern, and south Asia and the Western Pacific until the late 20th century. During the 1990s, JEV spread into countries such as Pakistan in 1992 (Igarashi et al., 1994) and Australia in 1995 (Ewald et al., 1996). In Australia, during the original outbreak, the virus never established into mainland and was not detected in mosquitoes since the early 2000s (van den Hurk et al., 2019). However, after more than two decades, the virus reemerged and was detected in mainland Australia during an outbreak in 2022. That outbreak involved both human and swine cases, with over 40 human cases and 73 piggeries affected (Williams et al., 2022). This recent outbreak demonstrates the virus can reemerge. The virus has not been detected in the US making it a foreign disease of concern.

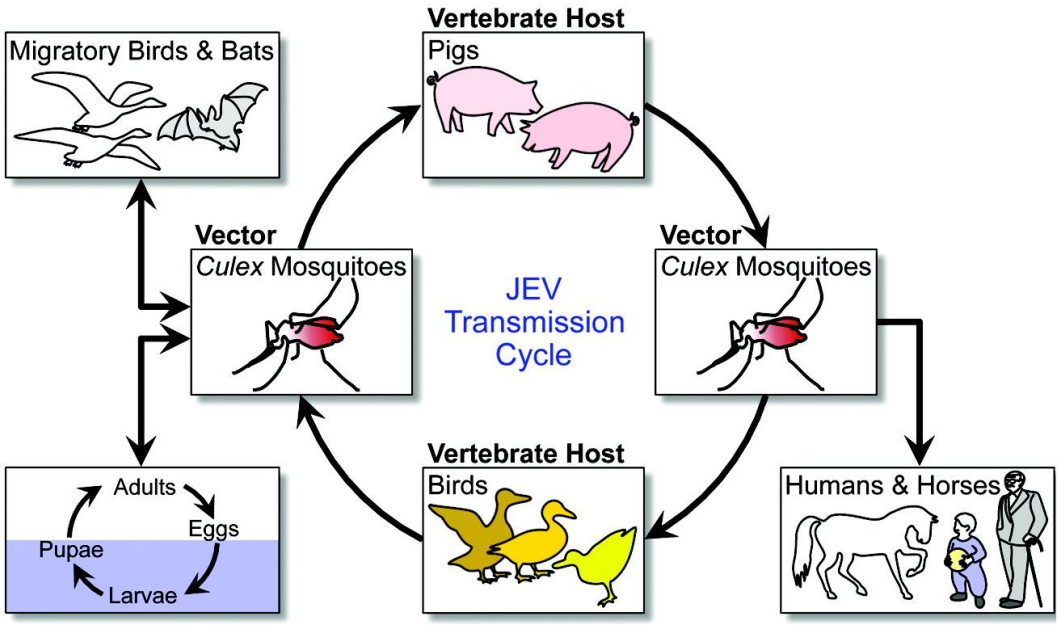

Figure 1.1 JEV transmission cycle

The transmission cycle of JEV involving an enzootic cycle between mosquitoes, birds, and swine. Image taken from Yun & Lee 2014; published under the Creative Commons Attribution License.

JEV infection in swine

JEV is an emerging pathogen of concern that could have a great impact on the U.S. swine industry if introduced. Clinical disease in pigs is varied and age-specific. In adult pigs, clinical signs are often nonapparent, with reproductive failure being the major clinical sign. Abortions, abnormal farrowing, mummified fetuses, and weak piglets are the most commonly observed reproductive signs of infection (Shimizu et al., 1954). In young pigs, clinical signs can be varied from fever, anorexia, and depression to neurologic signs such as hind limb tremors, ataxia or seizures (Yamada et al., 2009). JEV can progress in young pigs to more severe disease such as nonsuppurative encephalitis (Yamada et al., 2004).

Swine are amplifying hosts that produce high levels of viremia (Park et al., 2018; Ricklin, Garcia-Nicolas, Brechbuhl, Python, Zumkehr, Posthaus, et al., 2016). Viral shedding is abundant

in oronasal secretions that can contribute to vector free transmission between pigs that are cohoused (Ricklin, Garcia-Nicolas, Brechbuhl, Python, Zumkehr, Nougairede, et al., 2016). This suggests pigs could maintain the transmission of JEV without the presence of the mosquito vector. JEV transmission can be overlapped in areas with close proximity to rice irrigation and pig rearing as an example of sympatric overlap between humans and pigs (Erlanger et al., 2009). The close contact between humans and pigs in rearing practices can create an increased risk of transmission through infected mosquitoes or the potential of viral shedding in swine oral fluids.

Introduction to generation of cell-mediated immunity

Cell-mediated immunity is an immune response that involves the activation of macrophages and natural killer (NK) cells, the production of antigen-specific cytotoxic T lymphocytes (CTLs), and the release of cytokines. The response is not dependent on antibodies produced by plasma cells. Generation of cell-mediated immunity involves bridging the innate and adaptive immune system. When a foreign antigen enters the organism, it first triggers the innate immune system. Dendritic cells (DCs) are important in bridging innate immune responses that can lead to T cell activation to generate adaptive responses. DCs capture antigens in peripheral tissues via phagocytosis. DCs with antigens will migrate through the lymphatic vessels into the lymph node. Once in the lymph node, they present antigens to naïve T cells and provide co-stimulatory signals, such as CD80 and CD86, along with cytokines, to determine T cell fate (Alraies et al., 2024). If the naïve T cell recognizes the antigen, it will activate and begin to differentiate. The fate of the T cell depends on the strength of exposure to surrounding cytokines.

CD4+ T cells or T helper (Th) cells play a central role in immune responses. Naïve CD4+ T cells can differentiate into several subsets, such as Th1, Th2, regulatory T cells, follicular helper

T cells, Th17, Th9, Th22, and CD4+ cytotoxic T lymphocytes (Cenerenti et al., 2022). Upon T cell receptor (TCR) stimulation, the naïve CD4+ T cell will differentiate into a Th1 T cell if interferon gamma (IFNγ) and interleukin (IL) 12 are present (Sun et al., 2023). Th2 differentiation is driven by cytokine IL-2 and IL-4 signaling (Sun et al., 2023). Th1 T cells are primarily involved in cell-mediated responses, while Th2 T cells primarily stimulate B cells to produce antibodies.

CD8+ naïve T cells can differentiate into effector and memory T cells. Effector CD8+ T cells or CTLs can be distinguished into categories: short-live effector CD8+ T cells (TE), exhausted CD8+ T cells (Tex), long-lived memory CD8+ T cells (TM), memory precursor CD8+ T cells (TMP), central and effector memory CD8+ T cells (TCM and TEM), and tissue-resident memory (TRM) cells (Sun et al., 2023).

After T cells have differentiated, they leave the lymph node to traffic to the site of infection or to patrol the periphery. During acute viral infection, CD8+ T cells are the main responders. T effector cells will secrete pro-inflammatory cytokines such as IFNγ and tumor necrosis factor (TNF-α) to inhibit viral replication and attract other inflammatory cells to the sites of infection. CTLs will patrol for infected cells, and if they recognize an infected cell, the activated CTLs will release cytotoxins, perforin, and granzymes to penetrate the infected cell and trigger the caspase cascade, leading to the infected cell's apoptosis (Chang et al., 2017). CD4+ Th1 cells are associated with the cell-mediated immune response. Once activated, Th1 cells produce IFNγ, TNF-β, and IL-2 to promote the proliferation of CD8+ T cells and enhance killing efficacy (Bos & Sherman, 2010).

After the infection is cleared, the effector T cell populations will begin to wane, and some will become long-lived memory T cells. CD4+ T cells are required for generating long-lasting memory CD8+ T cells (Shedlock & Shen, 2003). CD4+ T cells promote IL-15 signaling to

maintain TCM. In the absence of CD4+ T cell help, memory CTLs exhibit impaired recall response (Ahrends et al., 2019).

A controlled Th1/Th2 response is needed to maintain T-cell balance. T cells have been implicated in the pathogenesis of a range of viruses and autoimmune diseases. Dysregulation of T cells can lead to T cell exhaustion, in which sustained TCR activation renders CD8+ T cells unresponsive (Appleman & Boussiotis, 2003).

Interferon gamma immunity

Interferon gamma (IFNγ) was first characterized in 1983 as an antiviral factor released by antigen stimulated lymphocytes that can activate macrophages to kill pathogens (Rothermel et al., 1983). This discovery led to IFNγ as the first cytokine used for host directed therapy with U.S. Food and Drug Administration (FDA) approval in 1991 (Casanova et al., 2024). IFNs can be grouped into three major families: type I, type II, and type III. IFNγ is a type II IFN known for its effector actions on the immune system. CD8+ T cells, Th1 T cells, and natural killer cells (NK) are known producers of IFNγ. IFNγ released by activated lymphocytes can act on cells expressing IFNγ receptors and transcription factors for interferon-stimulated genes (ISGs). ISGs encode a variety of proteins, including enzymes, ion channels, and pumps. These molecules can aid host defense against intracellular pathogens by restricting metabolite availability, disrupting microbial membranes, and promoting the eviction of microbial pathogens (Casanova et al., 2024). A major role of IFNγ signaling is the reprogramming of ISGs expression. These gene expression changes can act as direct effectors to restrict pathogens by orchestrating immune responses, such as promoting antigen presentation and regulating immune cell infiltration into the sites of infection.

In a primed IFNγ environment, JEV replication is significantly reduced in HeLa cells (Chhabra et al., 2022).

Immune responses to JEV infection

The immunological response to JEV infection has been partially characterized through animal models and human case studies of survivors and those who succumb to natural infection. In mouse models, the crucial importance of the humoral immune response in recovery from JEV has been demonstrated. Mice lacking B cells develop high levels of viremia that persist until they succumb to infection (Larena et al., 2011). There have been conflicting reports on the role of CD8+ T cells in recovery from JEV. Mice depleted of CD8+ T cells or defective CD8+ T cells lacking the Fas receptor or granzymes exhibit increased viral burden in the central nervous system (CNS) (Larena et al., 2013). Loss of cytotoxic function in CD8+ T cells increased mortality and compromised the blood-brain barrier, leading to neuronal damage in TCRβ-null mice (Jain et al., 2017). The transfer of purified naïve T cells into TCRβ-null mice protects mice from lethal challenge, highlighting that the essential role T cells play in providing protection (Jain et al., 2017). CTLs raised against JEV can specifically target JEV infected cells for destruction (Murali-Krishna et al., 1994). Adoptively transferred JEV-specific CTLs protect adult mice from a lethal intracerebral challenge (Murali-Krishna et al., 1996). Neutrophil depletion, combined with CD8+ T cell depletion, led to enhanced viral replication and increased mortality (Soni et al., 2025). Taken together, neutrophils reach the brain and recruit CD8+ T cells to clear the virus from the brain. JEV can induce the expansion of Myeloid-Derived Suppressor Cells (MDSCs), which secrete immunosuppressive factors, such as IL-10 (Zhang et al., 2024). These factors affect the function of CD8+ T cells by suppressing TCR signaling and activation, leading to decreased cytokine

production, including IL-2, IFNγ, and TNF-α (Zhang et al., 2024). Ultimately contributing to an exhaustion-like state in CD8+ T cells, increased viral loads, and increased mortality. In contrast, it has been reported that CD8+ T cells do not enhance survival and that CNS infiltration correlates with the severity of encephalitis and inflammation. An overabundance of CD8+ effector T cells infiltrating the brain was associated with increased encephalitis severity (Zhang et al., 2019). In sublethal models, CD8+ depletion delayed death compared with wild-type (WT) mice, suggesting that T cells contribute to pathology (Soni et al., 2025).

In humans, memory T cell responses have been studied in JEV-endemic areas among exposed individuals, survivors of JE, and those who succumbed to JE. Individuals who were exposed to JEV but did not develop clinical cases had memory CD8+ T cell responses against NS3, NS4, and NS5 (Turtle et al., 2016). These CD8+ T cells were cross reactive to other flaviviruses antigens (Turtle et al., 2016). Recovered JE patients had a predominately CD4+ T cell response to JEV structural proteins and NS1(Turtle et al., 2016). Individuals with poor outcomes had an unbalanced CD4+ T cell response that was predominantly pro-inflammatory, with increased TNF-α secretion (Turtle et al., 2016). A reduction in IFNγ and IL-12 produced from T cells was strongly associated with poor outcomes from JE (Turtle et al., 2016). Similarly, surviving JE patients had higher CD4+ T cell counts than fatal cases, while the percentage of CD8+ T cells did not differ (Zhou et al., 2022). Convalescent JE patients had reduced Th1 T cell responses upon stimulation with NS3. JE Patients with maximum neurological defects had no secretion of IFNγ from NS3-specific T cells (Kumar et al., 2004). Patients with IFNγ levels>300 pg/mL had near-total recovery from encephalitis with minimal neurological sequelae (Kumar et al., 2004). These results indicate the importance of JEV-specific T cells in producing a Th1 response that upregulates IFNγ, which ultimately correlates with better survival and clinical outcomes.

T cell epitopes specific to JEV have been identified. T cells from JEV-vaccinated individuals strongly responded to three regions of the JEV envelope, as well as to NS4B and NS2a (Pushpakumara et al., 2020). Other epitopes were found to cross-react with DENV and WNV envelope proteins, as certain JEV envelope regions share 35% homology with DENV and up to 75% with WNV (Pushpakumara et al., 2020). This homology could induce cross-reactive T cells from vaccination or natural exposure.

As seen with IFNγ, cytokines play an important role during JEV infection. Studies in JEV infected mice have shown increased levels of IFNγ, TNF-α, IL-10, and IL-4 in serum, brain, and spleen (Saxena et al., 2008b). IFNγ and IL-10 together can modulate inducible nitric oxide synthase (iNOS), increasing nitric oxide (NO) production to combat viruses and dilating blood vessels to increase immune cell trafficking. The antiviral effects of NO on JEV have been documented, as it can inhibit replication in treated macrophages (Lin et al., 1997). Pro-inflammatory cytokines can act as immunoregulators during infection. Loss of TNF-α reduced survival rates in mice, and exaggerated inflammatory responses were observed in the brain (Hayasaka et al., 2013). JEV infection has been shown to suppress immune responses by downregulating cytokines and IFNs (Zhou et al., 2018). After infection, there is a progressive decline in IL-4 and IL-10; the loss of these anti-inflammatory cytokines leads to dysregulation of pro-inflammatory cytokines (Saxena et al., 2008a). In the absence of IL-4, TNF-α and INF-γ can increase unchecked, and an overabundance of these cytokines is associated with increased brain tissue pathology and viral load (Saxena et al., 2008a). JE patients had higher levels of IL-18 and IL-6 than healthy adults (Zhou et al., 2022). IL-6 is a pro-inflammatory cytokine that can inhibit TNF-α and activate IL-10; its role in JEV infection is unclear, but it may suppress CD8+ T cell immunity.

Immune responses to JEV infection in swine

Immune responses to JEV infection in swine are poorly understood and uncharacterized. JEV in swine has tropism to the CNS and tonsils. Within the tonsils, the virus can persist for long periods after infection, with high titers up to 21 days post-infection (Redant et al., 2022; Ricklin, Garcia-Nicolas, Brechbuhl, Python, Zumkehr, Posthaus, et al., 2016). One study has demonstrated limited infiltration of CD3+ T cells into the cerebrum after infection, but the effect was not seen in all pigs (Redant et al., 2020). IFN-stimulated genes were moderately upregulated in the cerebellum, cerebrum, and olfactory bulb after JEV RNA was detected. IFNγ expression was upregulated in some animals in the olfactory bulb. Interestingly, there was no change in IL-10 expression in any of the pigs (Redant et al., 2020). Taken together, there seems to be suppression of JEV replication in the brain due to upregulation of IFN-stimulated genes. If viral replication cannot be controlled, then T cells will infiltrate and express IFNγ to suppress JEV replication.

One study has examined the immune cell responses in swine after JEV infection and restimulation (Redant et al., 2022). After infection, a decrease in CD8+ and CD4+CD8+ double-positive T cells was observed from day 14 through day 21, while an increase in CD4+ T cells was observed within peripheral blood mononuclear cells (PBMCs) from day 14 onward. There was no significant change in NK cells or γδ T cells over infection. Dendritic cell populations did not change over infection, but CD14+ monocytes increased at day 7 postinfection and then significantly decreased starting at day 10 postinfection. In the tonsils, CD8+ and CD4+ T cells decreased by day 3 and remained decreased until day 5. CD4+CD8+ double-positive T cells were significantly decreased from the beginning of infection through day 21. NK cells also diminished by the end of the experiment. Pro-inflammatory cytokines, such as TNF-α and IL-6, in the tonsils were downregulated during infection. Upon restimulation with NS3, there was no detectable IFN-

γ production in either the CD4+ or γδ T cell populations in the PBMCs. NK cells, CD8+ T cells, and CD4+CD8+ double-positive T cells showed a marked increase in IFNγ production after NS3 stimulation. The major findings of this study indicate that JEV infection in pigs elicits a weak inflammatory response, allowing the virus to replicate without immune cell-mediated interference that would normally suppress viral replication. The weak induction of the immune system and the absence of IFNγ may explain why clinical signs are subdued despite high viral titers in pigs.

Immune responses to WNV infection

The role of cellular immunity in controlling WNV infection has been well documented through animal models and human patient data. Similarly to JEV, in the absence of CD8+ T cells, higher viral burdens are observed in the CNS, leading to poor survival (Shrestha & Diamond, 2004). CD4+ T cells are required to sustain the CD8+ T cell response for clearance of virus from the CNS (Sitati & Diamond, 2006). Mice with depleted CD4+ T cells had increased viral loads and blunted antibody responses to WNV (Sitati & Diamond, 2006). Human responses after WNV infection are predominately CD4+ (Koblischke et al., 2020). Stimulation of these T cells was specific to the E protein and induced pro-inflammatory cytokines (IL-2, TNF-α, and IFNγ) (Koblischke et al., 2020). Like JEV, IFN can be a potent antiviral against WNV replication. Lack of IFNγ production or signaling resulted in increased lethality, viremia, and greater viral replication in the lymphoid tissues (Shrestha et al., 2006). Primary dendritic cells treated with IFNγ also reduced WNV replication by 130-fold (Shrestha et al., 2006).

Similarly to JEV an unbalanced T cell response as be implicated to contributing to pathogenic effects during infection. Neuroinvasive WN infections in humans have elevated WNV specific T cells that co-produced IFNγ and IL-4 and reduced T regulatory cells (James et al., 2016).

Patients who succumb to WNV had CD8+ T cell infiltration into parenchymal and glial nodules in the brain (Sampson & Armbrustmacher, 2001).

Antibodies play an important role in defense against WNV by neutralizing invading virus before it can infect host cells. As the passive transfer of antibodies to WNV have been shown to be protective against WNV challenge (Roehrig, 2009). Monoclonal antibodies (Mab) against the E protein such as Mab E16, have also been demonstrated to be protective and can be used for therapeutic treatment (Oliphant et al., 2005). Broadly cross reactive Mabs that target conserved structural proteins can be leveraged for protection against viruses in the same serogroup like WNV and JEV. Cross reactive Flavivirus Mabs that target the fusion loop domains or the nonstructural proteins can be protective against infection. An example is anti-NS1 Mabs that are cross reactive to WNV and JEV. These antibodies were protective against WNV challenge (Wessel Alex et al., 2021).

Current JEV and WNV vaccines approved for human or veterinary use

Currently, multiple human vaccines are approved for JE prevention. Vaccination programs to prevent JE are included in childhood immunization schedules in Asia (Heffelfinger et al., 2017). Approved licensed JE vaccines include inactivated and live attenuated vaccines that provide effective protection against infection and JE disease (Chen et al., 2015). The three types of licensed vaccines are inactivated Vero cell-derived vaccines, live attenuated vaccines, and live chimeric vaccines. The vaccines have demonstrated the ability to induce protective neutralizing antibodies after receiving two doses, with an efficacy of 91% (Hoke et al., 1988). Table 1-1 summarizes available human vaccines for JEV.

Despite pigs being an important amplifying host for JEV and a potential source of spillover into humans, there are currently no licensed veterinary vaccines. There are regionally approved swine vaccines in Japan, China, and Korea, but they have been reported to have low immunogenicity (Nah et al., 2015).

Table 1-1 Currently licensed human vaccines for JEV worldwide

Vaccine Type	Vaccine name	Country
Inactivated Vero cell-derived vaccine	JEIMMUNUGEN, TC-JEV (Beijing-1)	Japan, Korea
	IXIARO/JESPECT (SA14-14-2)	Europe, USA, Canada, Switzerland, Singapore, Hong Kong, and Australia
Live attenuated vaccine	CDJEVAX (SA14-14-2)	China, Japan, Thailand, Hong Kong
Live chimeric vaccine	IMOJEV (ChimeriVax-JE)	Australia, Thailand

References:(Chen et al., 2015)

In contrast, WNV has no FDA-approved vaccines for human use, despite the occurrence of occasional outbreaks in North America. However, veterinary vaccines are available to prevent West Nile neuroinvasive disease in horses. Currently, there are five licensed WNV equine vaccines in the United States, including inactivated whole-virus vaccines, a killed Flavivirus chimera, and a live, non-replicating, recombinant canarypox vector vaccine. Seasonal WNV vaccination in horses has been shown to be effective in reducing neurologic disease cases. For example, a three-year study in California found that vaccinated horses did not developed neurologic disease, compared to 2.5% of unvaccinated horses in the cohort (Gardner et al., 2007). Table 1-2 summarizes available veterinary vaccines for WNV.

Table 1-2 Currently available equine vaccines for WNV

Vaccine Type	Vaccine name	Manufacturer	Dose regimen
Inactivated whole virus with adjuvant	West Nile Innovator	Zoetis	Two 1 mL doses, 3 to 6 weeks apart. Annual revaccination with a single dose is recommended.
	Vetera WNV	Boehringer Ingelheim	
	Equi-Jec WNV	Boehringer Ingelheim	
Inactivated flavivirus chimera vaccine with adjuvant	Prestige WNV	Merck Animal Health	Two 1 mL doses, 3 to 4 weeks apart. Annual revaccination with a single dose is recommended.
Live non-replicating recombinant canarypox vector vaccine	Recombitek Equine WNV	Merial	Two 1 mL doses, 4 to 6 weeks apart. Annual revaccination with a single dose is recommended.

References: (Practitioners, 2023; USDA, 2026)

Demonstration of cross protection between JEV and WNV

Flavivirus cross-reactive immunity has been demonstrated in many publications. Many of these studies examine whether antibodies or memory T cells acquired through vaccination can cross-react with other viruses in the family. A publication in 1956, demonstrated that sublethal infection of JEV provides protection against subsequent WNV challenge. Hamsters immunized with sublethal doses of JEV were cross-protected against lethal WNV challenge with high neutralizing antibody titers (Hammon & Sather, 1956). Similar results were obtained in bonnet macaques, with JEV immunization conferring full protection against WNV challenge. The reverse was tested with WNV immunization first, then JEV challenge. The monkeys showed a lower degree of protection against the JEV challenge, but clinical signs were mild, and 3 out of 5 survived

(Goverdhan et al., 1992). Interestingly, the surviving monkeys had low titers of neutralizing antibodies, suggesting that T cells may play a protective role.

T cells have been examined for their capacity to cross-react with members of the viral family. There have been conflicting results on whether T cells can be cross-reactive, but the majority of findings suggest cross-reactivity is possible, depending on the primary immunization. The degree of cross-reactivity depends on the immunizing virus, as YF17D vaccination shows limited T-cell cross-reactivity to heterologous peptides (Grifoni et al., 2020). Specific lysis of viral targets (WNV and JEV) from memory flavivirus-immune T cells acquired through vaccination has been demonstrated (Regner et al., 2001). Anti-WNV T cells can recognize target cells treated with JEV peptides and lyse them (Regner et al., 2001).

Cross-reactive T cells have been studied in humans following flavivirus immunization. For example, individuals vaccinated for JEV develop CD4+ T memory cells that respond to YF and ZIKV antigens (Saron et al., 2018). These memory T cells produce IFNγ when exposed to other viral antigens, suggesting that cross-reactive T cells can be protective. In addition, JEV vaccination has been shown to induce cross-reactivity to ZIKV in CD8+ memory cells. Specifically, central memory CD8+ T cells, along with CD4+ effector and central memory T cells, are upregulated in response to ZIKV antigens (Wang et al., 2022).

Rational for research

Due to the potential threat of JEV introduction into the US swine industry, we examined current commercially available WNV vaccines for their ability to generate memory immunity to WNV and for potential cross-reactivity with JEV. Memory T cells responding to *in vitro* viral stimulation were assessed by flow cytometry. Expression of IFNγ was used as a marker of

activation and as a measure of the potential of these T cells to be protective against future viral challenge. **The hypothesis to be tested is that vaccination with equine WNV vaccines (West Nile Innovator (WNI), Vetera WNV (VET), Prestige WNV (PRES)) would generate memory T cells to WNV.** To test this hypothesis the potential protective functionality of T cells was assessed by intracellular IFNγ expression. Memory subsets were analyzed from CD4+, CD8+, and CD4+CD8+ T cells and further classified as either central memory or effector memory T cells. **A secondary hypothesis was that WNV vaccination could elicit cross-reactive T cells against JEV due to structural protein similarities between the viruses.** To test this hypothesis PBMCs from WNV-vaccinated pigs were stimulated *in vitro* with the vaccine strain of JEV to evaluate if a memory response was generated that was potentially cross-reactive.

Chapter 2 - Materials and Methods

Introduction

Three commercially available equine vaccines for WNV were evaluated in swine for the generation of cellular immunity to WNV and potential cross reactivity to JEV. The commercial vaccines were as follows, West Nile Innovator (Zoetis), Vetera WNV (Boehringer Ingelheim), and Prestige WNV (Merck). PBMCs from vaccinated swine were stimulated *in vitro* with virus then stained for antibody markers for flow cytometric analysis. This chapter details the material and methods used in the experiments.

Cell lines

African green monkey Vero cells were maintained at 37ºC in Leibovitz-15 (L-15) media supplemented with 10% fetal bovine serum (FBS), tryptose phosphate broth, L-glutamine, penicillin, and streptomycin. The Vero cells were maintained on a routine passage cycle and passed once confluency was reached in the flask. These cells were used for the propagation of all stock viruses prior to my involvement following similar methods (Higgs et al., 2006).

Virus

WNV strain NY99 was originally isolated from the brain of an infected Chilean flamingo in 1999 in New York City (Lanciotti et al., 1999). The virus strain was purchased from BEI Resources then propagated for laboratory stocks. The virus stocks were used for *in vitro* stimulation of PBMCs.

JEV strain SA 14-14-2 is the attenuated vaccine strain derived from wild type JEV SA14. The wild type strain was isolated from a pool of *Culex pipiens* mosquito larvae in China. The virus

was attenuated by serial passages in primary hamster kidney (PHK) cells (Yu, 2010). The virus was acquired through the World Reference Center for Emerging Viruses and Arboviruses managed by The University of Texas Medical Branch. The virus was used for propagation and *in vitro* stimulation of PBMCs.

Plaque assay

Plaque assays conducted in Vero cells were used to determine viral titers of working virus stocks after propagation. 50 μL of differing 10-fold serial dilutions of virus were inoculated into 24-well plates. Then placed into an incubator at 37°C for 60 minutes to allow for adsorption. After the adsorption period, the inoculum was removed then 1 ml of 1.5% methyl cellulose overlay was added per well. The plates were maintained at 37°C for 5 days. After incubation plates were fixed with 1 ml of 10% formalin solution then stained with 1% crystal violet solution. The plaques were counted and the titers of infectious viruses were calculated in plaque forming units (PFU)/ml. Plaque assays were used to calculate viral stock titers prior to my involvement.

Animals

The purpose of the animal experiments was to evaluate the immunogenicity of three commercially available equine WNV vaccines in domestic swine. Two separate vaccination experiments took place in March and September 2025 at the Large Animal Research Center at Kansas State University. The animal experiments followed an approved protocol from the Institutional Animal Care and Use Committee. Twenty-eight three-week-old pigs (Yorkshire cross) purchased from Oak Hill Genetics were acclimated to their environment for one week. Pigs were kept on an industry standard Ad Libitum diet. The pigs were randomly assigned into control

or vaccine groups to normalize the average starting weight between the groups. Each group had an equal number of males and females. The vaccine group pigs were separated into six groups of four pigs each, with three groups receiving a full dose of their assigned vaccine (1 mL) and the remaining three groups receiving a half dose (0.5 mL). The selected vaccines were all commercially available equine WNV vaccines as follows, West Nile Innovator (Zoetis), Vetera WNV (Boehringer Ingelheim), and Prestige WNV (Merck). After the acclimation period, pigs were bled by blind stick into jugular groove (jugular vein) for serum and whole blood then vaccinated intramuscularly (i.m.) in the semimembranosus muscle with the selected vaccine as either a whole or half dose. Control pigs were mock vaccinated i.m. in the semimembranosus muscle (1 mL) with phosphate buffered saline (PBS). On day twenty-one each pig received a second i.m. dose of the same vaccine and dosage they received on day zero. Pigs were bled by blind stick into jugular groove (jugular vein) on days 0, 7, 11, 14, 18, 21, 28, 32, and 35. On day thirty-five the pigs were administered tiletamine and zolazepam (Telazol) i.m. into the neck as a sedative. After sedation the pigs were terminated by intravenous administration of pentobarbital sodium solution (Fatal Plus Solution) into the jugular vein or vena cava. Figure 2.1 summarizes the study schedule.

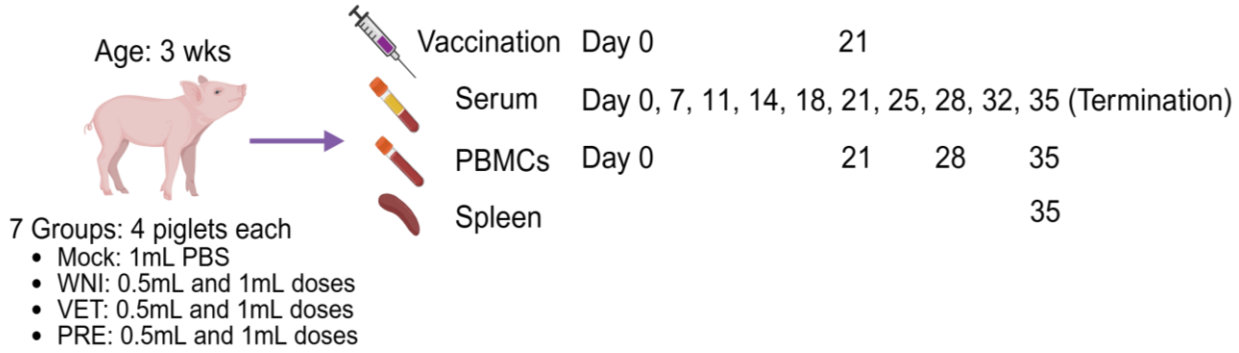

Figure 2.1 Animal study timeline

This graphic details the study design and sampling timepoints of this project. Pigs were separated into 7 groups of 4 pigs each. The vaccinated groups received one of the following commercial WNV vaccines as a half dose (0.5 mL) or a full dose (1 mL), West Nile Innovator (WNI), Vetera WNV (VET), Prestige WNV (PRE). Pigs were bled for serum and whole blood for PBMC isolation on the set days after vaccination. Animals were terminated on day 35 and spleens were collected. Created in BioRender. Matheny, A. (2026) https://BioRender.com/nlzwsfn. Permission granted for use from Matheny, A.

Blood collection

Venous blood samples were collected by blind stick into jugular groove (jugular vein) on days 0, 21, 28, and day 35 in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes as detailed in the above animal experiments. The tubes were inverted serval times to prevent blood clotting. The blood was transferred to the laboratory for PBMC isolation as detailed in the section below.

Isolation of peripheral blood mononuclear cells and cryopreservation

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood samples by gradient separation. Blood samples were diluted 1:1 with PBS then carefully layered over Ficoll-Paque (Cytiva) then centrifuged at 400 x *g* for 30 minutes (Eppendorf swing bucket centrifuge 5810R) at room temperature with acceleration set to a minimum with the break off. PBMCs were aspirated from the plasma-Ficoll interface with a Pasteur pipette and transferred to a new tube. PBMCs were washed two times in PBS by centrifugation at 500 x *g* for 8 minutes. Leftover red

blood cells were lysed by incubation with ACK lysis buffer (Gibco) for one minute then washed in PBS two times. PBMCs were resuspended in complete RPMI media (RMPI 1640, 10% FBS, penicillin, and streptomycin) (Corning) for quantitation of cell density with Trypan Blue on a Countess 3 FL Automated Cell Counter (ThermoFisher). PBMCs were adjusted to a density of 8 x 10^6 cells/mL then resuspended in cold freezing media (90% FBS, 10% dimethyl sulfoxide (DMSO)) and transferred to cryotubes. The tubes were immediately transferred to a controlled-rate freezing container (Mr. Frosty) (Fisher) containing 250 mL of 100% isopropyl alcohol then placed into a -80ºC freezer for 20 to 24 hours. The cryovials were then transferred to a -150ºC freezer for storage.

Stimulation of peripheral blood mononuclear cells

To resuscitate cryopreserved PBMCs, cryovials were rapidly thawed in 37ºC water bath then PBMCs were transferred to new tubes containing warmed complete RPMI media with a 1mL FBS underlay. Cells were washed by centrifugation at 1200 rpm for 5 minutes. Cells were resuspended in 1 to 5 mLs of complete RPMI for quantitation of cell density with Trypan Blue on a Countess 3 FL Automated Cell Counter. Cell density was adjusted to 500,000 to 1.5 x 10^6 cells per well then 100 µl was transferred to a 96-well plate in duplicates. PBMCs were stimulated with 100 µl of WNV NY99 or JEV SA 14-14-2 at a multiplicity of infection (MOI) of 1. The MOI was calculated using the formula MOI = number of infectious units (V) / number of host cells (C). The number of infectious units used in the calculation was the virus titer in plaque-forming units. A MOI of 1 was selected based off current literature on JEV stimulation which ranges from a MOI of 0.1 to 10 (Fang et al., 2022; Jain et al., 2017; Patabendige et al., 2018). A comparison between stimulation with a MOI of 0.5 and 1 showed no difference in viability resulting in a MOI of 1

being selected, data not shown. Positive control wells were stimulated with 5 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Invivogen) and 1 µg/ml of Ionomycin (Invivogen). Negative control wells were left unstimulated. Cells were placed in an incubator set at 37ºC with 5% CO_2 for overnight stimulation (14 to 16 hours) based on similar methods (Attreed et al., 2022; Franzoni et al., 2013; Redant et al., 2022). Brefeldin A (BFA/GolgiPlug) (BD Biosciences) was added after the overnight stimulation to block protein transport and cells were further incubated for six hours. After six hours staining procedures immediately began.

Antibodies and flow cytometric analysis

Cells were transferred to 96-well v bottom plate (ThermoFisher) then centrifuged at 500 x *g* for 5 minutes to pellet the cells. Cell supernatant was collected and transferred to new 96-well plate then placed at -20ºC. Cell pellets were resuspended in PBS for one wash. After washing, cells were resuspended in 50 µl of FC Block (Innovex Biosciences) for 15 minutes on ice and kept in the dark. After blocking cells were washed then resuspended in Live/Dead viability dye (ThermoFisher) for 20 minutes on ice and kept in the dark to stain for viability. Cells were washed twice with PBS then stained with monoclonal antibodies specific for surface markers listed in Table 2-1 in FACS buffer (PBS without Mg^{2+} and Ca^{2+}, 2% FBS, and 0.05% sodium azide) for 45 minutes to 1 hour on ice and kept in the dark. After cell surface staining, cells were washed twice in FACS buffer. Surface-stained cells were fixed and permeabilized using CytoFix/CytoPerm solution (BD Biosciences) for 20 minutes on ice and kept in the dark. Cells were washed twice with BD Perm/Wash buffer (BD Biosciences) then incubated with IFNγ monoclonal antibody for intracellular staining for 25 minutes on ice and kept in the dark. After the final staining step cells were washed twice with BD Perm/Wash buffer (BD Biosciences) then transferred to FACS buffer.

Stained PBMCs were placed in the fridge overnight, then analyzed on a Cytek Aurora full spectrum flow cytometer (Cytek Biosciences) the following morning.

Table 2-1 Monoclonal antibodies used for flow cytometric analysis of porcine T cell responses

Mab Marker	Fluorochrome Labeling	Clone	Species	Working dilution
CD3	PerCP-Cy5.5	BB23-8E6-8C8	Anti-Pig	1:50
CD4a	PE-Cy7	74-12-4	Anti-Pig	1:50
CD8a	PE-Cy5	76-2-11	Anti-Pig	1:50
CD44	BV421	IM7	Anti-Human (cross reactive)	1:200
CD62L	Alexa Fluor 647	CC32	Anti-Bovine (cross reactive)	1:10
CCR7 (CD197)	PE-eFluor 610	3D12	Anti-Human (cross reactive)	1:50
CD25	SBV760	K231.3B2	Anti-Pig	1:20
CD27	APC	B30C7	Anti-Pig	1:20
TCR1 Delta	PE	MAC320	Anti-Pig	1:100
IFNγ (intracellular)	Alexa Fluor 488	CC302	Anti-Bovine (cross reactive)	1:50
Live Dead	Live Dead Yellow or Zombie Aqua	N/A	N/A	1:1000

The morning of analysis, flow cytometry compensation beads (ThermoFisher) were stained with each single color fluorochrome for calibration of compensation to minimize fluorescence spillover. Unstained cells were used for setting the voltage of forward scatter (FSC) and side scatter (SSC) and for autofluorescence extraction. Cells stained only with live/dead marker were used for setting the viability gate. The number of events collected per sample ranged from 100,000 to 200,000. After sample data was acquired on the flow cytometer, data analysis was done in FlowJo software (BD Biosciences).

Gating strategy for flow cytometric analysis

The gating strategy for analysis is seen in Figure 2.2. Memory T cells were identified as those single positive CD4+ or CD8+ or double positive CD4+CD8+ cells that positively express CD44 and had low expression of CD25 and CD27. As T cells downregulate CD25 and CD27 upon differentiation into long lived memory T cells (Schiött et al., 2004). The memory cell population was distinguished as central memory or effector memory. Central memory cells had positive expression of CD62L and CCR7 (CD44+CD25-CD27-CD62L+CCR7+). Effector memory cells had low expression of CD62L and CCR7 (CD44+CD25-CD27-CD62L-CCR7-). Populations then were assessed for IFNγ positive cells. Gating strategy followed similar strategies in these papers (Attreed et al., 2022; Franzoni et al., 2013).

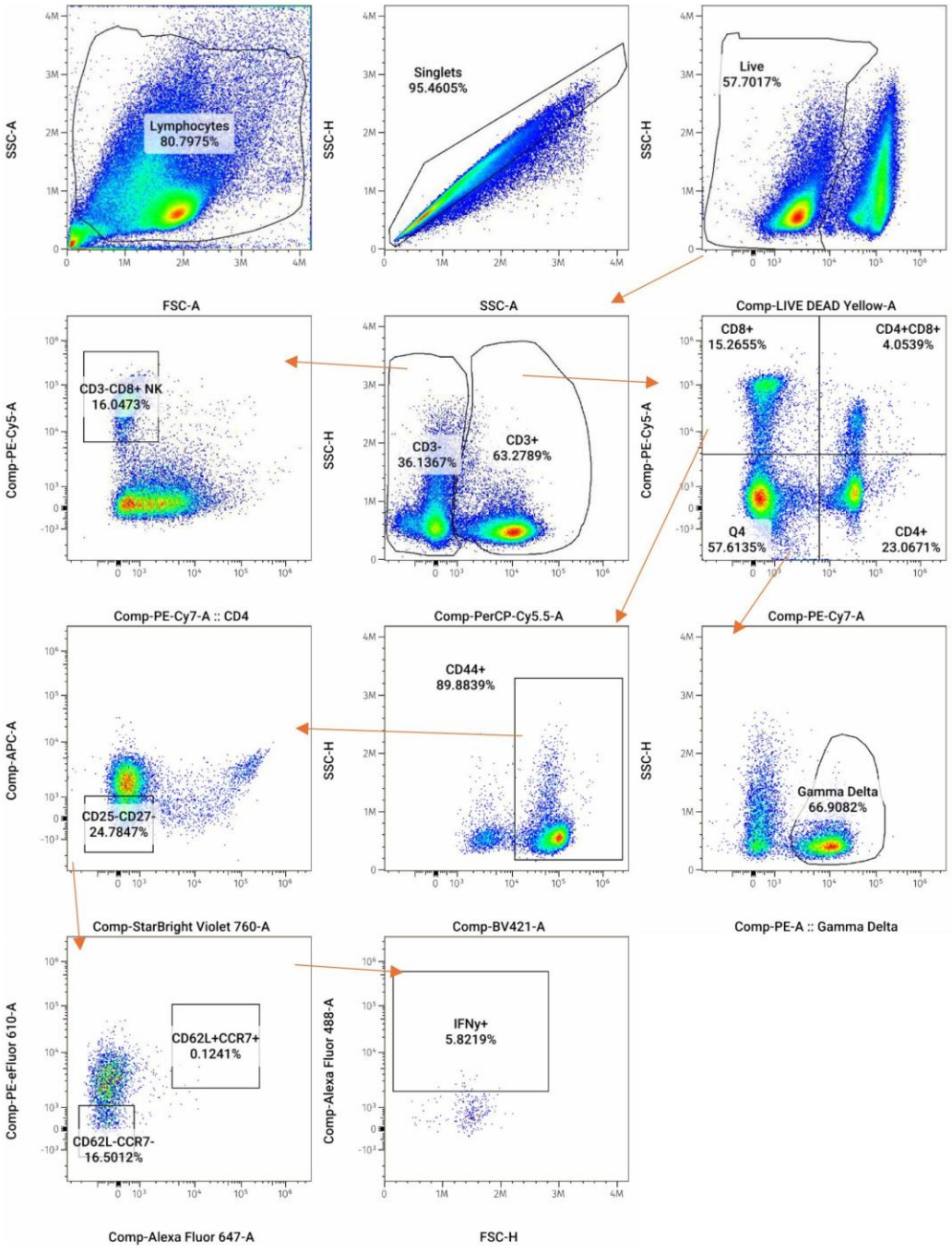

Figure 2.2 Gating Strategy for Flow Cytometric Analysis

Gating strategy for identifying memory T cell subsets in PBMC samples. Lymphocytes were identified from forward and side scatter then singlets to exclude doublets. Live cells were determined by the viability dye then gated on CD3 marker for positive and negative populations. CD3+ cells were gated on CD4+, CD8+, or CD4+CD8+ cells. CD44+ cells were gated on CD25-CD27- for memory cell populations then further gated into effector memory (CD62L-CCR7-) or central memory (CD62L+CCR7+). Then the IFNγ+ cells were identified.

Statistical analysis

Stimulated samples were subtracted from their unstimulated wells to calculate change after stimulation based on similar methods (Attreed et al., 2022; Fang et al., 2022; Lee et al., 2024) Percentages of cells were calculated from their parent populations. Comparisons of cell populations from flow cytometric analysis were analyzed by 2-way ANOVA followed by Tukey HSD for multiple comparisons. Statistical analyses were completed using GraphPad Prism (GraphPad Software Inc.). *p-value <0.03, **p-value 0.002, *** p-value 0.0002, **** p-value 0.0001.

Chapter 3 - Assessment of Cellular Immune Response After *In Vitro* Stimulation with WNV or JEV SA 14-14-2

Introduction

We hypothesized that vaccination with equine WNV vaccines (West Nile Innovator (WNI), Vetera WNV (VET), Prestige WNV (PRES)) could induce memory T cells in swine. Though pigs are not an important host to WNV, the memory response was examined to see if WNV vaccination could induce T cell memory before examining if cross reactive memory to JEV was generated. A secondary hypothesis was that WNV vaccination could elicit cross-reactive T cells against JEV due to structural protein similarities between the viruses. To test this hypothesis PBMCs from WNV-vaccinated pigs were stimulated *in vitro* with the vaccine strain of JEV to evaluate if a memory response was generated that was potentially cross-reactive. Lastly, we hypothesized cross-reactive memory T cell responses to JEV would be lower compared to primary responses to WNV. To test this the data from samples from day 28 stimulation with WNV or JEV were compared. A measurable increase in IFNγ expression after *in vitro* stimulation with WNV or JEV indicates these cells are responding to stimulation. Upregulation of IFNγ leads to many defensive downstream processes such as activation of ISGs which can promote antigen presentation and immune cell infiltration into the sites of infection. These defensive mechanisms can limit pathogen replication in host cells. Therefore, IFNγ was used as a measure of activation to viral stimulation that could potentially aid in protection against infection. Data in this chapter were analyzed on memory subsets from CD4+, CD8+, and CD4+CD8+ T cells and further classified as either central memory or effector memory T cells. The chapter is spilt into subchapters with: the CD4+ T cell response in chapter 3.a, the CD8+ T cell response in 3.b, the CD4+CD8+ T cell response in 3.c, the NK cell and γδ T cell response in 3.d.

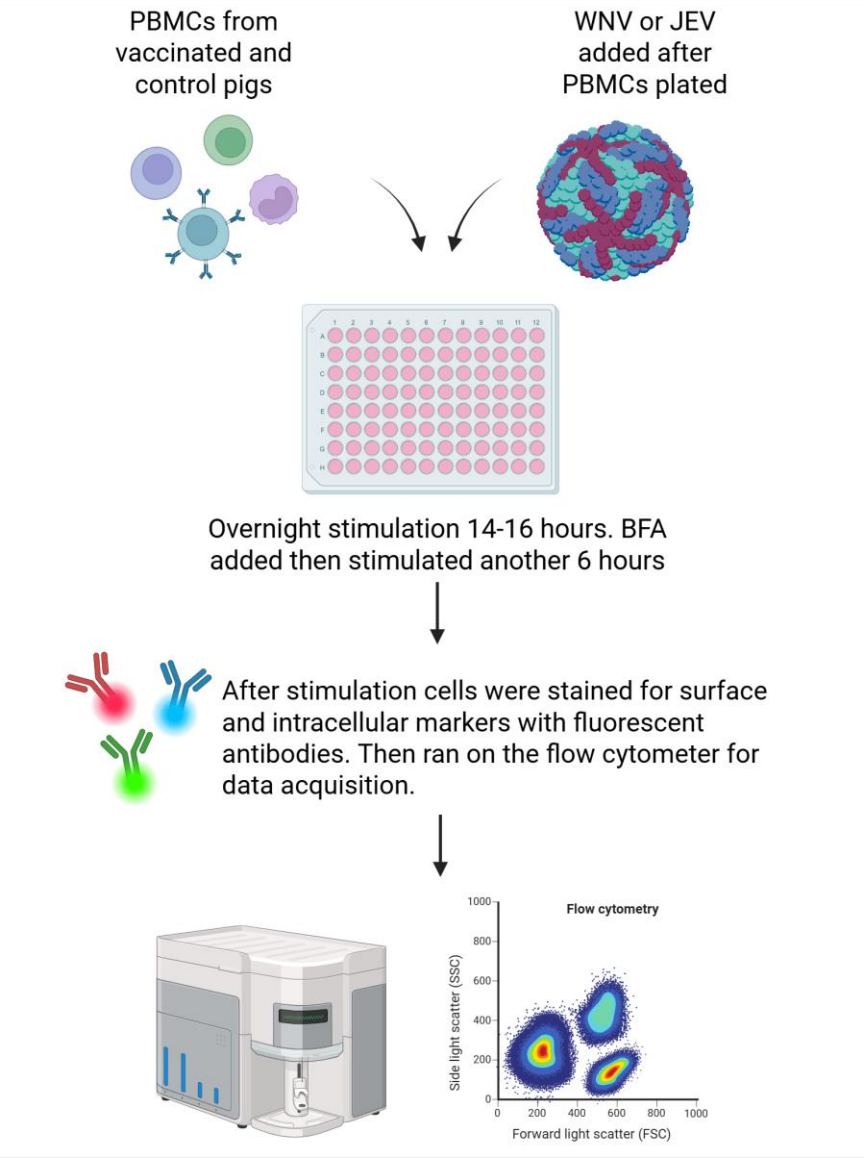

Figure 3.1 Graphical outline of data acquisition

PBMCs were isolated from vaccinated and control pigs then plated into 96 well plates. The PBMCs were then stimulated with WNV or JEV for 14 to 16 hours then BFA was added and incubated for another 6 hours. After stimulation cells were stained for surface and intracellular markers with fluorescent antibodies. The samples then were run on the flow cytometer for data acquisition. Created in BioRender. Matheny, A. (2026) https:// BioRender.com/068lyy7. Permission granted for use from Matheny, A.

3.a Memory CD4+ T cell responses

Memory CD4+ T cells were examined after stimulation with WNV or JEV in the vaccinated and control samples. CD4+ T cells can aid in defense against pathogens through helper and effector functions. CD4+ T helper cells are essential for B cell activation to help sustain antibody responses. These helper T cells help B cells in isotype switching, affinity maturation, and sustaining long-lived plasma cells and memory B cells (Mitchison, 2004). CD4+ T cells can have effector functions through production of cytokines that directly activate antimicrobial mechanisms in infected tissues (Mosmann & Coffman, 1989).

3.a.1 Memory CD4 + T cells to WNV

Memory CD4+ T cell populations to WNV were analyzed in Figures 3.2 to 3.3 by comparing PBMCs from day 0 (pre-vaccination) and day 28 (post-prime vaccination). On day 28, central memory T cells (TCM) were present for all three vaccines, as shown in Figure 3.1. WNI and VET groups showed a significant increase in TCMs from their day 0 samples, while PRES did not. However, the increase in TCMs after vaccination was not significantly greater than in the control group. No significant differences were observed between half- and full-dose vaccines in generating TCMs on day 28, although half-doses of WNI and VET showed a significant increase from day 0. The percentage of effector memory T cells (TEM) in stimulated samples was reduced compared to their unstimulated samples resulting in a negative change reported. Though it should be stated that expression of the effector phenotype (CD62L-CCR7-) in the stimulated samples was not truly negative. WNV stimulation did not induce a memory recall in CD4+ TEMs.

Next, the activation of the memory phenotype was assessed by intracellular IFNγ expression. In Figure 3.2, on day 28, WNI and VET vaccination showed a higher percentage of TCMs that

had IFNγ positive expression after WNV stimulation. WNI vaccination led to a significant increase in TCMs that had positive expression for IFNγ compared to their day 0 samples. VET vaccination increased the proportion of TCMs expressing IFNγ, but this difference was not significant. PRES vaccination did not induce an activated TCM response, with no increase in IFNγ expression on day 28. Vaccination with WNI or PRES vaccines did not induce an activated TEM response as IFNγ expression was not increased in response to stimulation as seen in Figure 3.3.

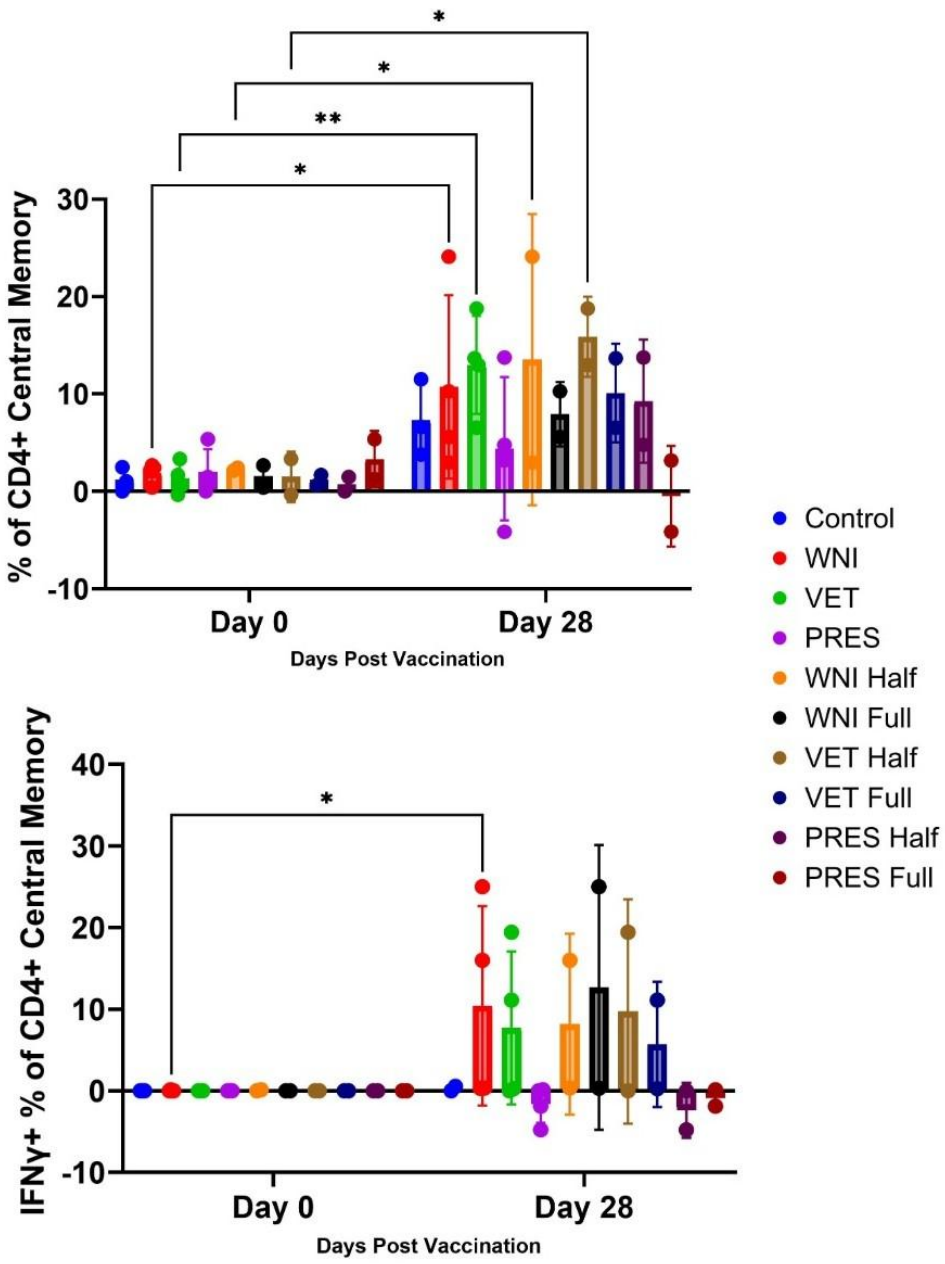

Figure 3.2 Vaccination with WNI and VET increases CD4+ central memory T cells after WNV stimulation

PBMCs from vaccinated and control pigs were stimulated with WNV then analyzed for central memory T cells. The central memory T cells were then analyzed for IFNγ expression. Vaccination with WNI (red) and VET (green) increased the percentage of CD4+ central memory T cells on day 28 after WNV stimulation. Vaccination with WNI (red) and VET (green) increased the percentage of CD4+ central memory T cells expressing IFNγ on day 28 after WNV stimulation. **p*-value <0.03, ***p*-value 0.002.

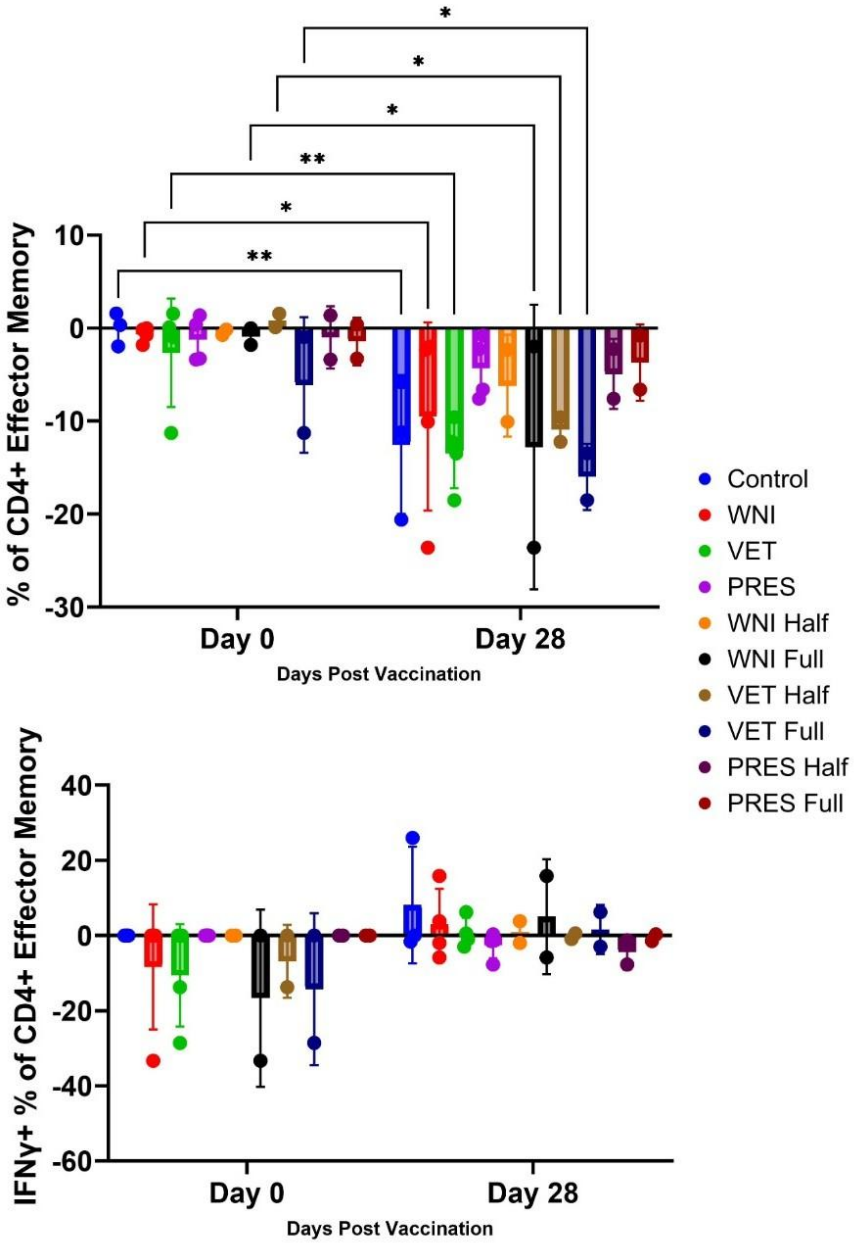

Figure 3.3 Vaccination did not increase CD4+ effector memory T cells after WNV stimulation

PBMCs from vaccinated and control pigs were stimulated with WNV then analyzed for effector memory T cells. The effector memory T cells were then analyzed for IFNγ expression. None of the vaccines increased the percentage of CD4+ effector memory T cells on day 28 after WNV stimulation. Vaccination with WNI (red) increased the percentage of CD4+ effector memory T cells expressing IFNγ on day 28 after WNV stimulation but was not significant. *p-value <0.03, **p-value 0.002.

3.a.2 Memory CD4 + T cells to JEV

Memory CD4+ T cell populations were analyzed in Figures 3.4 to 3.5. Day 0 PBMCs before vaccination were compared with their day 28 post-prime vaccination samples. Central memory T cells were observed in pigs vaccinated with VET, specifically in the half-dose group, compared to their day 0 samples (Figure 3.4). Populations reported as a negative change had less recorded memory cells in their simulated samples then their unstimulated samples resulting in the negative value. The stimulated populations had reportable memory populations but was not specific to JEV recall. The percentage of effector memory CD4+ T cells in the JEV stimulated samples were less than their unstimulated samples. Resulting in populations reported as a negative change. The stimulated populations had reportable memory populations but was not specific to JEV recall.

Next, the activation of the memory phenotype was assessed by intracellular IFNγ expression. Specifically, VET vaccination induced IFNγ expression in TCMs after JEV stimulation. In contrast, neither WNI nor PRES induced a reactive response to JEV stimulation (Figure 3.4). Turning to effector memory CD4+ T cells, these showed minimal IFNγ response to JEV stimulation, as seen in Figure 3.5. Notably, VET vaccination was again the only vaccine to induce a reactive response to stimulation. Furthermore, VET at full dose showed a significantly higher response in TEMs expressing IFNγ compared to their day 0 samples.

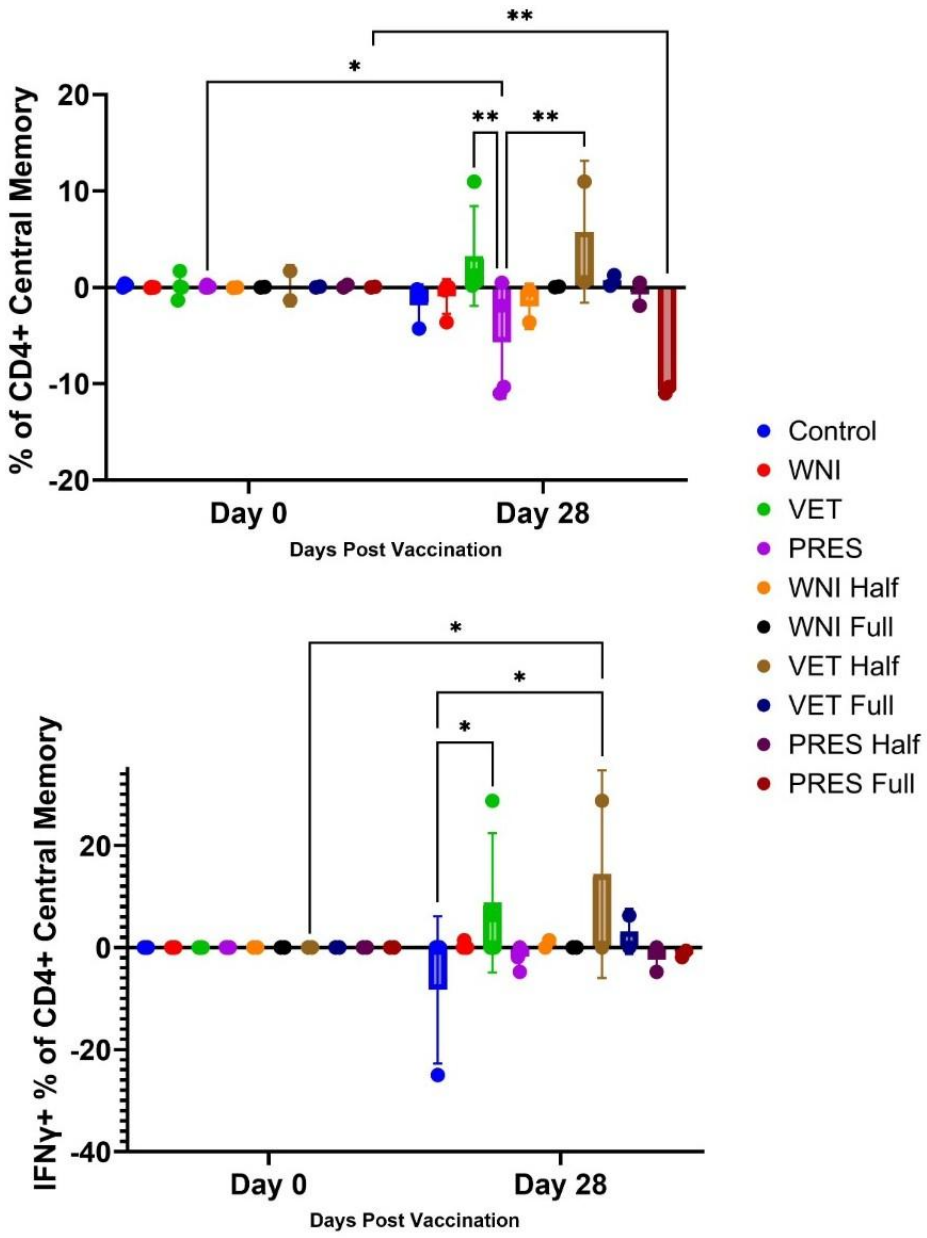

Figure 3.4 Vaccination with VET increases CD4+ central memory T cells after JEV stimulation

PBMCs from vaccinated and control pigs were stimulated with JEV then analyzed for central memory T cells. The central memory T cells were then analyzed for IFNγ expression. Vaccination with VET (green) increased the percentage of CD4+ central memory T cells on day 28 after JEV stimulation. Vaccination with VET (green) increased the percentage of CD4+ central memory T cells expressing IFNγ on day 28 after WNV stimulation. *p-value <0.03, **p-value 0.002.

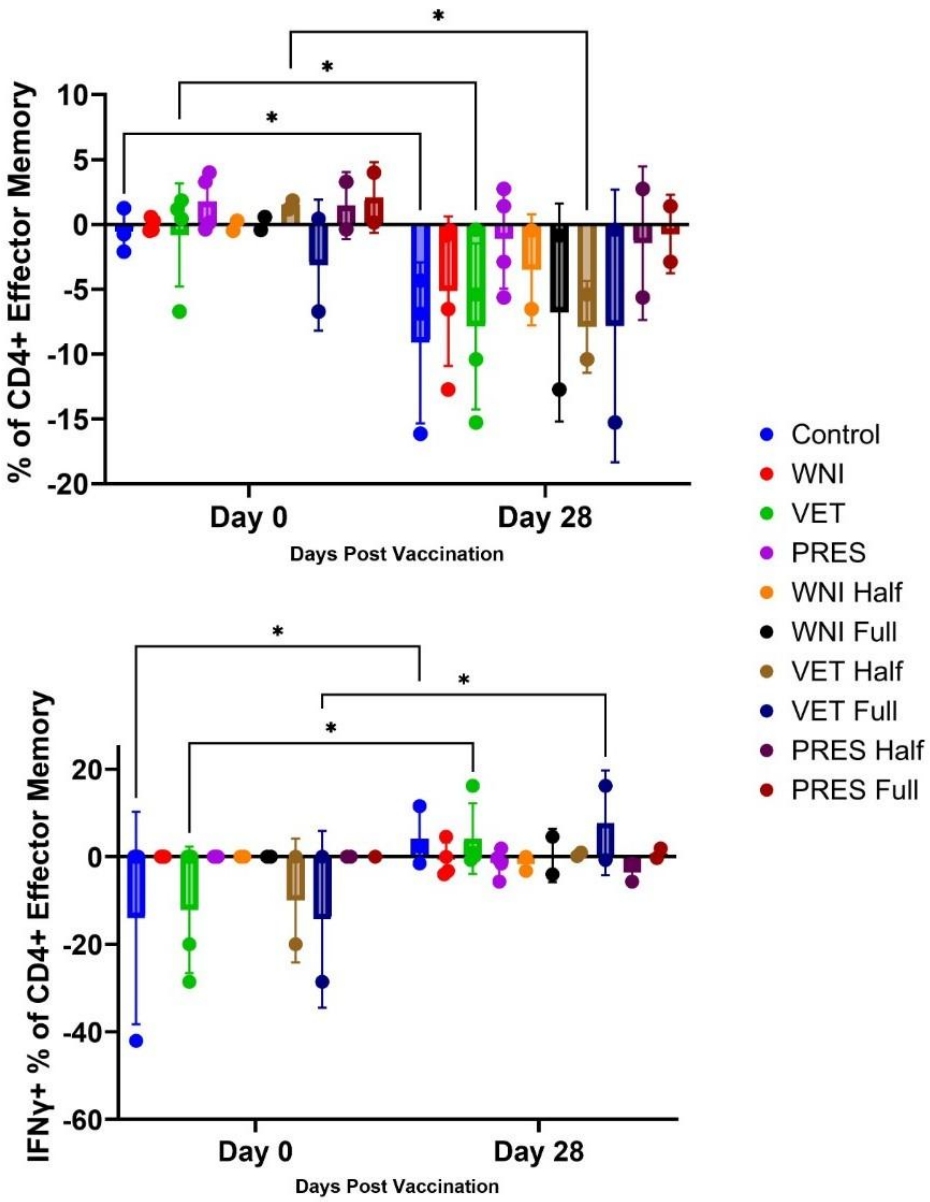

Figure 3.5 Vaccination did not increase CD4+ effector memory T cells after JEV stimulation

PBMCs from vaccinated and control pigs were stimulated with JEV then analyzed for effector memory T cells. The effector memory T cells were then analyzed for IFNγ expression. None of the vaccines increased the percentage of CD4+ effector memory T cells on day 28 after JEV stimulation. Vaccination with VET (green) increased the percentage of CD4+ effector memory T cells expressing IFNγ on day 28 after WNV stimulation. *p-value <0.03.

3.a.3 Comparisons of memory CD4 + T cells to WNV vs JEV

Central memory CD4+ cells showed higher percentages in response to recall WNV stimulation than to JEV (Figure 3.6). VET vaccination seemed to induce potential cross-reactive CD4+ TCMs. As VET was the only vaccine that have an increased percentage of memory cells after JEV stimulation. Populations reported as a negative change had less recorded memory cells in their simulated samples then their unstimulated samples resulting in the negative value.

IFNγ expression in the TCMs was upregulated after WNV and JEV stimulation in WNI and VET samples. VET samples had similar responses to WNV and JEV, suggesting these T cells may be cross-reactive. IFNγ expression in the CD4+ TEMs was variable with the control pigs showing a reaction (Figure 3.7). There were no significant differences in IFNγ expression between WNV or JEV stimulation in the TCMs or TEMs but reported measures were low so it should be interpreted with caution.

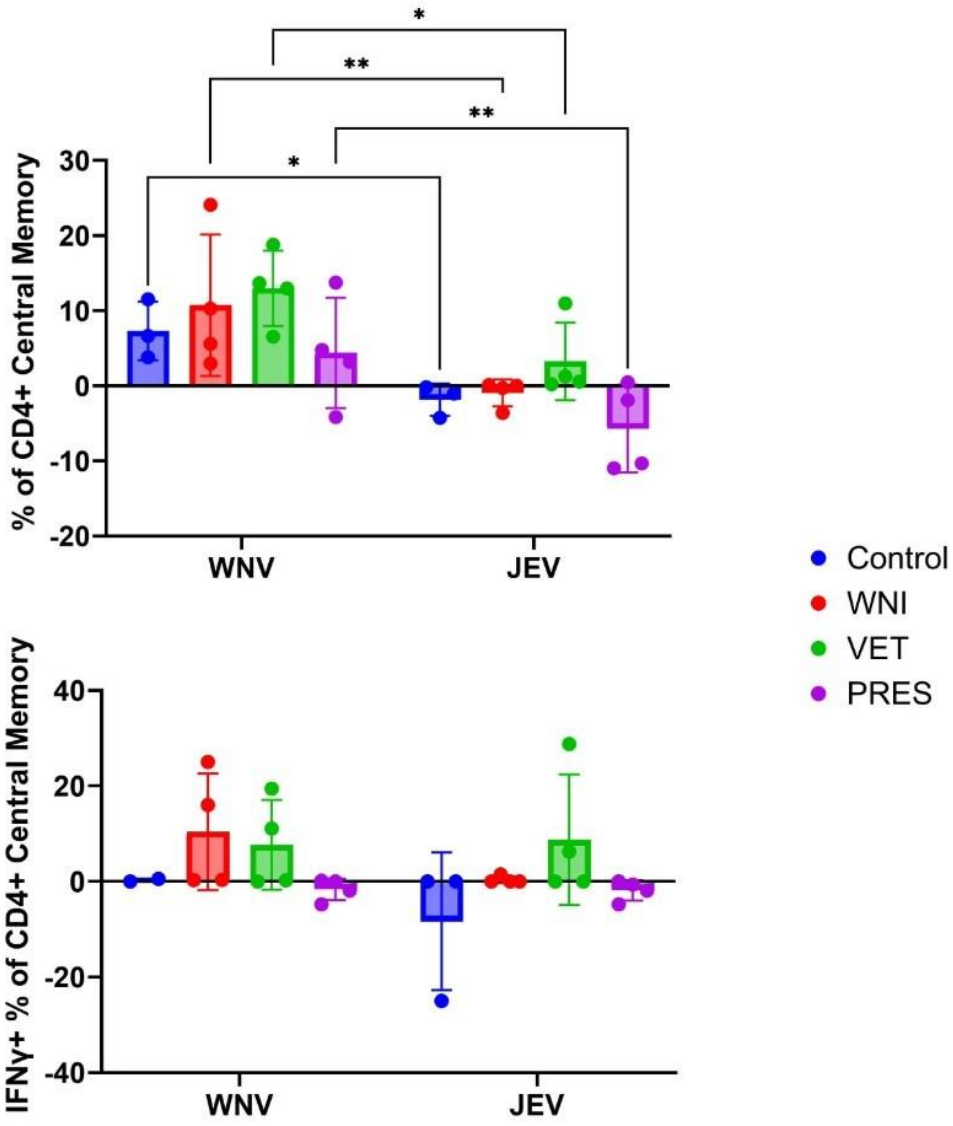

Figure 3.6 Comparisons of CD4+ central memory T cells in response to WNV vs JEV

Data from day 28 stimulated PBMC samples were compared to evaluate responses to viral stimulation. Vaccination with WNI (red), VET (green), and PRES (purple) had increased percentage of CD4+ central memory T cells to WNV stimulation. Vaccination with VET (green) had increased memory cells to JEV compared to WNI and PRES. Vaccination with VET (green) had similar percentages of CD4+ central memory T cells expressing IFNγ to WNV and JEV stimulation. **p*-value <0.03, ***p*-value 0.002.

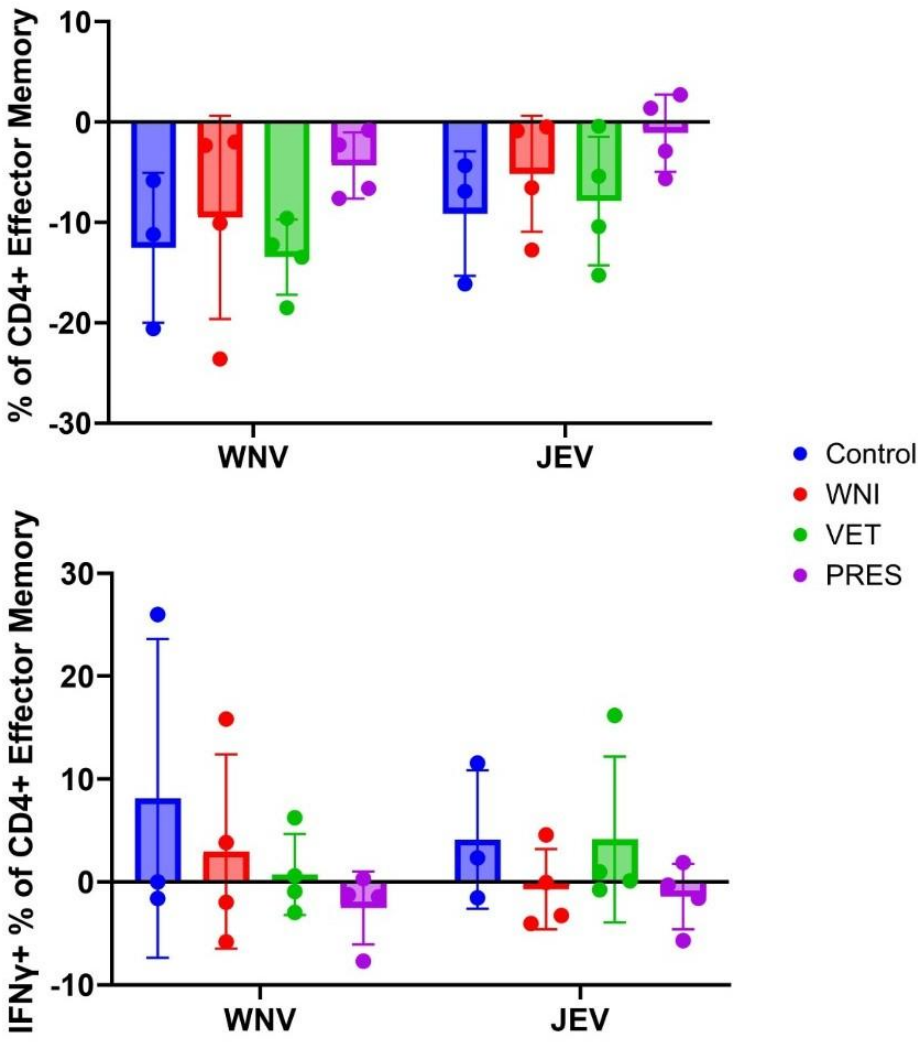

Figure 3.7 Comparisons of CD4+ effector memory T cells in response to WNV vs JEV

Data from day 28 stimulated PBMC samples were compared to evaluate responses to viral stimulation. Vaccination did not increase the percentage of CD4+ effector cells for either stimulation. Vaccination with VET (green) had increased percentages of CD4+ effector memory T cells expressing IFNγ to JEV stimulation.

3.b Memory CD8+ T cell responses

Memory CD8+ T cells were examined after stimulation with WNV or JEV in the vaccinated and control samples. Memory CD8+ T cells can serve as effector cells against pathogens. CTLs are a type of memory CD8+ T cell that limit viral replication by targeting infected host cells for destruction by recognizing viral antigen presented (Zinkernagel & Doherty, 1974).

3.b.1 Memory CD8+ T cells to WNV

Memory CD8+ T cell populations were analyzed in Figures 3.8 to 3.9. Day 0 PBMCs before vaccination were compared to their day 28 post-prime vaccination. Vaccination with WNI induced TCMs upon recall with WNV stimulation, as seen in Figure 3.8. The percent of TCMs was significantly higher than in their day 0 samples, and the half-dose WNI also showed a significant increase. Vaccination with VET and PRES did not induce a percent increase of TCMs upon WNV recall. Populations reported as a negative change had less recorded memory cells in their simulated samples then their unstimulated samples resulting in the negative value. The stimulated populations had reportable memory populations but was not specific to WNV recall. Effector memory CD8+ T cells were not reliably identified, as a portion of cells stained for this phenotype on day 0, inferring the staining could have been nonspecific, as seen in Figure 3.9. Populations reported as a negative change had less recorded memory cells in their simulated samples then their unstimulated samples resulting in the negative value. The stimulated populations had reportable memory populations but was not specific to WNV recall.

Next, the activation of the memory phenotype was assessed by intracellular IFNγ expression. Central memory T cells on day 28 responded to WNV stimulation with a percentage increase in cells expressing IFNγ (Figure 3.8). Vaccination with WNI and VET induced an

increase in the percentage of TCMs expressing IFNγ after WNV stimulation, but this increase was not significant compared to their day 0 samples or to the control samples. Vaccination with PRES did not induce an increased percentage of TCMs expressing IFNγ after WNV stimulation. There were no significant differences in percentage of TCMs expressing IFNγ between the vaccine dosages after stimulation. Vaccination with WNI and VET induced an increase in the percentage of TEMs expressing IFNγ after WNV stimulation on day 28 but the increase was not significantly different from their day 0 samples. As control samples also had an increased percentage of cells expressing IFNγ after WNV stimulation.

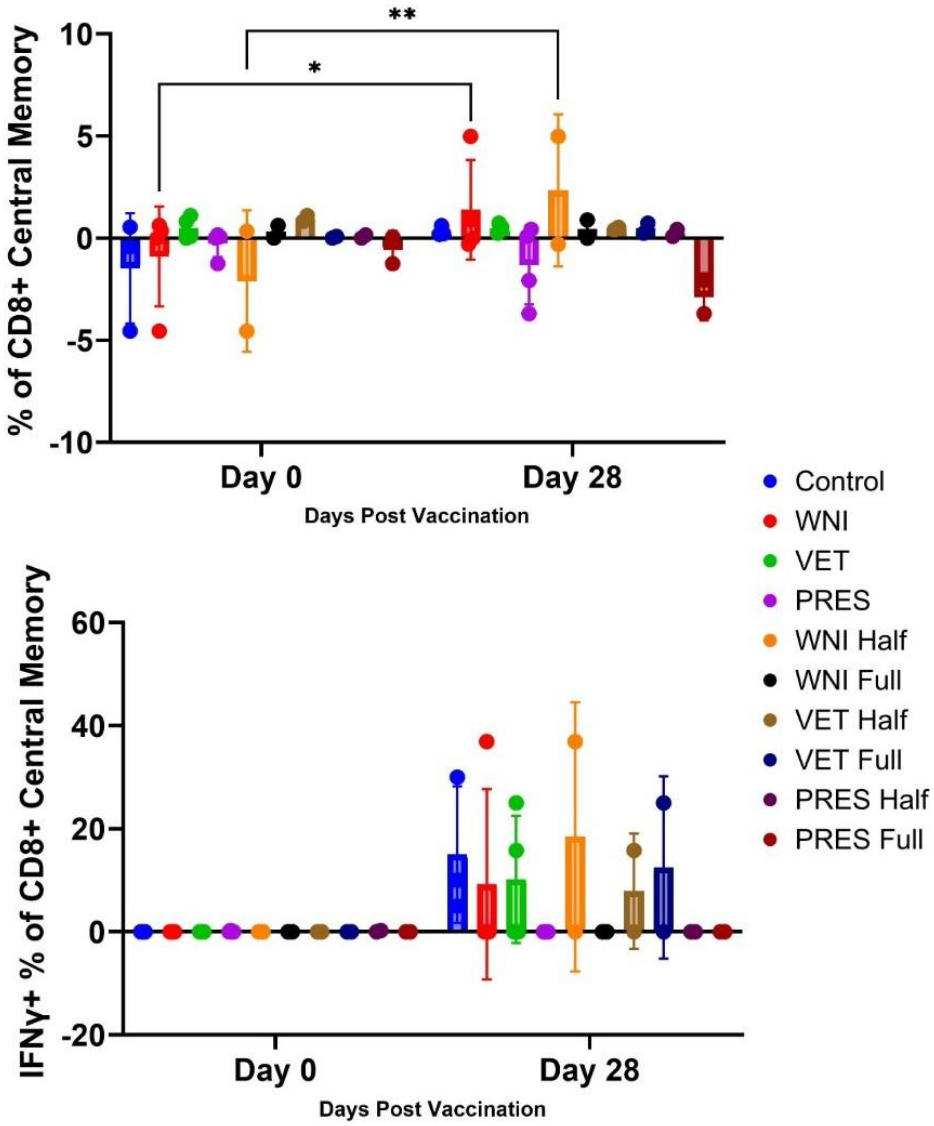

Figure 3.8 Vaccination with WNI increases CD8+ central memory T cells after WNV stimulation

PBMCs from vaccinated and control pigs were stimulated with WNV then analyzed for central memory T cells. The central memory T cells were then analyzed for IFNγ expression. Vaccination with WNI (red) increased the percentage of CD8+ central memory T cells on day 28 after WNV stimulation. Vaccination with WNI (red) and VET (green) increased the percentage of CD8+ central memory T cells expressing IFNγ on day 28 after WNV stimulation. **p*-value <0.03, ***p*-value 0.002.

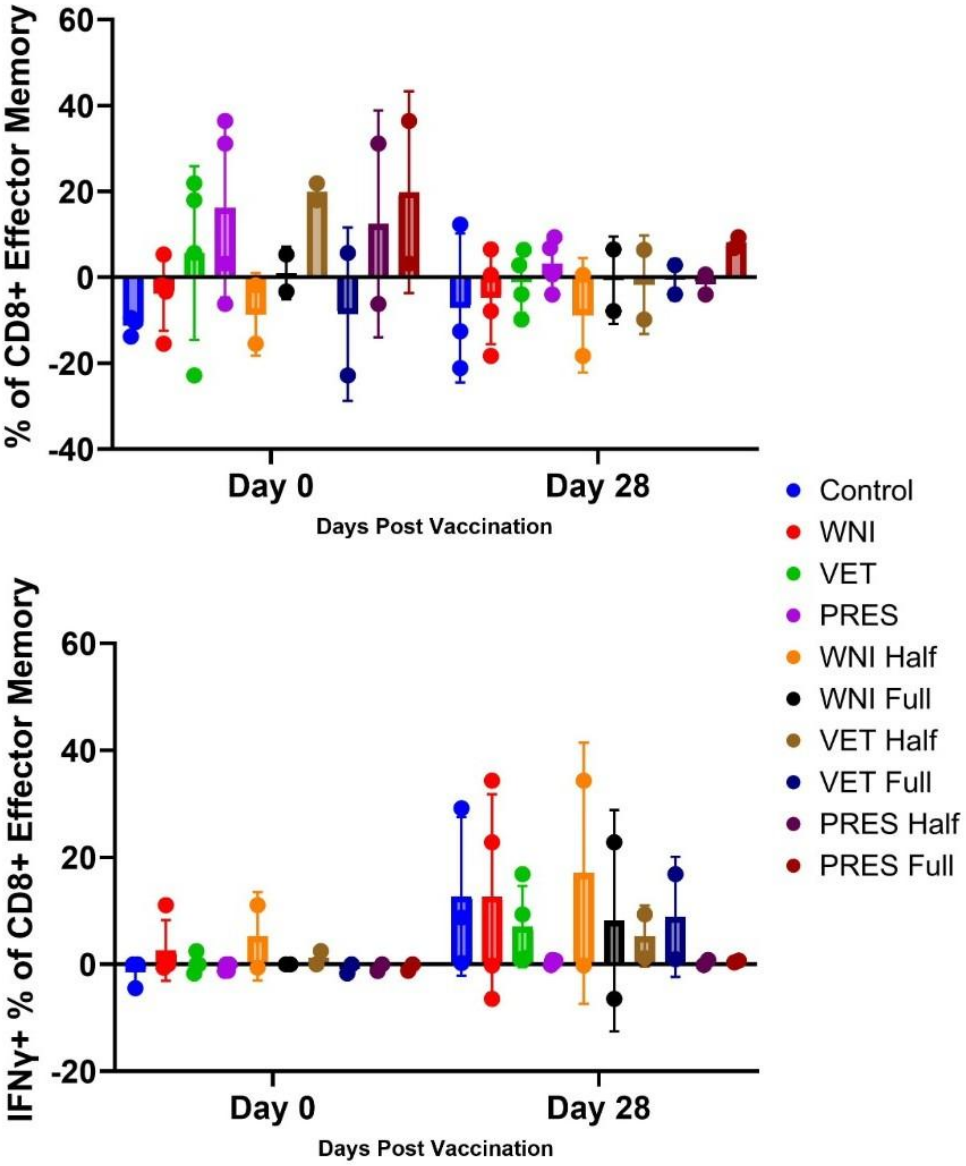

Figure 3.9 Vaccination did not increase CD8+ effector memory T cells after WNV stimulation

PBMCs from vaccinated and control pigs were stimulated with WNV then analyzed for effector memory T cells. The effector memory T cells were then analyzed for IFNγ expression. None of the vaccines increased the percentage of CD8+ effector memory T cells on day 28 after WNV stimulation. Vaccination with WNI (red) increased the percentage of CD8+ effector memory T cells expressing IFNγ on day 28 after WNV stimulation but was not significant.

3.b.2 Memory CD8+ T cells to JEV

Memory CD8+ T cell populations were analyzed in Figures 3.10 to 3.11. Day 0 PBMCs before vaccination were compared with their day 28 post-prime vaccination samples. TCMs and TEMS in response to JEV stimulation were varied across timepoints and treatments. A large portion of cells stained for this phenotype on day 0, inferring the staining could have been nonspecific for the true phenotype. As a result, it is difficult to determine whether vaccinated samples are true memory subtypes. There was no significant difference between timepoints or treatments for the generation of CD8+ TCMs or TEMs. Populations reported as a negative change had less recorded memory cells in their simulated samples then their unstimulated samples resulting in the negative value. The stimulated populations had reportable memory populations but was not specific to JEV recall.

The activation of CD8+ memory T cells was evaluated by intracellular IFNγ expression following JEV stimulation. CD8+ TCMs responded to JEV stimulation in the day 28 samples (Figure 3.10). Vaccination with WNI or VET induced activated TCMs in response to JEV stimulation, with an increased percentage of IFNγ-expressing cells. Though these results were not significant compared to their day 0 samples or control samples. CD8+ TEMs followed a similar pattern to the TCMs. Vaccination with WNI or VET increased the percentage of IFNγ-expressing cells, though not significantly, compared to their day 0 samples (Figure 3.11). PRES vaccination did not induce activated TCMs or TEMs to JEV stimulation.

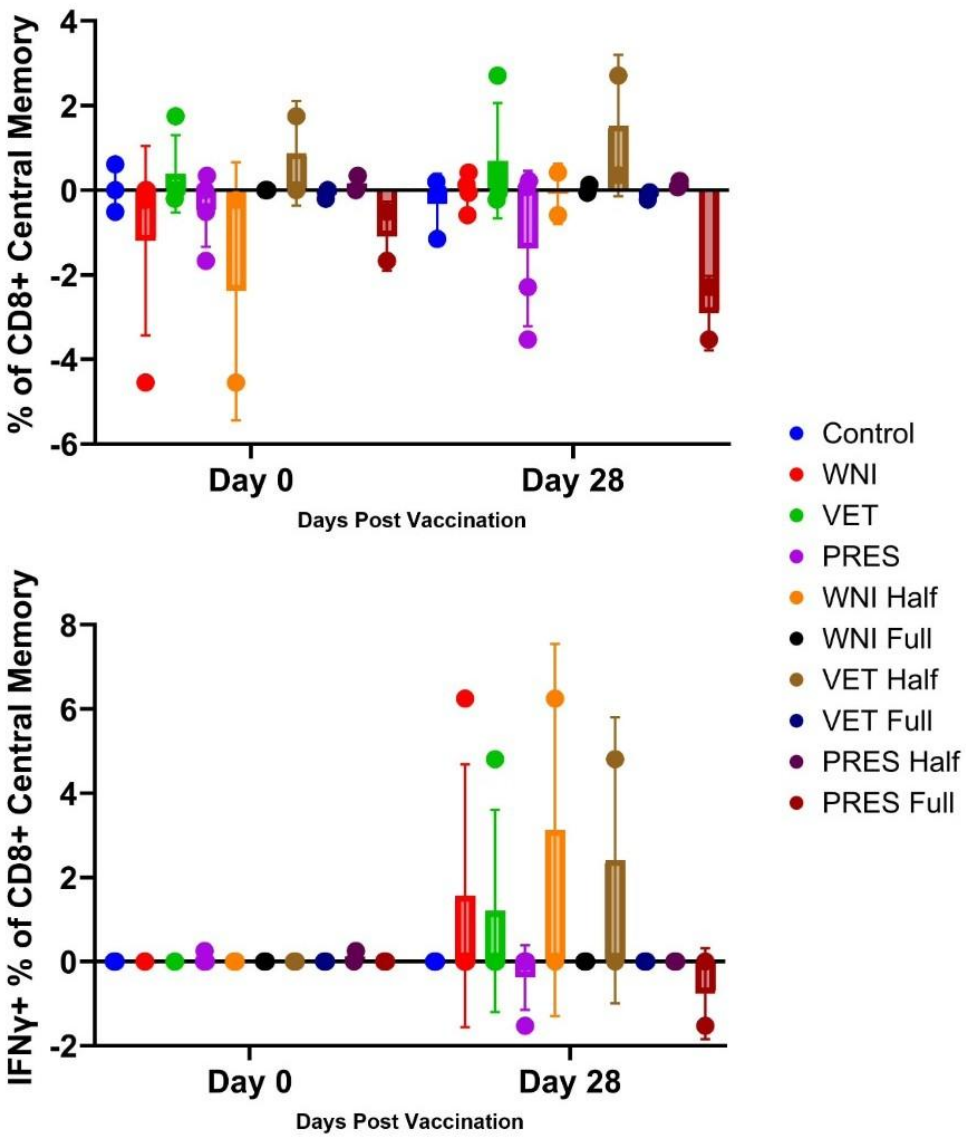

Figure 3.10 Vaccination with VET increases CD8+ central memory T cells after JEV stimulation

PBMCs from vaccinated and control pigs were stimulated with JEV then analyzed for central memory T cells. The central memory T cells were then analyzed for IFNγ expression. Vaccination with VET (green) increased the percentage of CD8+ central memory T cells on day 28 after WNV stimulation but was not significant compared to day 0. Vaccination with WNI (red) and VET (green) increased the percentage of CD8+ central memory T cells expressing IFNγ on day 28 after WNV stimulation, but was not significant.

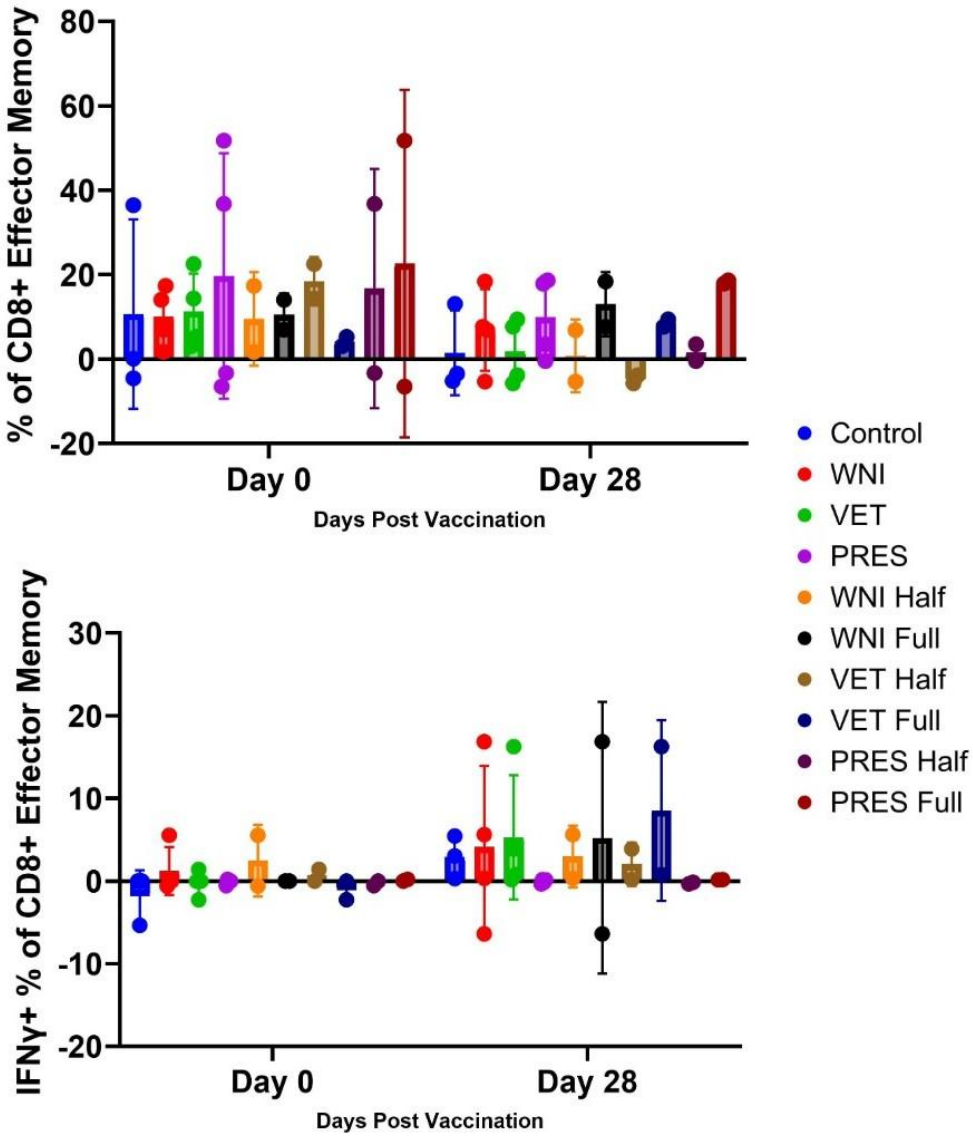

Figure 3.11 Vaccination did not increase CD8+ effector memory T cells after JEV stimulation

PBMCs from vaccinated and control pigs were stimulated with JEV then analyzed for effector memory T cells. The effector memory T cells were then analyzed for IFNγ expression. None of the vaccines increased the percentage of CD8+ effector memory T cells on day 28 after WNV stimulation. Vaccination with WNI (red) and VET (green) increased the percentage of CD8+ effector memory T cells expressing IFNγ on day 28 after WNV stimulation but was not significant.

3.b.3 Comparisons of memory CD8 + T cells to WNV vs JEV

CD8+ TCMs had a greater percentage in WNI samples in response to WNV compared to JEV, but the difference was not statistically significant. VET samples showed comparable TCMs percentages between WNV and JEV (Figure3.12). TEMs seemed to better respond to JEV but comparisons should be cautioned with populations have a negative change in the WNV stimulated group (Figure 3.13). Populations reported as a negative change had less recorded memory cells in their simulated samples then their unstimulated samples resulting in the negative value.

CD8+ TCMs had greater percentages of cells expressing IFNγ in response to WNV stimulation compared to JEV. WNI and VET samples showed similar responses, with only a small percentage of cells positive for IFNγ expression in response to JEV stimulation but was not significant (Figure 3.12). CD8+ TEMs followed a similar pattern with a reduced response in IFNγ expression to JEV stimulation (Figure 3.13)

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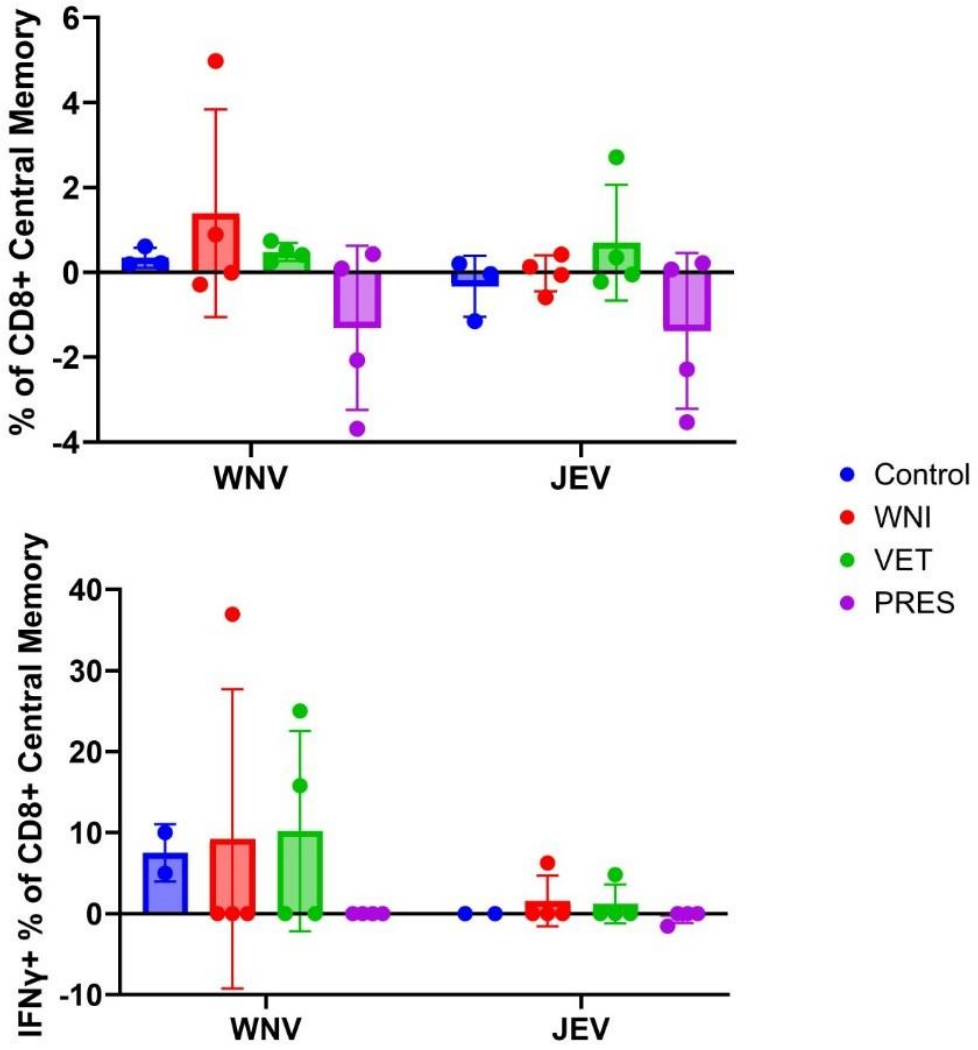

Figure 3.12 Comparisons of CD8+ central memory T cells in response to WNV vs JEV

Data from day 28 stimulated PBMC samples were compared to evaluate responses to viral stimulation. Vaccination with WNI (red), VET (green), had increased percentage of CD8+ central memory T cells to WNV stimulation. Vaccination with VET (green) had increased memory cells to JEV compared to WNI and PRES. Vaccination with WNI (red) and VET (green) had increased percentages of CD8+ central memory T cells expressing IFNγ to WNV. IFNγ expression from JEV stimulation was limited.

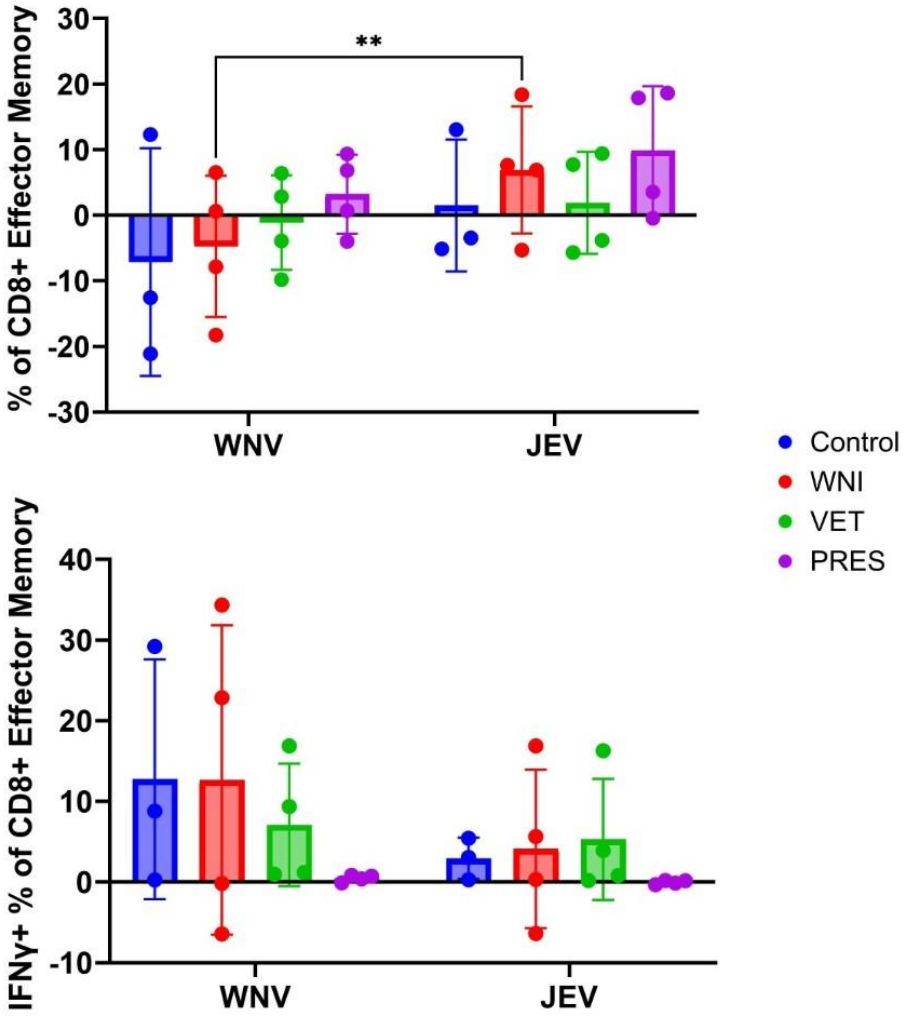

Figure 3.13 Comparisons of CD8+ effector memory T cells in response to WNV vs JEV

Data from day 28 stimulated PBMC samples were compared to evaluate responses to viral stimulation. Vaccination did not increase the percentage of CD8+ effector cells after WNV stimulation. Vaccination with WNI (red) and PRES (purple) did have increased effector memory cells after JEV stimulation. Vaccination with VET (green) had similar percentages of CD8+ effector memory T cells expressing IFNγ to WNV and JEV stimulation. ***p*-value 0.002.

3.c Memory CD4+CD8+ T cell responses

In pigs, $CD4^{+}CD8^{+}$ double-positive T cells are major source of antigen-experienced effector and cytotoxic lymphocytes (Zuckermann & Husmann, 1996).An example of this are pigs that recover from Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) infection develop cytotoxic $CD4^{+}CD8^{+}$ T cells that kill virus-infected macrophages (Chung et al., 2018).Therefore they are important immune population to examine.

3.c.1 Memory CD4+CD8+ T cells to WNV

Double-positive CD4+CD8+ memory T cells were analyzed after WNV stimulation. Comparing the day 0 samples to day 28 samples there was no increase in the percentages of TCMs across the vaccines (Figure 3.14). Populations reported as a negative change had less recorded memory cells in their simulated samples then their unstimulated samples resulting in the negative value. The stimulated populations had reportable memory populations but was not specific to WNV recall. WNI and VET vaccination did not induce CD4+CD8+ TEMs after WNV stimulation (Figure 3.15). Interestingly, PRES full-dose vaccination led to a significant increase in the percentage of TEMs compared to day 0 samples. The PRES full-dose samples showed a significant increase in the percentage of memory cells compared to the half-dose samples on day 28. Populations reported as a negative change had less recorded memory cells in their simulated samples then their unstimulated samples resulting in the negative value. The stimulated populations had reportable memory populations but was not specific to WNV recall.

Next, the activation of the memory phenotype was assessed by intracellular IFNγ expression. Central memory CD4+CD8+ T cells showed increased IFNγ expression in response to WNV stimulation on day 28 in WNI and VET vaccinated samples (Figure 3.14). WNI

vaccination, including the half-dose, led to a significant increase in cells expressing IFNγ compared to day 0 samples after stimulation. VET vaccination induced a response, but it was not significant compared to day 0 samples. PRES vaccination did not have an increase in TCMs expressing IFNγ after stimulation. IFNγ expression in the effector memory CD4+CD8+ T cells was seen in both day 0 and day 28 samples, inferring that this IFNγ expression may have been nonspecific to memory recall from WNV stimulation (Figure 3.15).

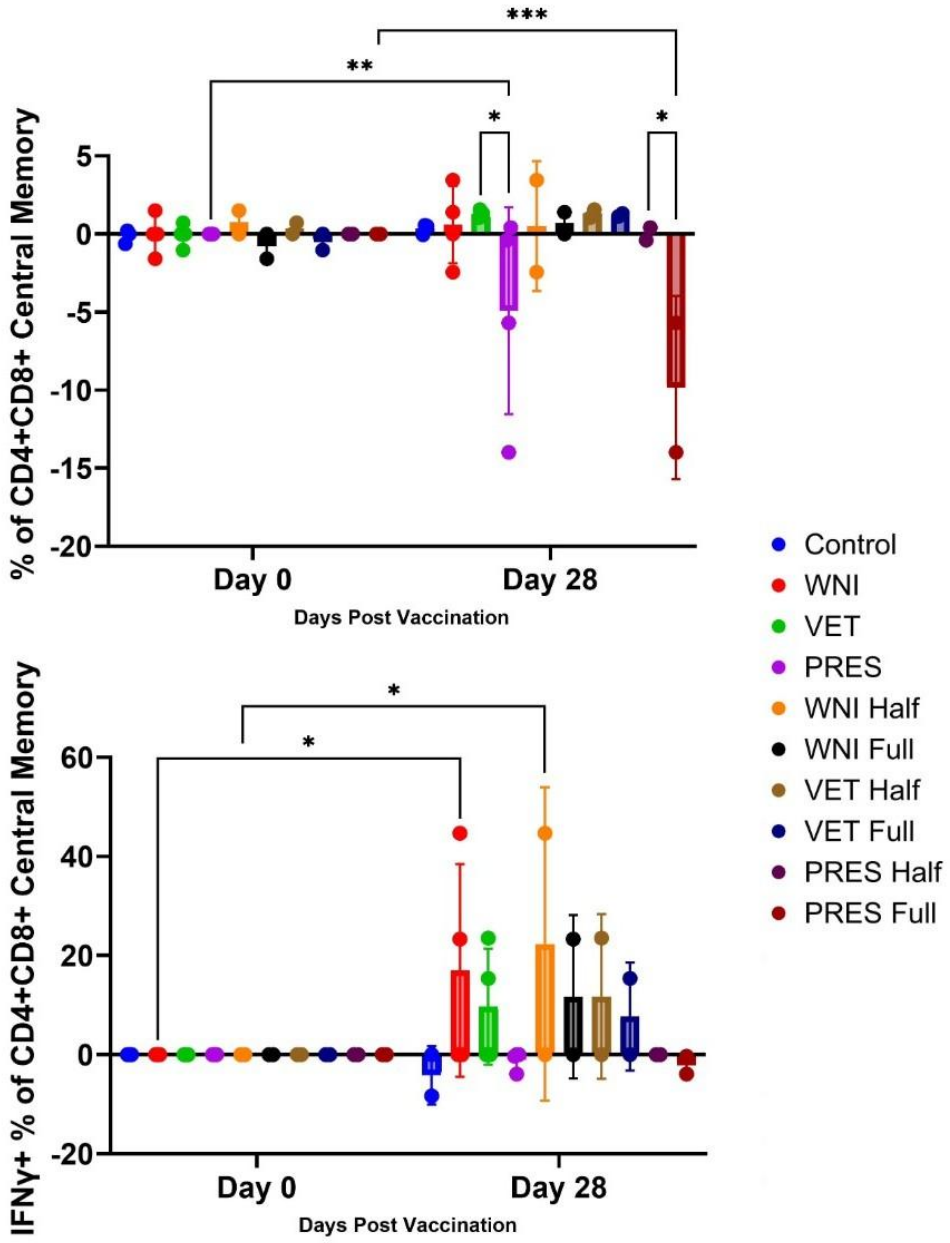

Figure 3.14 Vaccination with WNI and VET increased IFNγ+ CD4+CD8+ central memory T cells after WNV stimulation

PBMCs from vaccinated and control pigs were stimulated with WNV then analyzed for central memory T cells. The central memory T cells were then analyzed for IFNγ expression. None of the vaccines increased the percentage of CD4+CD8+ central memory T cells. Vaccination with WNI (red) and VET (green) increased the percentage of CD4+CD8+ central memory T cells expressing IFNγ on day 28 after WNV stimulation. *p-value <0.03, **p-value 0.002, *** p-value 0.0002.

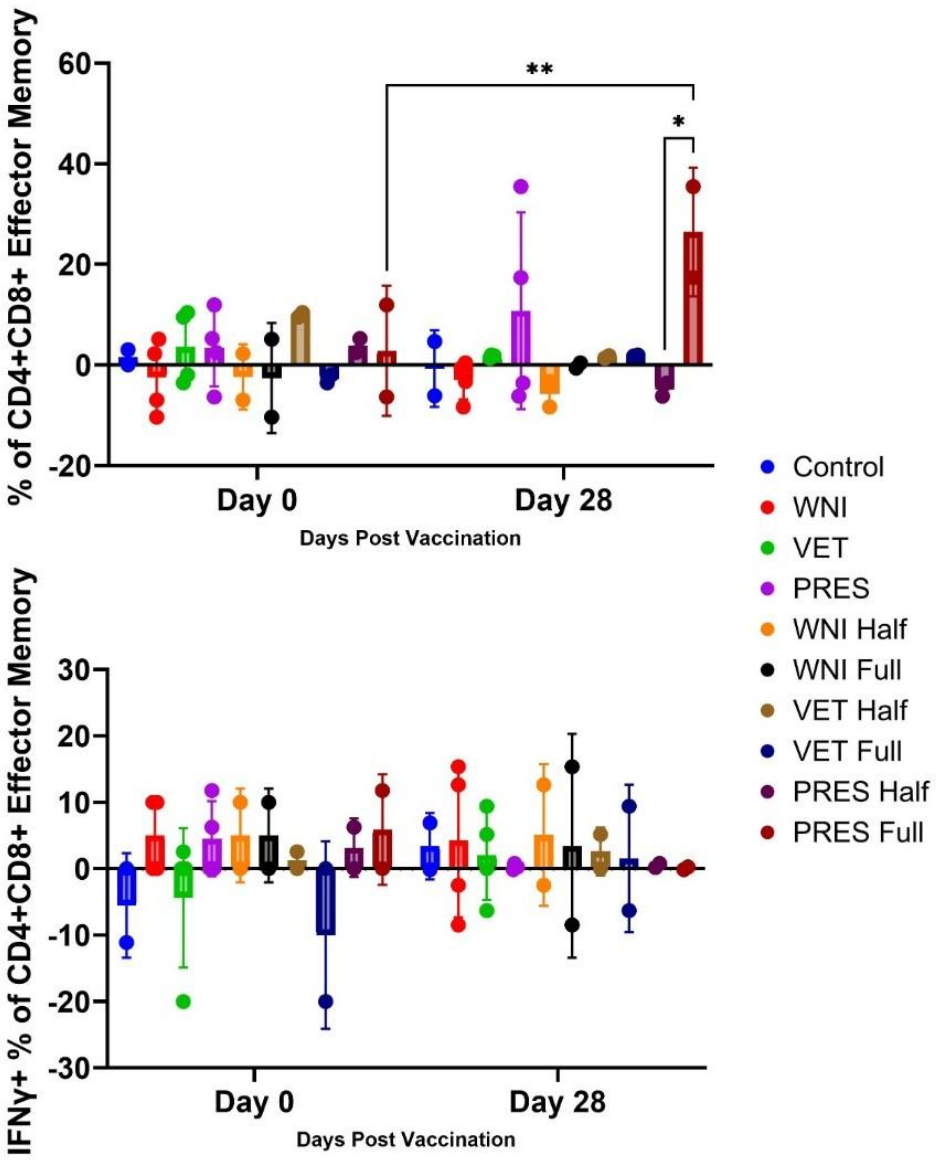

Figure 3.15 Vaccination with PRES increased CD4+CD8+ effector memory T cells after WNV stimulation

PBMCs from vaccinated and control pigs were stimulated with WNV then analyzed for central memory T cells. The central memory T cells were then analyzed for IFNγ expression. Vaccination with PRES increased the percentage of CD4+CD8+ effector memory T cells. None of the vaccines increased the percentage of CD4+CD8+ effector memory T cells expressing IFNγ on day 28 after WNV stimulation. ***p*-value 0.002.

3.c.2 Memory CD4+CD8+ T cells to JEV

Double-positive CD4+CD8+ memory T cells were analyzed after JEV stimulation. VET vaccinated samples had a slight increase in TCMs but was not significant compared to their day 0 samples. Populations reported as a negative change had less recorded memory cells in their simulated samples then their unstimulated samples resulting in the negative value. The stimulated populations had reportable memory populations but was not specific to JEV recall. Similar to the CD8+ TEMs, the CD4+CD8+ TEMs showed varied responses (Figure 3.16. Full-dose vaccination with PRES increased the percentage of TEMs compared with their day 0 samples. Neither WNI nor VET vaccination had a significant impact on TEMs.

Central memory CD4+CD8+ T cells showed an increased percentage of IFNγ expressing cells after JEV stimulation (Figure 3.17). Vaccination with WNI and VET had increased activated TCMs to JEV stimulation. The WNI half-dose group had the highest IFNγ response in TCMs, which was significantly higher percentage than their day 0 samples. Effector memory CD4+CD8+ T cells were varied in their IFNγ response to JEV stimulation, Figure 3.17. Populations reported as a negative change had less recorded IFNγ positive cells in their simulated samples then their unstimulated samples resulting in the negative value. The stimulated populations had reportable populations but was not specific to JEV recall.

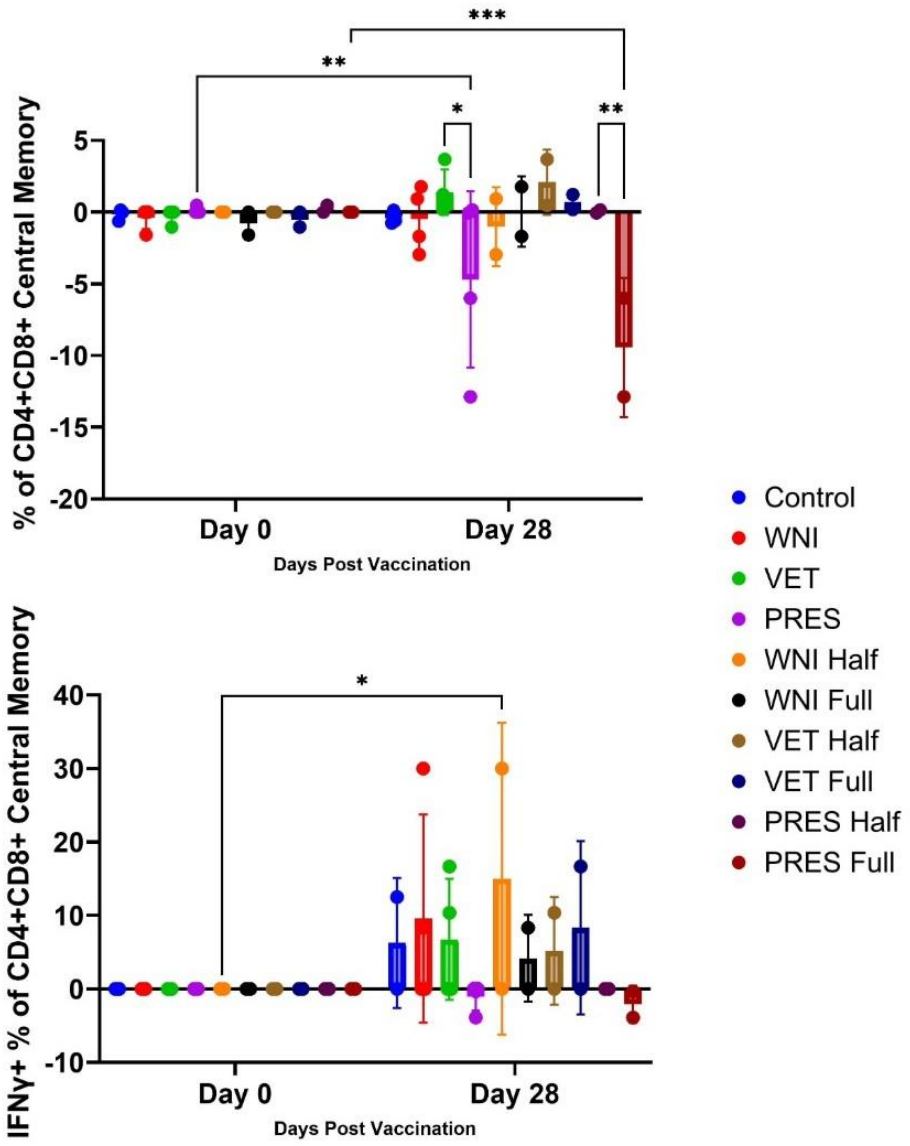

Figure 3.16 Vaccination did not increase CD4+CD8+ central memory T cells after JEV stimulation

PBMCs from vaccinated and control pigs were stimulated with JEV then analyzed for central memory T cells. The central memory T cells were then analyzed for IFNγ expression. None of the vaccines increased the percentage of CD4+CD8+ central memory T cells. Vaccination with WNI (red) and VET (green) increased the percentage of CD4+CD8+ central memory T cells expressing IFNγ on day 28 after WNV stimulation. *p-value <0.03, **p-value 0.002, *** p-value 0.0002.

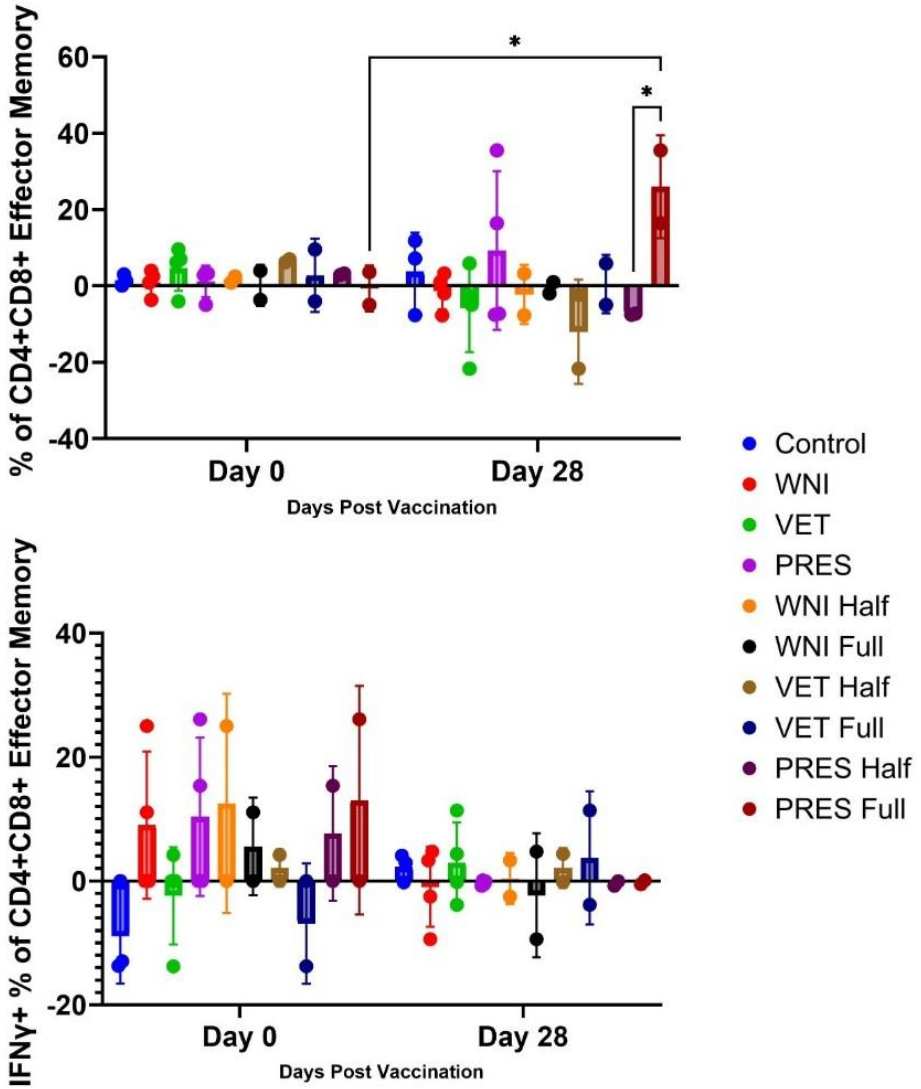

Figure 3.17 Vaccination with PRES increases CD4+CD8+ effector memory T cells after JEV stimulation

PBMCs from vaccinated and control pigs were stimulated with JEV then analyzed for effector memory T cells. The effector memory T cells were then analyzed for IFNγ expression. Vaccination with PRES increased the percentage of CD4+CD8+ effector memory T cells. None of the vaccines increased the percentage of CD4+CD8+ effector memory T cells expressing IFNγ on day 28 after WNV stimulation. *p-value <0.03.

3.c.3 Comparisons of memory CD4+CD8 + T cells to WNV vs JEV

Vaccination induced very few CD4+CD8+ TCMs to either virus. VET samples had a small percentage of memory cells to both WNV and JEV but was not significant (Figure 3.18). Populations reported as a negative change had less recorded memory cells in their simulated samples then their unstimulated samples resulting in the negative value. The percentages of CD4+CD8+ TEMs were similar across the viruses, and PRES samples responded positively to stimulation, Figure 3.19.

IFNγ expression for both TCMs and TEMs was similar between the two viral stimulations. TCMs and TEMs from WNI samples had greater percentages of IFNγ-expressing cells in response to WNV stimulation. PRES samples did not induce activated TCMs or TEMs in response to either viral stimulus.

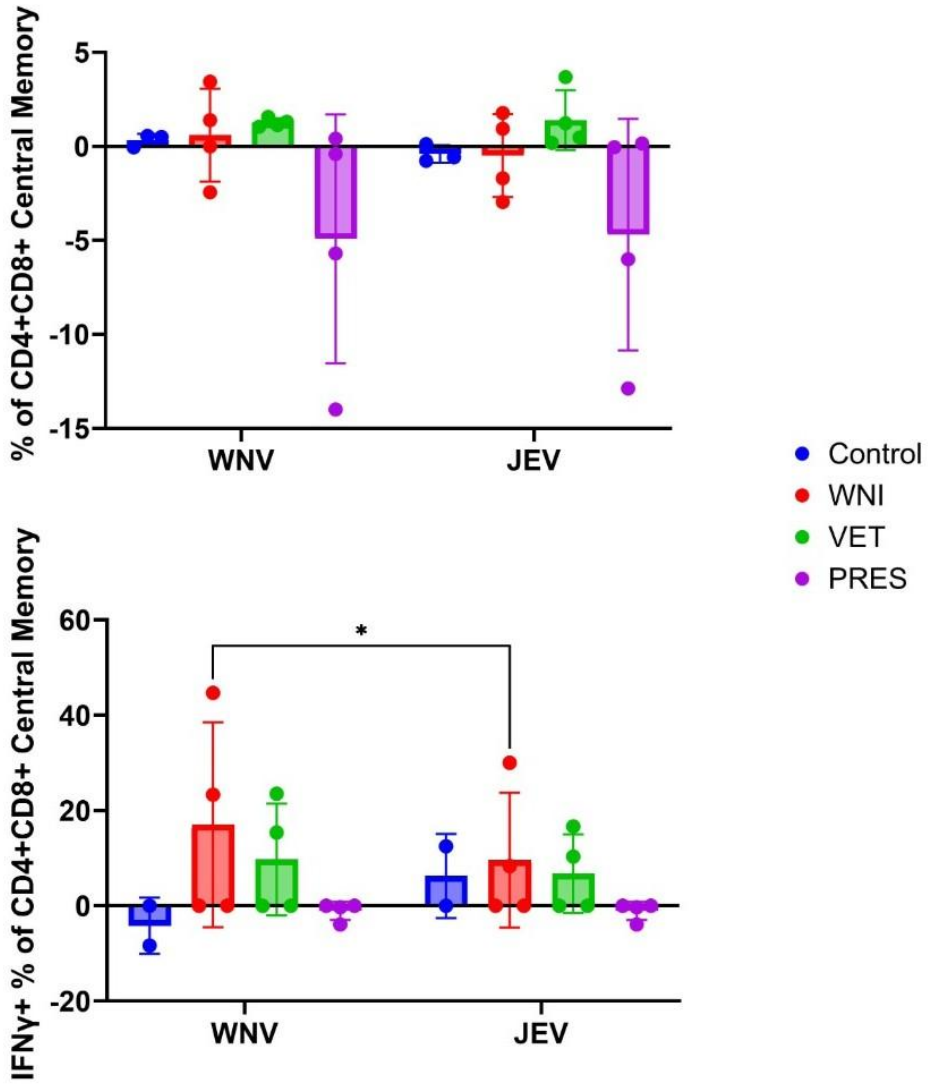

Figure 3.18 Comparisons of CD4+CD8+ central memory T cells in response to WNV vs JEV

Data from day 28 stimulated PBMC samples were compared to evaluate responses to viral stimulation. Vaccination with VET (green), had increased percentage of CD4+CD8+ central memory T cells to WNV and JEV stimulation. Vaccination with VET (green) had increased memory cells to JEV compared to WNI and PRES. Vaccination with WNI (red) had increased percentages of CD4+CD8+ central memory T cells expressing IFNγ to WNV. *p-value <0.03.

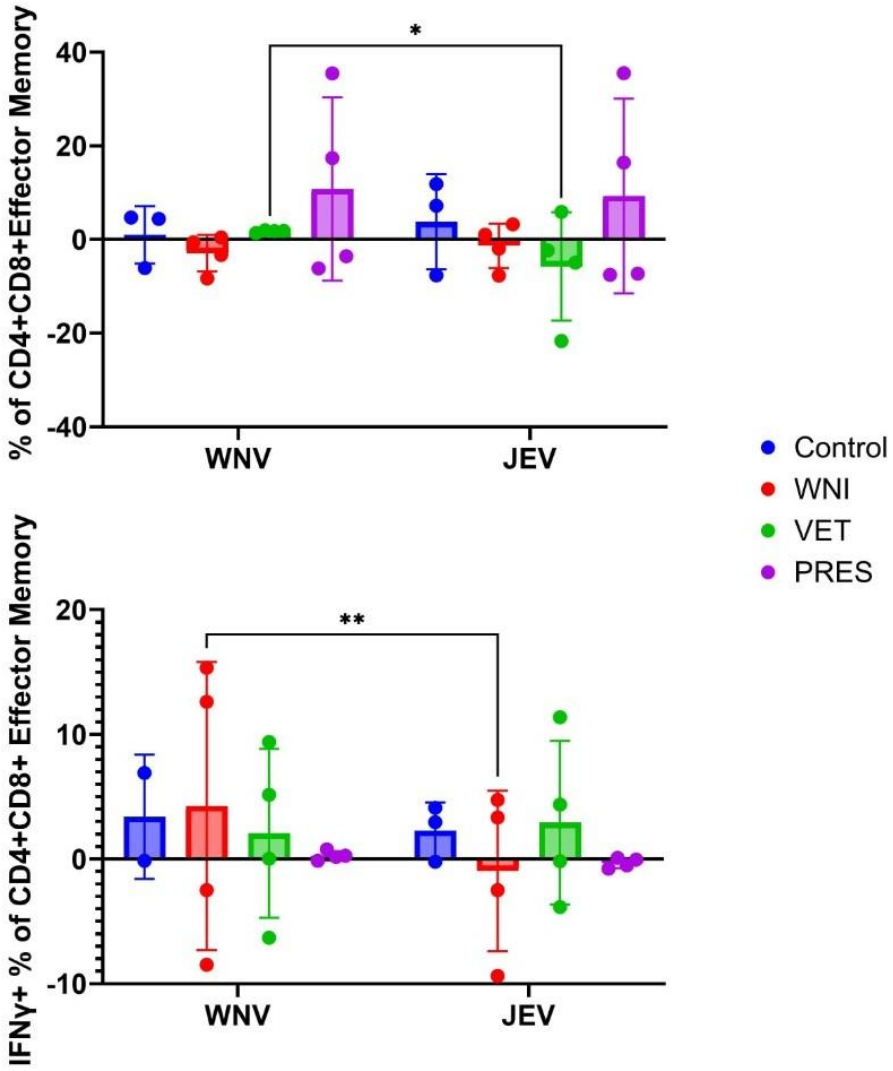

Figure 3.19 Comparisons of CD4+CD8+ effector memory T cells in response to WNV vs JEV

Data from day 28 stimulated PBMC samples were compared to evaluate responses to viral stimulation. Vaccination with PRES (purple), had increased percentage of CD4+CD8+ effector memory T cells to WNV and JEV stimulation. Vaccination with WNI (red) had increased percentages of CD4+CD8+ effector memory T cells expressing IFNγ to WNV. **p*-value <0.03, ***p*-value 0.002.

3.d NK cell and γδ T cell responses

Swine naturally have a large portion of gamma delta T cells (γδ T cells) that play a role in immune responses. They have been identified as cytotoxic, like CD8+ CTLs, and can provide protection, but the specific mechanisms by which they recognize target cells remain unknown (Bettin et al., 2024). NK cells are a type of cytotoxic lymphocyte that can recognize and kill virally infected cells through the presentation of stressed induced ligands by the infected cells or the down regulation of MHC-I on the infected cell (Quatrini et al., 2021). Both of these lymphocyte populations are important to examine along with the memory T cell subsets. Though γδ T cells and NK cells are not memory T cells, they are important in bridging the response between the innate and adaptive immune system. As they can also secrete cytokines that aid in defense mechanisms against pathogens.

3.d.1 NK cells and γδ T cells responses to WNV

The percentage of γδ T cells was analyzed in Figure 3.20. VET and PRES vaccination induced a higher percentage of γδ T cells than their day 0 samples. The full dose of PRES significantly increased the percentage compared to the half dose. As expected, the percentage of NK cells did not change between vaccine groups.

The expression of IFNγ was examined in both the γδ T cell and NK cell populations after WNV stimulation, Figure 3.20. IFNγ expression in γδ T cells varied across time points and vaccine treatments. Based on the data, mock-vaccinated pigs showed a natural response to WNV stimulation in the γδ T cell population. PRES vaccination did not induce γδ T cells to increase IFNγ expression after WNV stimulation. The NK cell population responded to WNV stimulation in control, WNI and VET samples (Figure 3.21). Though we cannot conclude this response is

specific to the vaccines or WNV stimulation as NK cells do not specifically recognize viral antigens.

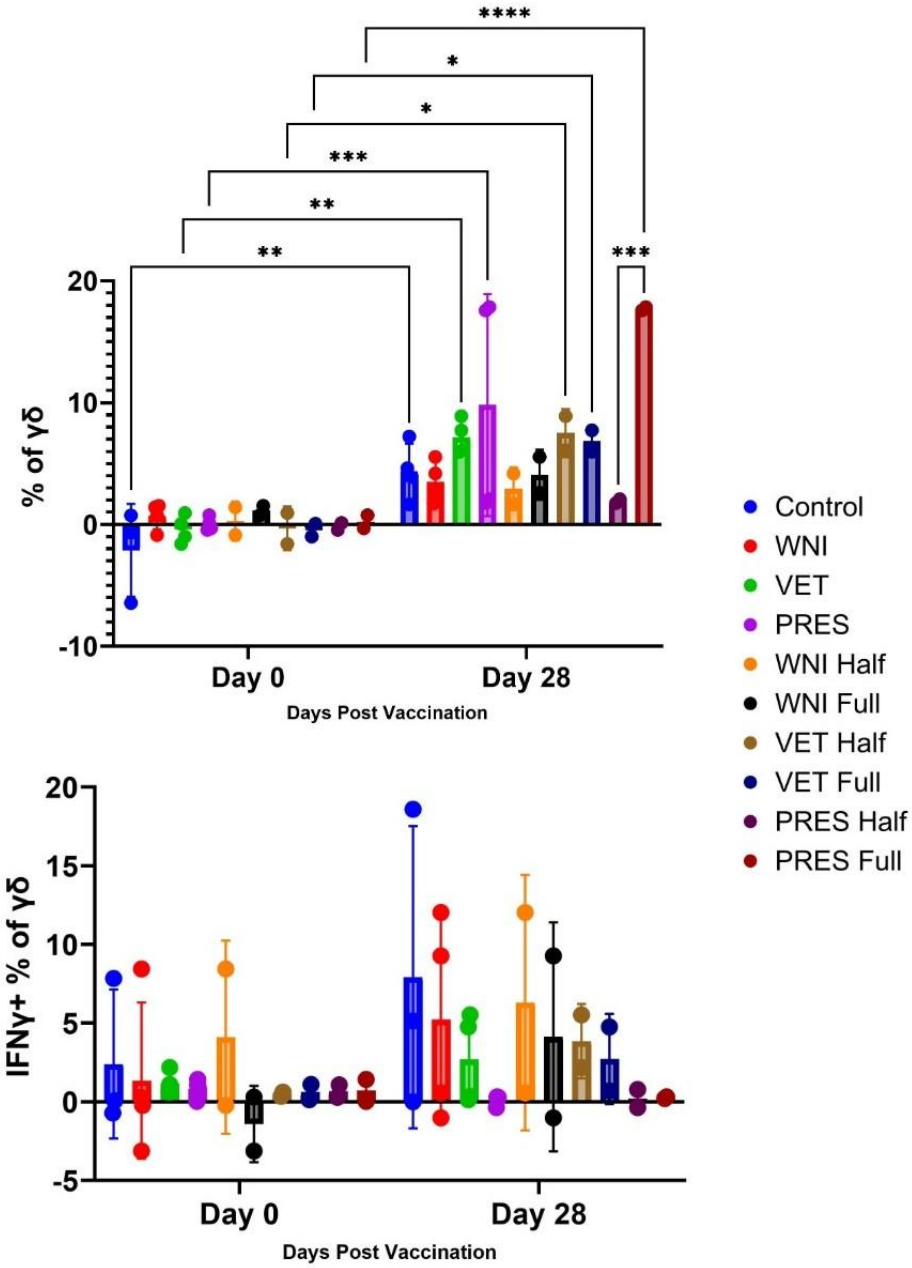

Figure 3.20 Vaccination increased % of γδ T cells after WNV stimulation

PBMCs from vaccinated and control pigs were stimulated with WNV then analyzed for gamma delta T cells. Gamma delta T cells were then analyzed for IFNγ expression. Vaccination with VET (green) and PRES (purple) increased the percentage of gamma delta T cells. The percentage of gamma delta T cells expressing IFNγ on day 28 after WNV stimulation was varied. *p-value <0.03, **p-value 0.002, *** p-value 0.0002, **** p-value 0.0001.

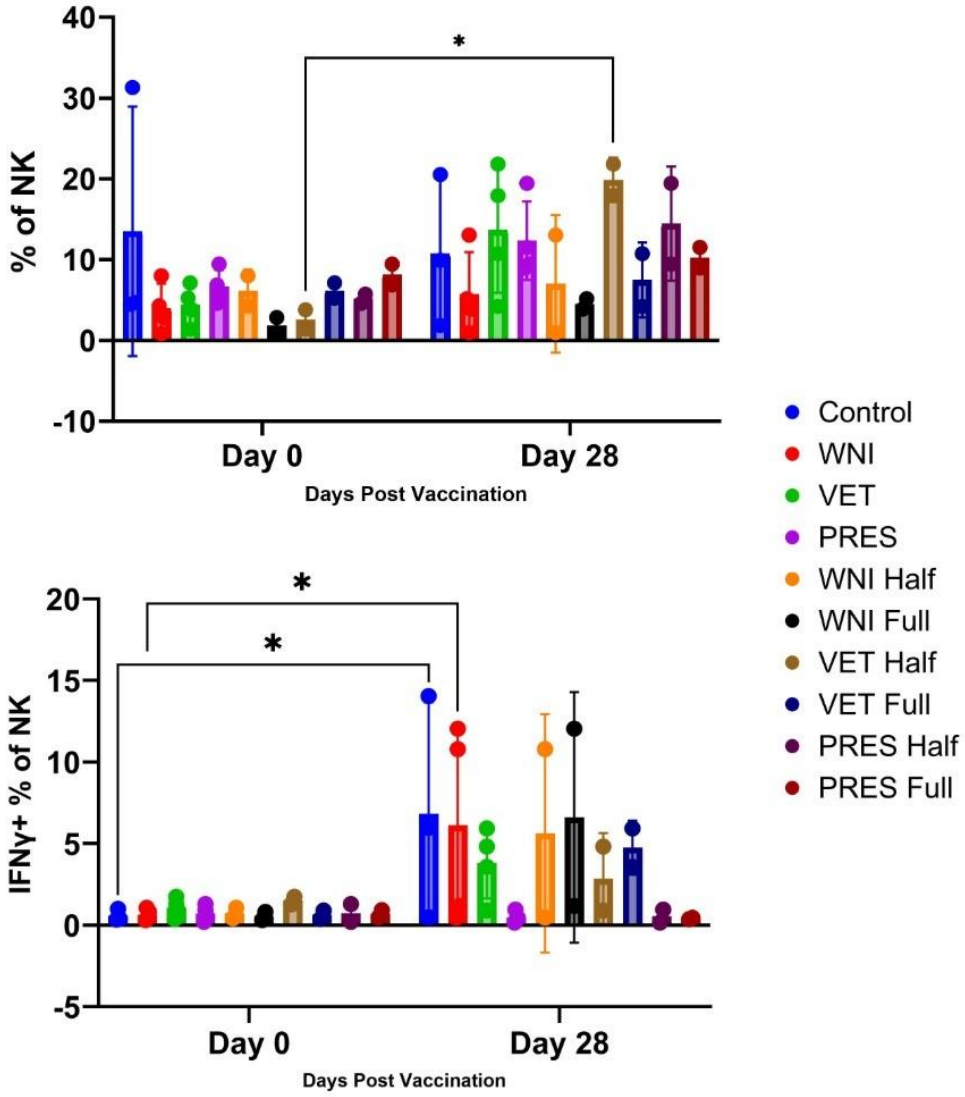

Figure 3.21 NK cell responses after WNV stimulation

PBMCs from vaccinated and control pigs were stimulated with WNV then analyzed for NK cells. NK cells were then analyzed for IFNγ expression. NK cell responses were consistent across the groups. Control and WNI samples had increased IFNγ expression on day 28. *p-value <0.03.

3.d.2 NK cells and γδ T cells responses to JEV

The percentage of gamma delta T cells was higher in day 28 PRES vaccinated samples after JEV stimulation, Figure 3.22. VET samples also showed a slight increase, but it was not significant. Interestingly, PRES vaccination did not induce activated γδ T cells upon JEV stimulation, even though it increased the percentage of γδ T cells. WNI or VET vaccination resulted in a higher percentage of γδ T cells expressing IFN-γ after JEV stimulation than in their day 0 samples. The percentages of NK cells were varied across time point and treatment. IFNγ expression after JEV stimulation was increased for WNI and VET groups, Figure 3.23. Though this increase is likely not due to the vaccine as NK cells are an innate immune cell population and do not have memory. Populations reported as a negative change had less recorded memory cells in their simulated samples then their unstimulated samples resulting in the negative value. The stimulated populations had reportable memory populations but was not specific to JEV recall.

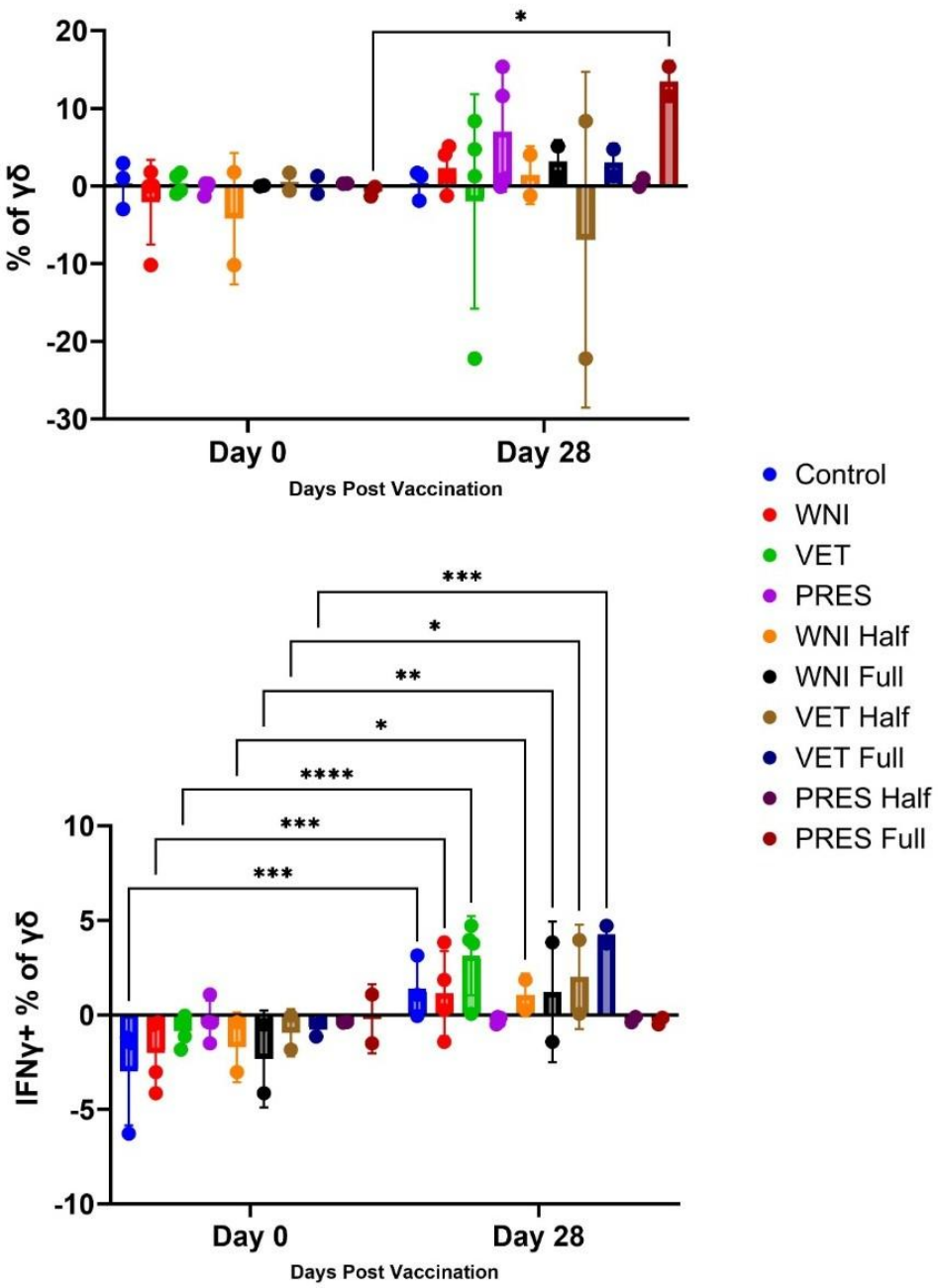

Figure 3.22 Vaccination with PRES increases % of γδ T cells after JEV stimulation

PBMCs from vaccinated and control pigs were stimulated with JEV then analyzed for gamma delta T cells. Gamma delta T cells were then analyzed for IFNγ expression. Vaccination with PRES (purple) increased the percentage of gamma delta T cells. The percentage of gamma delta T cells expressing IFNγ on day 28 after JEV stimulation was increased in control (blue), WNI (red), and VET (green) samples. **p*-value <0.03, ***p*-value 0.002, *** *p*-value 0.0002, **** *p*-value 0.0001.

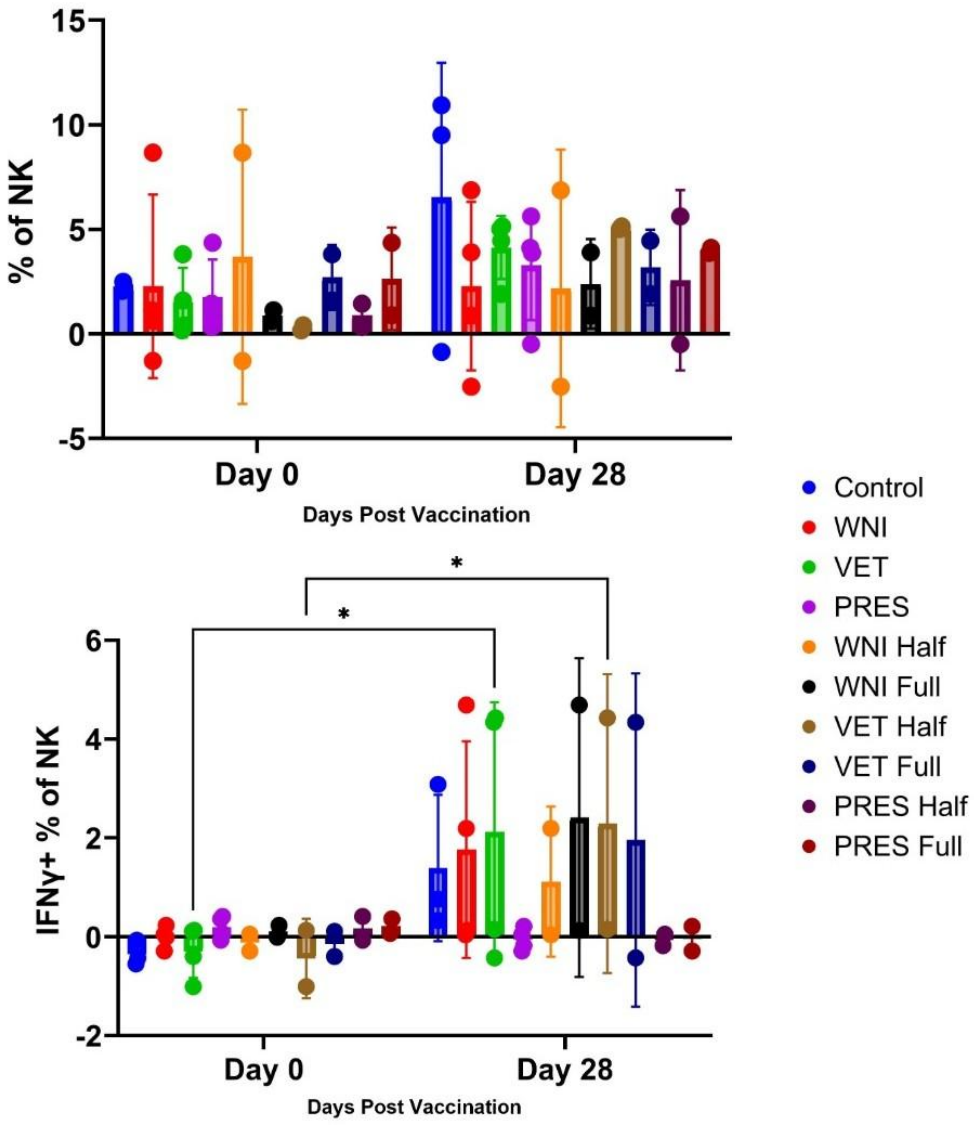

Figure 3.23 NK cell populations after JEV stimulation

PBMCs from vaccinated and control pigs were stimulated with JEV then analyzed for NK cells. NK cells were then analyzed for IFNγ expression. NK cell responses were consistent across the groups. WNI and VET samples had increased IFNγ expression on day 28. *p-value <0.03.

3.d.3 Comparisons of NK cells and γδ T cells responses to WNV vs JEV

The activation of γδ T cells and NK cells was similar across all viral stimulations. Vaccination with WNI or VET induced activated γδ T cells to either virus. Though stimulation with WNV induced a greater percentage of IFNγ-expressing cells in the WNI samples than JEV stimulation (Figure 3.24). NK cell activation was similar to the viruses which was expected because NK cells are an innate immune cell that do not have memory.

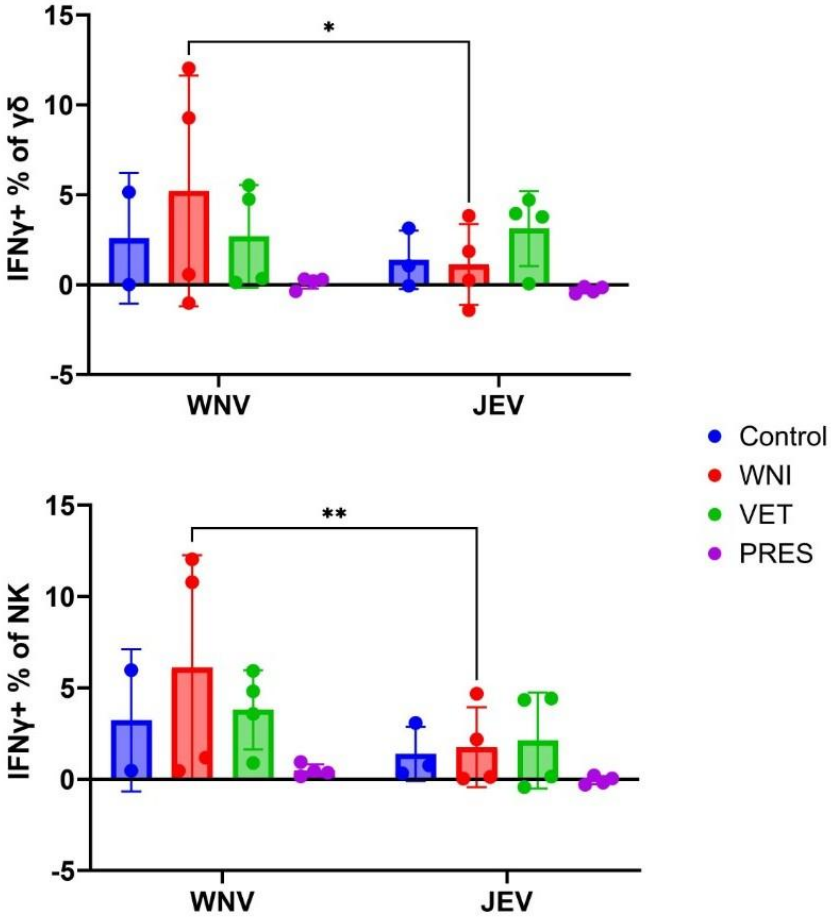

Figure 3.24 Comparisons of IFNγ % of γδ T cells and NK cells in response to WNV vs JEV

Data from day 28 stimulated PBMC samples were compared to evaluate responses to viral stimulation. Vaccination with WNI (red) had increased percentages of gamma delta T cells expressing IFNγ to WNV. Vaccination with VET (green) had similar percentages of cells expressing IFNγ to WNV and JEV. The percentage of NK cells expressing IFNγ was reduced to JEV stimulation. **p*-value <0.03, ***p*-value 0.002.

Discussions

Memory WNV discussion

Overall, each of the three WNV vaccines induced varying T cell memory responses to recall upon *in vitro* WNV stimulation. Memory generation enables a faster, more robust response upon recall following virus reinfection. WNI and VET vaccination induced greater central memory T cell generation in the CD4+ and CD8+ T cell populations than PRES vaccination. The TCM population on day 28 across the T cell phenotypes had increased IFNγ expression in the WNI and VET vaccinated groups. The increase in CD4+ TCMs could help stimulate faster antibody responses after infection with WNV. As CD4+ TCMs can help drive helper T cell functions in activating B cells. Gamma delta T cells responded to vaccination with VET and PRES samples, having an increased percentage on day 28. Though this response may not be driven by vaccination alone as gamma delta T cells are more an innate immune population. There has been evidence that gamma delta T cells isolated from individuals vaccinated with an inactivated influenza vaccine could respond to restimulation and proliferate (Re et al., 2006). There was no significant difference in the NK cell population between vaccinated and control samples after WNV stimulation. As this was expected because NK cells have no memory response. Evaluation of the effector memory phenotype seemed to be nonspecific across all the T cell phenotypes. As seen with cells staining for the markers on day zero, when there should be in theory no memory response. IFNγ expression within the effector memory subset was also varied. It should also be noted that the control pigs showed varying degrees of memory response by this assay. In theory they should not have memory responses as they did not receive a vaccine nor are pigs natural hosts of WNV. This memory response is likely nonspecific staining, so the data should be interpreted with caution. Confirmatory testing of expression IFNγ could be done by testing the cell supernatant after

stimulation for confirmatory secretion by ELISA for IFNγ. Proliferation of T cells after WNV stimulation could be another confirmatory test for more supporting evidence.

Memory JEV discussion

Overall, memory generation to JEV from WNV vaccination was limited. VET samples had the highest percentage of CD4+ TCMs among the vaccine groups. These TCMs from VET showed significant increased IFNγ expression in response to JEV stimulation compared to day 0 samples. The increase in IFNγ suggests these T cells could potentially respond to JEV infection in a helper T cell function by activating B cells. VET also increased CD8+ TCMs on day 28, but this was not significant compared to their day 0 samples. The CD8+ TCMs from VET vaccination responded to JEV stimulation with a small increase in the percentage of IFNγ expressing cells but was not significant. IFNγ expression in the gamma delta T cells and NK cells populations was increased in response to JEV stimulation across the groups. Vaccination with WNI and VET both increased IFNγ expression in gamma delta T cells and NK cells. Though the response in NK cell population is not a memory response from vaccination. Similar to WNV results, evaluation of the effector memory phenotype was nonspecific across all the T cell phenotypes. As seen with cells staining for the markers on day zero, when there should in theory be no memory response. IFNγ expression within the effector memory subset was also varied. It should also be noted that the control pigs showed varying degrees of memory response. This memory response is likely nonspecific staining, so the data should be interpreted with caution. Confirmatory testing of expression IFNγ could be done by testing the cell supernatant after stimulation for confirmatory secretion by ELISA for IFNγ. Proliferation of T cells after JEV stimulation could be another confirmatory test for more supporting evidence.

Comparisons discussion

VET vaccination may have to induce potential cross-reactive CD4+ TCMs. Specifically, VET samples had similar responses to WNV and JEV, suggesting these T cells may be cross-reactive. Overall, compared to JEV stimulation, WNV stimulation elicited greater memory recall responses. Control pigs showed varying degrees of memory response. It is important to note that this memory response is likely due to nonspecific staining, so the data should be interpreted with caution. This data should also be interpreted with caution as some samples were reported as negative changes after stimulation. As the percentage of memory cells from stimulation was less than their unstimulated samples.

Chapter 4 - Discussion and Future Directions

In this study, we examined whether or not commercially available equine WNV vaccines could generate memory T cells in swine to WNV and JEV. The generation of CD4+ central memory T cells against WNV and JEV following vaccination of swine with VET provides promising evidence that memory cells could be cross reactive to JEV. Vaccination with WNI vaccine was more specific to WNV stimulation and less cross-reactive with JEV. PRES vaccination induced CD4+CD8+ memory T cells, but they did not activate on stimulation. The selected WNV vaccines are all inactivated vaccines which rely on adjuvants in the vaccine to stimulate the immune system. Adjuvants antagonize Toll-like receptors to cause the immune system to respond (Hemmi et al., 2000). While adjuvants induce the immune system, they do not induce a strong T cell response, a better T cell memory response is seen in live replicating vaccines (Ahmed & Gray, 1996). Vaccination with an inactivated vaccine mainly drives CD4+ T helper responses not CD8+ responses which was observed in these data (Yewdell & Bennink, 1999).

Memory responses to WNV

Each of the three WNV vaccines induced varying memory T cell response to WNV in swine. WNI and VET vaccination induced greater central memory T cell generation in the CD4+ and CD8+ T cell populations than PRES vaccination. Central memory T cells can aid in protection by differentiating into helper effector cells upon reinfection (Sallusto et al., 2004). They can help sustain long-term immunity and provide support along with neutralizing antibodies to clear viral infections. CD4+CD8+ double-positive T cells exhibit properties of mature, antigen-experienced cells and can be recalled by antigen stimulation (Saalmüller et al., 1999). Vaccination did not appear to increase the number of memory CD4+CD8+ cells in response to WNV stimulation. WNI,

however, induced central memory CD4+CD8+ T cells to express IFNγ after stimulation. Swine naturally have a large proportion of γδ T cells that play a role in immune responses by producing IFNγ (Comeau et al., 2020). Vaccination with VET and PRES increased the percentage of γδ T cells on day 28. IFNγ expression in γδ T cells varied across time points and vaccine treatments. NK cells showed a strong IFNγ response to WNV stimulation in WNI and VET samples. Their stimulation could limit viral replication upon infection by destroying early-infected cells that lose MHC-I expression (Zhang et al., 2010). The three WNV vaccines induced memory responses, demonstrating that these equine vaccines can be used off-label to generate immunity in swine.

Memory response to JEV

Memory generation to JEV from WNV vaccination was limited. VET samples had the highest percentage of CD4+ central memory T cells among WNI, PRES, and VET. These TCMs showed increased IFNγ expression in response to JEV stimulation. The increase in IFNγ suggests these T cells could potentially respond to JEV infection in helper T cell functions by secreting cytokines and stimulating B cells. IFNγ had been shown to play a protective role during JEV infection by protecting the blood-brain barrier (Li et al., 2015). Polyfunctional CD4+ memory T cells, along with IFNγ and TNF-α, correlated with better clinical recovery in patients (Turtle et al., 2016). Similarly to WNV stimulation, γδ T cells showed increased IFNγ expression in VET and WNI samples. γδ T cells expand rapidly and early after JEV infection and are shown to suppress viral replication through IFNγ (Li et al., 2025). NK cells and γδ T cells could play a protective and defensive helper role against JEV infection. These results are similar to other findings that immunization with WNV first confers weaker protection against JEV (Martina et al., 2008; Petrovsky et al., 2013). Immunization with JEV first seems to confer greater protection against

WNV (Goverdhan et al., 1992). Among the three WNV vaccines, VET had the most evidence of inducing T cells that could be cross-reactive with JEV.

Limitations and Future directions

The current study has limitations that should be considered when interpreting these data. Stimulated samples were subtracted from their unstimulated samples to calculate the percentage change from stimulation as similar methods (Attreed et al., 2022; Cuesta-Martín de la Cámara et al., 2024; Ebner et al., 2017; Franzoni et al., 2013; Lee et al., 2024). This approached caused negative values to be recorded in some of the memory populations. It is not to say these memory cells had negative expression of memory markers, but a reduction in total populations after stimulation. This approach was taken to eliminate basal cytokine expression and existing surface markers from memory T cells that are not specific to the viruses of interest, allowing for observation of differences in the populations resulting from viral stimulation. Activation-Induced Marker (AIM) assays detect antigen-specific T cells by flow cytometry based on upregulation of surface activation markers after short-term antigen stimulation (Dan et al., 2016). AIM assays also take a similar approach to analyzing stimulated populations subtracted from their unstimulated samples to measure activation markers (Dan et al., 2016). In swine AIM assays have been developed to examine activated T cells after stimulation in African swine fever virus, PRRSV, and classical swine fever virus (Ebner et al., 2017; Moorton et al., 2024). The data could also be reported as percentage of the total lymphocyte population without subtraction of unstimulated controls. The unstimulated controls would be reported next to their unstimulated samples. This is would not result in negative values being reported.

The flow cytometric panel used in this study has some limitations with emission overlap in the selected fluorochromes used for marker selection. Compensation was applied to help control fluorescence spillover with single color controls. To eliminate fluorescence spillover the selected panel could be modified by conjugation of a different fluorochrome to some of the antibody markers. A new fluorochrome would allow more distance between the emission overlap limiting spillover. Commercially available antibodies for swine are limited, which impacts marker selection (Lunney, 2025). The use of a cross-reactive monoclonal IFNγ antibody may have impacted results. As this antibody may have not bound as effectively as a swine specific antibody would. Using a species-specific antibody may have captured more cells expressing IFNγ. IFNγ was selected as a marker of activation in this assay but there are limitations in the interpretation. Immune cells can naturally express IFNγ and is not a specific effector molecule to measure memory responses (Tau & Rothman, 1999). However, IFNγ is important in aiding host defenses through activation of ISGs which can promote antigen presentation and immune cell infiltration into the sites of infection. IFNγ was selected due to an inactivated vaccine being used to generate memory as inactivated vaccines drive more CD4+ T cells then CD8+ T cells. As both T cell subsets can secrete IFNγ while CD4+ cells secrete limited amounts of granzyme B compared to CD8+ T cells (Grossman et al., 2004). IFNγ is also commonly selected as an activation marker in other methods in flow cytometric analysis (Attreed et al., 2022; Franzoni et al., 2013; Lee et al., 2024; Redant et al., 2022). Though a more robust choice to measure activation from viral stimulation would be to measure perforin or granzyme B expression as these effectors are specifically secreted by activated CTLs to lyse infected cells (Waterhouse et al., 2006). An increase in either would be stronger evidence that memory cells induced from vaccination could play a defensive role against the virus they were stimulated with. The panel could be modified by eliminating IFNγ as a marker and the

addition of a granzyme B antibody instead. Though with the current method used, perforin or granzyme B expression may not be captured as the percentages of memory CD8+ T cells was quite low which in theory would result in reduced CTLs that could secrete perforin or granzyme B.

The measured memory responses reported are very low when put into the context of the total lymphocyte populations as they would be less than 1% when converted. This may be from the selected stimulation time, as overnight stimulation may have been too short to activate memory cells. A higher MOI or longer stimulation time may allow a more robust memory cell response (Linder et al., 2024). Though the stimulation time selected in our methods was in the context of other *in vitro* JEV stimulation publications (Fang et al., 2022; Redant et al., 2022). The use of peptides for stimulation may also have greater impacts on the memory response. Peptide stimulation can have greater sensitivity than whole antigens, as peptides bypass antigen processing and presentation steps (Inoue et al., 2013). The use of peptides pools to WNV or JEV epitopes can identify specific T cell responses to vaccination or infection. JEV peptide pools have been used to identify T cell epitopes specific to JEV in human stimulated PBMC samples (Pushpakumara et al., 2020). Peptide pools were not used in our methods as the specific JEV epitopes that pigs respond to are unknown with only one publication reported using NS3 for *in vitro* stimulation in pigs (Redant et al., 2022).

Additionally, mock-vaccinated control pigs showed some degree of memory responses when virally stimulated especially in effector memory populations. This affects the data interpretation in determining whether observed results are solely due to vaccination alone or are natural responses. The control pig response could be due to nonspecific staining, which could be overcome by the addition of an isotype control during staining to aid in the gating analysis or through further titrations of the antibody markers selected (Robinson et al., 2026). An isotype

control is used to estimate the nonspecific binding in a sample. The control is an antibody that is the same species and isotype (e.g. anti-swine IgG1) and fluorophore as your test antibody but does not recognize your marker of interest. Any signal the isotype antibody gives reflects nonspecific binding. Nonspecific binding occurs when the marker antibodies are binding to the FC receptor and not the target antigen (Maecker & Trotter, 2006). Analyzing more of the PBMC samples from other pigs could help eliminate this bias and strengthen the patterns observed in the vaccinated samples. PBMCs were sampled on day 21 before booster vaccination, but viability was low during the flow cytometric analysis and not enough live lymphocytes were available further to gate on memory cell subsets.

To strengthen confidence in the data, proliferation of the PBMCs in response to viral stimulation could be measured by flow cytometry or ELISpot. Although proliferation is not a measure of direct memory it can help determine if vaccination primed the immune cells to respond and expand to the target antigen when compared to naïve controls (Todryk et al., 2009). As naïve controls should have little to no proliferation after stimulation to the antigen. IFNγ expression could be confirmed through ELISA of cell supernatant after longer stimulation times to strengthen the IFNγ data as seen in similar studies (Redant et al., 2022; Xu et al., 2019).

Final conclusions

The results of this study demonstrated that equine WNV vaccines can be used in swine to generate memory CD4+ T cells. These memory T cells were analyzed for their potential cross-reactivity to JEV. Swine vaccinated with the VET vaccine generated CD4+ central memory T cells that may be cross-reactive to JEV stimulation, as indicated by an increase in IFNγ expression. Further testing is needed to confirm if these T cells are truly memory subsets to WNV and JEV.

The neutralization antibody response should also be investigated to see if vaccination can generate protective immunity.

References

Ahmed, R., & Gray, D. (1996). Immunological memory and protective immunity: understanding their relation. *Science (New York, N.Y.)*, *272*(5258), 54-60. https://doi.org/10.1126/science.272.5258.54

Ahrends, T., Busselaar, J., Severson, T. M., Babala, N., de Vries, E., Bovens, A., Wessels, L., van Leeuwen, F., & Borst, J. (2019). CD4(+) T cell help creates memory CD8(+) T cells with innate and help-independent recall capacities. *Nat Commun*, *10*(1), 5531. https://doi.org/10.1038/s41467-019-13438-1

Alraies, Z., Rivera, C. A., Delgado, M. G., Sanseau, D., Maurin, M., Amadio, R., Maria Piperno, G., Dunsmore, G., Yatim, A., Lacerda Mariano, L., Kniazeva, A., Calmettes, V., Saez, P. J., Williart, A., Popard, H., Gratia, M., Lamiable, O., Moreau, A., Fusilier, Z., . . . Lennon-Dumenil, A. M. (2024). Cell shape sensing licenses dendritic cells for homeostatic migration to lymph nodes. *Nat Immunol*, *25*(7), 1193-1206. https://doi.org/10.1038/s41590-024-01856-3

Appleman, L. J., & Boussiotis, V. A. (2003). T cell anergy and costimulation. *Immunol Rev*, *192*, 161-180. https://doi.org/10.1034/j.1600-065x.2003.00009.x

Attreed, S. E., Silva, C., Abbott, S., Ramirez-Medina, E., Espinoza, N., Borca, M. V., Gladue, D. P., & Diaz-San Segundo, F. (2022). A Highly Effective African Swine Fever Virus Vaccine Elicits a Memory T Cell Response in Vaccinated Swine. *Pathogens*, *11*(12), 1438. https://doi.org/10.3390/pathogens11121438

Bettin, L., Darbellay, J., van Kessel, J., Dhar, N., & Gerdts, V. (2024). Porcine γδ T cells express cytotoxic cell-associated markers and display killing activity but are not selectively cytotoxic against PRRSV- or swIAV-infected macrophages [Original Research]. *Frontiers in Immunology*, *Volume 15 - 2024*. https://doi.org/10.3389/fimmu.2024.1434011

Bos, R., & Sherman, L. A. (2010). CD4+ T-Cell Help in the Tumor Milieu Is Required for Recruitment and Cytolytic Function of CD8+ T Lymphocytes. *Cancer Research*, *70*(21), 8368-8377. https://doi.org/10.1158/0008-5472.Can-10-1322

Brinton, M. A. (2002). The molecular biology of West Nile Virus: a new invader of the western hemisphere. *Annu Rev Microbiol*, *56*, 371-402. https://doi.org/10.1146/annurev.micro.56.012302.160654

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., & Ginsburg, A. S. (2011). Estimated global incidence of Japanese encephalitis: a systematic review. *Bull World Health Organ*, *89*(10), 766-774, 774A-774E. https://doi.org/10.2471/BLT.10.085233

Casanova, J.-L., MacMicking, J. D., & Nathan, C. F. (2024). Interferon-Y and infectious diseases: Lessons and prospects. *Science*, *384*(6693), eadl2016. https://doi.org/doi:10.1126/science.adl2016

Cenerenti, M., Saillard, M., Romero, P., & Jandus, C. (2022). The Era of Cytotoxic CD4 T Cells [Review]. *Frontiers in Immunology*, *Volume 13 - 2022*. https://doi.org/10.3389/fimmu.2022.867189

Chang, H. F., Bzeih, H., Chitirala, P., Ravichandran, K., Sleiman, M., Krause, E., Hahn, U., Pattu, V., & Rettig, J. (2017). Preparing the lethal hit: interplay between exo- and

endocytic pathways in cytotoxic T lymphocytes. *Cell Mol Life Sci*, *74*(3), 399-408. https://doi.org/10.1007/s00018-016-2350-7

Chen, H. L., Chang, J. K., & Tang, R. B. (2015). Current recommendations for the Japanese encephalitis vaccine. *J Chin Med Assoc*, *78*(5), 271-275. https://doi.org/10.1016/j.jcma.2014.12.009

Chhabra, S., Sharma, K. B., & Kalia, M. (2022). Human Guanylate-Binding Protein 1 Positively Regulates Japanese Encephalitis Virus Replication in an Interferon Gamma Primed Environment. *Front Cell Infect Microbiol*, *12*, 832057. https://doi.org/10.3389/fcimb.2022.832057

Chung, C. J., Cha, S.-H., Grimm, A. L., Ajithdoss, D., Rzepka, J., Chung, G., Yu, J., Davis, W. C., & Ho, C.-S. (2018). Pigs that recover from porcine reproduction and respiratory syndrome virus infection develop cytotoxic CD4+CD8+ and CD4+CD8- T-cells that kill virus infected cells. *PLOS ONE*, *13*(9), e0203482. https://doi.org/10.1371/journal.pone.0203482

Comeau, K., Paradis, P., & Schiffrin, E. L. (2020). Human and murine memory γδ T cells: Evidence for acquired immune memory in bacterial and viral infections and autoimmunity. *Cellular Immunology*, *357*, 104217. https://doi.org/https://doi.org/10.1016/j.cellimm.2020.104217

Cuesta-Martín de la Cámara, R., Torices-Pajares, A., Miguel-Berenguel, L., Reche-Yebra, K., Frauca-Remacha, E., Hierro-Llanillo, L., Muñoz-Bartolo, G., Lledín-Barbacho, M. D., Gutiérrez-Arroyo, A., Martínez-Feito, A., López-Granados, E., & Sánchez-Zapardiel, E. (2024). Epstein-Barr virus-specific T-cell response in pediatric liver transplant recipients: a cross-sectional study by multiparametric flow cytometry [Original Research]. *Frontiers in Immunology, Volume 15 - 2024*. https://doi.org/10.3389/fimmu.2024.1479472

Dan, J. M., Lindestam Arlehamn, C. S., Weiskopf, D., da Silva Antunes, R., Havenar-Daughton, C., Reiss, S. M., Brigger, M., Bothwell, M., Sette, A., & Crotty, S. (2016). A Cytokine-Independent Approach To Identify Antigen-Specific Human Germinal Center T Follicular Helper Cells and Rare Antigen-Specific CD4+ T Cells in Blood. *The Journal of Immunology*, *197*(3), 983-993. https://doi.org/10.4049/jimmunol.1600318

Dey, D., Poudyal, S., Rehman, A., & Hasan, S. S. (2021). Structural and biochemical insights into flavivirus proteins. *Virus Research*, *296*, 198343. https://doi.org/https://doi.org/10.1016/j.virusres.2021.198343

Ebner, F., Schwiertz, P., Steinfelder, S., Pieper, R., Zentek, J., Schütze, N., Baums, C. G., Alber, G., Geldhof, P., & Hartmann, S. (2017). Pathogen-Reactive T Helper Cell Analysis in the Pig [Original Research]. *Frontiers in Immunology, Volume 8 - 2017*. https://doi.org/10.3389/fimmu.2017.00565

Erlanger, T. E., Weiss, S., Keiser, J., Utzinger, J., & Wiedenmayer, K. (2009). Past, present, and future of Japanese encephalitis. *Emerg Infect Dis*, *15*(1), 1-7. https://doi.org/10.3201/eid1501.080311

Estell, K. E. (2025). *AAEP West Nile Virus Disease Guidelines*. https://aaep.org/wp-content/uploads/2017/01/West-Nile-Virus-Disease-Guidelines-final.pdf

Ewald, D., Hanna, J., & Barnett, D. (1996). Vaccination against Japanese encephalitis in the Torres Strait. *Communicable Diseases Intelligence*, *20*(8), 188. https://search.informit.org/doi/10.3316/informit.448204158448898

Fang, E., Liu, X., Liu, X., Li, M., Wang, L., Li, M., Zhang, Z., Li, Y., & Yu, Y. (2022). Investigation of immune response induction by Japanese encephalitis live-attenuated and

chimeric vaccines in mice. *MedComm (2020)*, *3*(2), e117. https://doi.org/10.1002/mco2.117

Franzoni, G., Kurkure, N. V., Edgar, D. S., Everett, H. E., Gerner, W., Bodman-Smith, K. B., Crooke, H. R., & Graham, S. P. (2013). Assessment of the Phenotype and Functionality of Porcine CD8 T Cell Responses following Vaccination with Live Attenuated Classical Swine Fever Virus (CSFV) and Virulent CSFV Challenge. *Clinical and Vaccine Immunology*, *20*(10), 1604-1616. https://doi.org/10.1128/cvi.00415-13

Gardner, I. A., Wong, S. J., Ferraro, G. L., Balasuriya, U. B., Hullinger, P. J., Wilson, W. D., Shi, P. Y., & MacLachlan, N. J. (2007). Incidence and effects of West Nile virus infection in vaccinated and unvaccinated horses in California. *Vet Res*, *38*(1), 109-116. https://doi.org/10.1051/vetres:2006045

Gaunt, M. W., Sall, A. A., Lamballerie, X. d., Falconar, A. K. I., Dzhivanian, T. I., & Gould, E. A. (2001). Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association and biogeography. *Journal of General Virology*, *82*(8), 1867-1876. https://doi.org/https://doi.org/10.1099/0022-1317-82-8-1867

Gould, E. A., & Solomon, T. (2008). Pathogenic flaviviruses. *The Lancet*, *371*(9611), 500-509. https://doi.org/https://doi.org/10.1016/S0140-6736(08)60238-X

Goverdhan, M. K., Kulkarni, A. B., Gupta, A. K., Tupe, C. D., & Rodrigues, J. J. (1992). Two-way cross-protection between West Nile and Japanese encephalitis viruses in bonnet macaques. *Acta virologica*, *36*(3), 277-283. http://europepmc.org/abstract/MED/1360756

Grifoni, A., Voic, H., Dhanda, S. K., Kidd, C. K., Brien, J. D., Buus, S., Stryhn, A., Durbin, A. P., Whitehead, S., Diehl, S. A., De Silva, A. D., Balmaseda, A., Harris, E., Weiskopf, D., & Sette, A. (2020). T Cell Responses Induced by Attenuated Flavivirus Vaccination Are Specific and Show Limited Cross-Reactivity with Other Flavivirus Species. *J Virol*, *94*(10). https://doi.org/10.1128/JVI.00089-20

Grossman, W. J., Verbsky, J. W., Tollefsen, B. L., Kemper, C., Atkinson, J. P., & Ley, T. J. (2004). Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood*, *104*(9), 2840-2848. https://doi.org/10.1182/blood-2004-03-0859

Hammon, W. M., & Sather, G. E. (1956). Immunity of Hamsters to West Nile and Murray Valley Viruses Following Immunization with St. Louis and Japanese B.∗. *Proceedings of the Society for Experimental Biology and Medicine*, *91*(3), 521-524. https://doi.org/10.3181/00379727-91-22314

Hayasaka, D., Shirai, K., Aoki, K., Nagata, N., Simantini, D. S., Kitaura, K., Takamatsu, Y., Gould, E., Suzuki, R., & Morita, K. (2013). TNF-α Acts as an Immunoregulator in the Mouse Brain by Reducing the Incidence of Severe Disease Following Japanese Encephalitis Virus Infection. *PLOS ONE*, *8*(8), e71643. https://doi.org/10.1371/journal.pone.0071643

Heffelfinger, J. D., Li, X., Batmunkh, N., Grabovac, V., Diorditsa, S., Liyanage, J. B., Pattamadilok, S., Bahl, S., Vannice, K. S., Hyde, T. B., Chu, S. Y., Fox, K. K., Hills, S. L., & Marfin, A. A. (2017). Japanese Encephalitis Surveillance and Immunization - Asia and Western Pacific Regions, 2016. *MMWR. Morbidity and mortality weekly report*, *66*(22), 579-583. Retrieved 2017/06//, from https://doi.org/10.15585/mmwr.mm6622a3

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., & Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. *Nature*, *408*(6813), 740-745. https://doi.org/10.1038/35047123

Higgs, S., Vanlandingham, D. L., Klingler, K. A., Mcelroy, K. L., Mcgee, C. E., Harrington, L., Lang, J., Monath, T. P., & Guirakhoo, F. (2006). Growth Characteristics Of Chimerivax-Den Vaccine Viruses In Aedes Aegypti And Aedes Albopictus From Thailand. *The American Journal of Tropical Medicine and Hygiene Am J Trop Med Hyg*, *75*(5), 986-993. https://doi.org/10.4269/ajtmh.2006.75.986

Hoke, C. H., Nisalak, A., Sangawhipa, N., Jatanasen, S., Laorakapongse, T., Innis, B. L., Kotchasenee, S.-o., Gingrich, J. B., Latendresse, J., Fukai, K., & Burke, D. S. (1988). Protection against Japanese Encephalitis by Inactivated Vaccines. *New England Journal of Medicine*, *319*(10), 608-614. https://doi.org/doi:10.1056/NEJM198809083191004

Igarashi, A., Tanaka, M., Morita, K., Takasu, T., Ahmed, A., Ahmed, A., Akram, D. S., & Waqar, M. A. (1994). Detection of west Nile and Japanese encephalitis viral genome sequences in cerebrospinal fluid from acute encephalitis cases in Karachi, Pakistan. *Microbiol Immunol*, *38*(10), 827-830. https://doi.org/10.1111/j.1348-0421.1994.tb01866.x

Inoue, S.-I., Niikura, M., Mineo, S., & Kobayashi, F. (2013). Roles of IFN-γ and γδ T Cells in Protective Immunity Against Blood-Stage Malaria [Review]. *Frontiers in Immunology*, *Volume 4 - 2013*. https://doi.org/10.3389/fimmu.2013.00258

Jain, N., Oswal, N., Chawla, A. S., Agrawal, T., Biswas, M., Vrati, S., Rath, S., George, A., Bal, V., & Medigeshi, G. R. (2017). CD8 T cells protect adult naive mice from JEV-induced morbidity via lytic function. *PLOS Neglected Tropical Diseases*, *11*(2). https://doi.org/10.1371/journal.pntd.0005329

James, E. A., Gates, T. J., LaFond, R. E., Yamamoto, S., Ni, C., Mai, D., Gersuk, V. H., O'Brien, K., Nguyen, Q. A., Zeitner, B., Lanteri, M. C., Norris, P. J., Chaussabel, D., Malhotra, U., & Kwok, W. W. (2016). Neuroinvasive West Nile Infection Elicits Elevated and Atypically Polarized T Cell Responses That Promote a Pathogenic Outcome. *PLoS Pathog*, *12*(1), e1005375. https://doi.org/10.1371/journal.ppat.1005375

Khare, B., & Kuhn, R. J. (2022). The Japanese Encephalitis Antigenic Complex Viruses: From Structure to Immunity. *Viruses*, *14*(10), 2213. https://www.mdpi.com/1999-4915/14/10/2213

Koblischke, M., Spitzer, F. S., Florian, D. M., Aberle, S. W., Malafa, S., Fae, I., Cassaniti, I., Jungbauer, C., Knapp, B., Laferl, H., Fischer, G., Baldanti, F., Stiasny, K., Heinz, F. X., & Aberle, J. H. (2020). CD4 T Cell Determinants in West Nile Virus Disease and Asymptomatic Infection. *Front Immunol*, *11*, 16. https://doi.org/10.3389/fimmu.2020.00016

Kumar, P., Sulochana, P., Nirmala, G., Chandrashekar, R., Haridattatreya, M., & Satchidanandam, V. (2004). Impaired T Helper 1 Function of Nonstructural Protein 3-Specific T Cells in Japanese Patients with Encephalitis with Neurological Sequelae. *The Journal of Infectious Diseases*, *189*(5), 880-891. https://doi.org/10.1086/381768

Lanciotti, R. S., Roehrig, J. T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K. E., Crabtree, M. B., Scherret, J. H., Hall, R. A., MacKenzie, J. S., Cropp, C. B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., . . . Gubler, D. J. (1999). Origin of the West Nile Virus Responsible for an Outbreak of Encephalitis in the Northeastern United States. *Science*, *286*(5448), 2333-2337. https://doi.org/doi:10.1126/science.286.5448.2333

Larena, M., Regner, M., Lee, E., & Lobigs, M. (2011). Pivotal Role of Antibody and Subsidiary Contribution of CD8+T Cells to Recovery from Infection in a Murine Model of Japanese

Encephalitis. *Journal of Virology*, *85*(11), 5446-5455. https://doi.org/10.1128/jvi.02611-10

Larena, M., Regner, M., & Lobigs, M. (2013). Cytolytic effector pathways and IFN-gamma help protect against Japanese encephalitis. *Eur J Immunol*, *43*(7), 1789-1798. https://doi.org/10.1002/eji.201243152

Lee, Y., Tarke, A., & Grifoni, A. (2024). In-depth characterization of T cell responses with a combined Activation-Induced Marker (AIM) and Intracellular Cytokine Staining (ICS) assay. *Oxford Open Immunology*, *5*(1). https://doi.org/10.1093/oxfimm/iqae014

Li, F., Wang, Y., Yu, L., Cao, S., Wang, K., Yuan, J., Wang, C., Wang, K., Cui, M., & Fu Zhen, F. (2015). Viral Infection of the Central Nervous System and Neuroinflammation Precede Blood-Brain Barrier Disruption during Japanese Encephalitis Virus Infection. *Journal of Virology*, *89*(10), 5602-5614. https://doi.org/10.1128/jvi.00143-15

Li, Q., Zhang, M., Kim, B., Soriano, S., Mishra, H., Wang, Q., Kain, K. C., & Wang, R. (2025). The role of γδ T cells in flavivirus infections: Insights into immune defense and therapeutic opportunities. *PLOS Neglected Tropical Diseases*, *19*(4), e0012972. https://doi.org/10.1371/journal.pntd.0012972

Liang, Y., & Dai, X. (2024). The global incidence and trends of three common flavivirus infections (Dengue, yellow fever, and Zika) from 2011 to 2021. *Front Microbiol*, *15*, 1458166. https://doi.org/10.3389/fmicb.2024.1458166

Lin, Y. L., Huang, Y. L., Ma, S. H., Yeh, C. T., Chiou, S. Y., Chen, L. K., & Liao, C. L. (1997). Inhibition of Japanese encephalitis virus infection by nitric oxide: antiviral effect of nitric oxide on RNA virus replication. *Journal of Virology*, *71*(7), 5227-5235. https://doi.org/doi:10.1128/jvi.71.7.5227-5235.1997

Linder, A., Portmann, K., & Eyer, K. (2024). The impact of cryopreservation on cytokine secretion and polyfunctionality in human PBMCs: a comparative study [Original Research]. *Frontiers in Immunology*, *Volume 15 - 2024*. https://doi.org/10.3389/fimmu.2024.1478311

Lord, J. S., Gurley, E. S., & Pulliam, J. R. (2015). Rethinking Japanese Encephalitis Virus Transmission: A Framework for Implicating Host and Vector Species. *PLoS Negl Trop Dis*, *9*(12), e0004074. https://doi.org/10.1371/journal.pntd.0004074

Lunney, J. K. (2025). Progress and challenges in developing swine immune reagents. *Veterinary Immunology and Immunopathology*, *286*, 110975. https://doi.org/https://doi.org/10.1016/j.vetimm.2025.110975

Maecker, H. T., & Trotter, J. (2006). Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry Part A*, *69A*(9), 1037-1042. https://doi.org/https://doi.org/10.1002/cyto.a.20333

Marino, A., Vitale, E., Maniaci, A., La Via, L., Moscatt, V., Spampinato, S., Senia, P., Venanzi Rullo, E., Restivo, V., Cacopardo, B., & Nunnari, G. (2025). West Nile Virus: Insights into Microbiology, Epidemiology, and Clinical Burden. *Acta Microbiologica Hellenica*, *70*(4). https://doi.org/10.3390/amh70040044

Martina, B. E., Koraka, P., van den Doel, P., van Amerongen, G., Rimmelzwaan, G. F., & Osterhaus, A. D. M. E. (2008). Immunization with West Nile virus envelope domain III protects mice against lethal infection with homologous and heterologous virus. *Vaccine*, *26*(2), 153-157. https://doi.org/10.1016/j.vaccine.2007.10.055

Mitchison, N. A. (2004). T-cell–B-cell cooperation. *Nature Reviews Immunology*, *4*(4), 308-312. https://doi.org/10.1038/nri1334

Monath, T. P. (1988). *The arboviruses: Epidemiology and ecology* (Vol. 1-5). CRC Press.
Moorton, M., Tng, P. Y. L., Inoue, R., Netherton, C. L., Gerner, W., & Schmidt, S. (2024). Investigation of activation-induced markers (AIM) in porcine T cells by flow cytometry [Original Research]. *Frontiers in Veterinary Science, Volume 11 - 2024*. https://doi.org/10.3389/fvets.2024.1390486
Mosmann, T. R., & Coffman, R. L. (1989). TH1 and TH2 Cells: Different Patterns of Lymphokine Secretion Lead to Different Functional Properties. *Annual Review of Immunology*, *7*(Volume 7, 1989), 145-173. https://doi.org/https://doi.org/10.1146/annurev.iy.07.040189.001045
Murali-Krishna, K., Ravi, V., & Manjunath, R. (1994). Cytotoxic T lymphocytes raised against Japanese encephalitis virus: effector cell phenotype, target specificity and in vitro virus clearance. *Journal of General Virology*, *75*(4), 799-807. https://doi.org/https://doi.org/10.1099/0022-1317-75-4-799
Murali-Krishna, K., Ravi, V., & Manjunath, R. (1996). 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. *Journal of General Virology*, *77*(4), 705-714. https://doi.org/https://doi.org/10.1099/0022-1317-77-4-705
Nah, J. J., Yang, D. K., Kim, H. H., & Song, J. Y. (2015). The present and future of veterinary vaccines for Japanese encephalitis in Korea. *Clin Exp Vaccine Res*, *4*(2), 130-136. https://doi.org/10.7774/cevr.2015.4.2.130
Oliphant, T., Engle, M., Nybakken, G. E., Doane, C., Johnson, S., Huang, L., Gorlatov, S., Mehlhop, E., Marri, A., Chung, K. M., Ebel, G. D., Kramer, L. D., Fremont, D. H., & Diamond, M. S. (2005). Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. *Nature Medicine*, *11*(5), 522-530. https://doi.org/10.1038/nm1240
Park, S. L., Huang, Y. S., Lyons, A. C., Ayers, V. B., Hettenbach, S. M., McVey, D. S., Burton, K. R., Higgs, S., & Vanlandingham, D. L. (2018). North American domestic pigs are susceptible to experimental infection with Japanese encephalitis virus. *Sci Rep*, *8*(1), 7951. https://doi.org/10.1038/s41598-018-26208-8
Patabendige, A., Michael, B. D., Craig, A. G., & Solomon, T. (2018). Brain microvascular endothelial-astrocyte cell responses following Japanese encephalitis virus infection in an in vitro human blood-brain barrier model. *Molecular and Cellular Neuroscience*, *89*, 60-70. https://doi.org/https://doi.org/10.1016/j.mcn.2018.04.002
Petersen, L. R., Brault, A. C., & Nasci, R. S. (2013). West Nile virus: review of the literature. *JAMA*, *310*(3), 308-315. https://doi.org/10.1001/jama.2013.8042
Petrovsky, N., Larena, M., Siddharthan, V., Prow, N. A., Hall, R. A., Lobigs, M., & Morrey, J. (2013). 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*, *87*(18), 10324-10333. https://doi.org/doi:10.1128/JVI.00480-13
Practitioners, A. A. o. E. (2023). *West Nile Virus Vaccination Guidelines*. American Association of Equine Practitioners. https://aaep.org/resource/west-nile-virus-vaccination-guidelines/
Pushpakumara, P. D., Jeewandara, C., Wijesinghe, A., Gomes, L., Ogg, G. S., Goonasekara, C. L., & Malavige, G. N. (2020). Identification of Immune Responses to Japanese

Encephalitis Virus Specific T Cell Epitopes. *Front Public Health*, *8*, 19. https://doi.org/10.3389/fpubh.2020.00019

Quatrini, L., Della Chiesa, M., Sivori, S., Mingari, M. C., Pende, D., & Moretta, L. (2021). Human NK cells, their receptors and function. *European Journal of Immunology*, *51*(7), 1566-1579. https://doi.org/https://doi.org/10.1002/eji.202049028

Re, F., Donnini, A., & Provinciali, M. (2006). Induction of αδ- and αβ-mediated T cell responses in healthy elderly subjects after influenza vaccination. *Biogerontology*, *7*(4), 249-259. https://doi.org/10.1007/s10522-006-9024-z

Redant, V., Favoreel, H. W., Dallmeier, K., Van Campe, W., & De Regge, N. (2020). Efficient control of Japanese encephalitis virus in the central nervous system of infected pigs occurs in the absence of a pronounced inflammatory immune response. *Journal of Neuroinflammation*, *17*(1). https://doi.org/10.1186/s12974-020-01974-3

Redant, V., Favoreel, H. W., Dallmeier, K., Van Campe, W., & De Regge, N. (2022). Japanese Encephalitis Virus Persistence in Porcine Tonsils Is Associated With a Weak Induction of the Innate Immune Response, an Absence of IFNγ mRNA Expression, and a Decreased Frequency of CD4+CD8+ Double-Positive T Cells. *Frontiers in Cellular and Infection Microbiology*, *12*. https://doi.org/10.3389/fcimb.2022.834888

Regner, M., Lobigs, M., Blanden, R. V., Milburn, P., & Müllbacher, A. (2001). Antiviral Cytotoxic T Cells Cross-Reactively Recognize Disparate Peptide Determinants from Related Viruses but Ignore More Similar Self- and Foreign Determinants. *The Journal of Immunology*, *166*(6), 3820-3828. https://doi.org/10.4049/jimmunol.166.6.3820

Ricklin, M. E., Garcia-Nicolas, O., Brechbuhl, D., Python, S., Zumkehr, B., Nougairede, A., Charrel, R. N., Posthaus, H., Oevermann, A., & Summerfield, A. (2016). Vector-free transmission and persistence of Japanese encephalitis virus in pigs. *Nat Commun*, *7*, 10832. https://doi.org/10.1038/ncomms10832

Ricklin, M. E., Garcia-Nicolas, O., Brechbuhl, D., Python, S., Zumkehr, B., Posthaus, H., Oevermann, A., & Summerfield, A. (2016). Japanese encephalitis virus tropism in experimentally infected pigs. *Vet Res*, *47*, 34. https://doi.org/10.1186/s13567-016-0319-z

Robinson, J. P., Gmyrek, G. B., & Rajwa, B. (2026). Flow Cytometry: Advances, Challenges and Trends. *BioEssays*, *48*(1), e70091. https://doi.org/https://doi.org/10.1002/bies.70091

Roehrig, J. T. (2009). Antibody Protection and Therapy for West Nile Virus Infections. In M. S. Diamond (Ed.), *West Nile Encephalitis Virus Infection: Viral Pathogenesis and the Host Immune Response* (pp. 417-425). Springer New York. https://doi.org/10.1007/978-0-387-79840-0_19

Rothermel, C. D., Rubin, B. Y., & Murray, H. W. (1983). Gamma-interferon is the factor in lymphokine that activates human macrophages to inhibit intracellular Chlamydia psittaci replication. *The Journal of Immunology*, *131*(5), 2542-2544. https://doi.org/10.4049/jimmunol.131.5.2542

Saalmüller, A., Pauly, T., Höhlich, B.-J., & Pfaff, E. (1999). Characterization of porcine T lymphocytes and their immune response against viral antigens. *Journal of Biotechnology*, *73*(2), 223-233. https://doi.org/https://doi.org/10.1016/S0168-1656(99)00140-6

Sallusto, F., Geginat, J., & Lanzavecchia, A. (2004). Central Memory and Effector Memory T Cell Subsets: Function, Generation, and Maintenance. *Annual Review of Immunology*, *22*(Volume 22, 2004), 745-763. https://doi.org/https://doi.org/10.1146/annurev.immunol.22.012703.104702

Sampson, B. A., & Armbrustmacher, V. (2001). West Nile Encephalitis. *Annals of the New York Academy of Sciences*, *951*(1), 172-178. https://doi.org/https://doi.org/10.1111/j.1749-6632.2001.tb02695.x

Saron, W. A. A., Rathore, A. P. S., Ting, L., Ooi, E. E., Low, J., Abraham, S. N., & St. John, A. L. (2018). Flavivirus serocomplex cross-reactive immunity is protective by activating heterologous memory CD4 T cells. *Science Advances*, *4*(7), eaar4297. https://doi.org/doi:10.1126/sciadv.aar4297

Saxena, V., Mathur, A., Krishnani, N., & Dhole, T. N. (2008a). An insufficient anti-inflammatory cytokine response in mouse brain is associated with increased tissue pathology and viral load during Japanese encephalitis virus infection. *Arch Virol*, *153*(2), 283-292. https://doi.org/10.1007/s00705-007-1098-7

Saxena, V., Mathur, A., Krishnani, N., & Dhole, T. N. (2008b). Kinetics of cytokine profile during intraperitoneal inoculation of Japanese encephalitis virus in BALB/c mice model. *Microbes Infect*, *10*(10-11), 1210-1217. https://doi.org/10.1016/j.micinf.2008.06.015

Schiött, Å., Lindstedt, M., Johansson-Lindbom, B., Roggen, E., & Borrebaeck, C. A. K. (2004). CD27− CD4+ memory T cells define a differentiated memory population at both the functional and transcriptional levels. *Immunology*, *113*(3), 363-370. https://doi.org/https://doi.org/10.1111/j.1365-2567.2004.01974.x

Sejvar, J. J. (2016). West Nile Virus Infection. *Microbiol Spectr*, *4*(3). https://doi.org/10.1128/microbiolspec.EI10-0021-2016

Shedlock, D. J., & Shen, H. (2003). Requirement for CD4 T Cell Help in Generating Functional CD8 T Cell Memory. *Science*, *300*(5617), 337-339. https://doi.org/doi:10.1126/science.1082305

Shimizu, T., Kawakami, Y., Fukuhara, S., & Matumoto, M. (1954). Experimental stillbirth in pregnant swine infected with Japanese encephalitis virus. *The Japanese journal of experimental medicine*, *24*(6), 363-375. http://europepmc.org/abstract/MED/13270968

Shrestha, B., & Diamond, M. S. (2004). Role of CD8+ T cells in control of West Nile virus infection. *J Virol*, *78*(15), 8312-8321. https://doi.org/10.1128/JVI.78.15.8312-8321.2004

Shrestha, B., Wang, T., Samuel, M. A., Whitby, K., Craft, J., Fikrig, E., & Diamond, M. S. (2006). Gamma interferon plays a crucial early antiviral role in protection against West Nile virus infection. *J Virol*, *80*(11), 5338-5348. https://doi.org/10.1128/JVI.00274-06

Sitati, E. M., & Diamond, M. S. (2006). CD4+ T-cell responses are required for clearance of West Nile virus from the central nervous system. *J Virol*, *80*(24), 12060-12069. https://doi.org/10.1128/JVI.01650-06

Soni, R., Jena, P., Kusuma, B., & Banerjee, A. (2025). Neutrophil depletion at the early stage of Japanese encephalitis virus infection affects CD8+ T cell infiltration into the mouse brain and causes severe encephalitis. *Front Immunol*, *16*, 1748085. https://doi.org/10.3389/fimmu.2025.1748085

Sun, L., Su, Y., Jiao, A., Wang, X., & Zhang, B. (2023). T cells in health and disease. *Signal Transduction and Targeted Therapy*, *8*(1), 235. https://doi.org/10.1038/s41392-023-01471-y

Tau, G., & Rothman, P. (1999). Biologic functions of the IFN-γ receptors. *Allergy*, *54*(12), 1233-1251. https://doi.org/https://doi.org/10.1034/j.1398-9995.1999.00099.x

Todryk, S. M., Pathan, A. A., Keating, S., Porter, D. W., Berthoud, T., Thompson, F., Klenerman, P., & Hill, A. V. S. (2009). The relationship between human effector and memory T cells measured by ex vivo and cultured ELISPOT following recent and distal

priming. *Immunology*, *128*(1), 83-91. https://doi.org/https://doi.org/10.1111/j.1365-2567.2009.03073.x

Turtle, L., Bali, T., Buxton, G., Chib, S., Chan, S., Soni, M., Hussain, M., Isenman, H., Fadnis, P., Venkataswamy, M. M., Satishkumar, V., Lewthwaite, P., Kurioka, A., Krishna, S., Shankar, M. V., Ahmed, R., Begum, A., Ravi, V., Desai, A., . . . Solomon, T. (2016). Human T cell responses to Japanese encephalitis virus in health and disease. *J Exp Med*, *213*(7), 1331-1352. https://doi.org/10.1084/jem.20151517

USDA. (2026). *Licensed Veterinary Biological Product Information*. U.S. Department of Agriculture. https://www.aphis.usda.gov/veterinary-biologics/licensed-products

van den Hurk, A. F., Pyke, A. T., Mackenzie, J. S., Hall-Mendelin, S., & Ritchie, S. A. (2019). Japanese Encephalitis Virus in Australia: From Known Known to Known Unknown. *Tropical Medicine and Infectious Disease*, *4*(1), 38. https://www.mdpi.com/2414-6366/4/1/38

Wang, R., Zhang, M., Zhang, L., Liu, M., Shan, C., An, J., & Xie, Z. (2022). Japanese Encephalitis Vaccine Generates Cross-Reactive Memory T Cell Responses to Zika Virus in Humans. *J Trop Med*, *2022*, 8379286. https://doi.org/10.1155/2022/8379286

Waterhouse , N. J., Sutton , V. R., Sedelies , K. A., Ciccone , A., Jenkins , M., Turner , S. J., Bird , P. I., & Trapani , J. A. (2006). Cytotoxic T lymphocyte–induced killing in the absence of granzymes A and B is unique and distinct from both apoptosis and perforin-dependent lysis. *Journal of Cell Biology*, *173*(1), 133-144. https://doi.org/10.1083/jcb.200510072

Webster , L. T. (1938). JAPANESE B ENCEPHALITIS VIRUS: ITS DIFFERENTIATION FROM ST. LOUIS ENCEPHALITIS VIRUS AND RELATIONSHIP TO LOUPING ILL VIRUS. *Journal of Experimental Medicine*, *67*(4), 609-618. https://doi.org/10.1084/jem.67.4.609

Wessel Alex, W., Doyle Michael, P., Engdahl Taylor, B., Rodriguez, J., Crowe James, E., & Diamond Michael, S. (2021). Human Monoclonal Antibodies against NS1 Protein Protect against Lethal West Nile Virus Infection. *mBio*, *12*(5), 10.1128/mbio.02440-02421. https://doi.org/10.1128/mbio.02440-21

Williams, C. R., Webb, C. E., Higgs, S., & van den Hurk, A. F. (2022). Japanese Encephalitis Virus Emergence in Australia: Public Health Importance and Implications for Future Surveillance. *Vector Borne Zoonotic Dis*, *22*(11), 529-534. https://doi.org/10.1089/vbz.2022.0037

Xu, Y., Wang, Q., Wei, B., Huang, X., Wen, Y., Yan, Q., Ma, X., Zhao, Q., Cao, S., Huang, Y., Wen, X., Han, X., Bai, Y., & Wu, R. (2019). Enhanced Immune Responses Against Japanese Encephalitis Virus Infection Using Japanese Encephalitis Live-Attenuated Virus Adjuvanted with Montanide GEL 01 ST in Mice. *Vector Borne Zoonotic Dis*, *19*(11), 835-843. https://doi.org/10.1089/vbz.2018.2419

Yamada, M., Nakamura, K., Yoshii, M., & Kaku, Y. (2004). Nonsuppurative Encephalitis in Piglets after Experimental Inoculation of Japanese Encephalitis Flavivirus Isolated from Pigs. *Veterinary Pathology*, *41*(1), 62-67. https://doi.org/10.1354/vp.41-1-62

Yamada, M., Nakamura, K., Yoshii, M., Kaku, Y., & Narita, M. (2009). Brain Lesions Induced by Experimental Intranasal Infection of Japanese Encephalitis Virus in Piglets. *Journal of Comparative Pathology*, *141*(2), 156-162. https://doi.org/https://doi.org/10.1016/j.jcpa.2009.04.006

Yewdell, J. W., & Bennink, J. R. (1999). Mechanisms of Viral Interference with MHC Class I Antigen Processing and Presentation1. *Annual Review of Cell and Developmental*

Biology, *15*(Volume 15, 1999), 579-606. https://doi.org/https://doi.org/10.1146/annurev.cellbio.15.1.579

Yu, Y. (2010). Phenotypic and genotypic characteristics of Japanese encephalitis attenuated live vaccine virus SA14-14-2 and their stabilities. *Vaccine*, *28*(21), 3635-3641. https://doi.org/10.1016/j.vaccine.2010.02.105

Yun, S.-I., & Lee, Y.-M. (2014). Japanese encephalitis. *Human Vaccines & Immunotherapeutics*, *10*(2), 263-279. https://doi.org/10.4161/hv.26902

Zhang, F., Qi, L., Li, T., Li, X., Yang, D., Cao, S., Ye, J., & Wei, B. (2019). PD1+CCR2+CD8+ T Cells Infiltrate the Central Nervous System during Acute Japanese Encephalitis Virus Infection. *Virologica Sinica*, *34*(5), 538-548. https://doi.org/10.1007/s12250-019-00134-z

Zhang, M., Daniel, S., Huang, Y., Chancey, C., Huang, Q., Lei, Y. F., Grinev, A., Mostowski, H., Rios, M., & Dayton, A. (2010). Anti-West Nile virus activity of in vitro expanded human primary natural killer cells. *BMC Immunology*, *11*(1), 3. https://doi.org/10.1186/1471-2172-11-3

Zhang, W., Yu, Q., Gao, X., Chen, H., Su, J., Chen, Y., Li, Y., Zhang, N., Fu, Z., & Cui, M. (2024). Myeloid-Derived Suppressor Cells Induce Exhaustion-Like CD8⁺ T Cells during JEV Infection [Research Paper]. *International Journal of Biological Sciences*, *20*(15), 5959-5978. https://doi.org/10.7150/ijbs.102372

Zhou, D., Jia, F., Li, Q., Zhang, L., Chen, Z., Zhao, Z., Cui, M., Song, Y., Chen, H., Cao, S., & Ye, J. (2018). Japanese Encephalitis Virus NS1′ Protein Antagonizes Interferon Beta Production. *Virologica Sinica*, *33*(6), 515-523. https://doi.org/10.1007/s12250-018-0067-5

Zhou, Y., Bian, P., Du, H., Wang, T., Li, M., Hu, H., Ye, C., Zheng, X., Zhang, Y., Lei, Y., Jia, Z., & Lian, J. (2022). The Comparison of Inflammatory Cytokines (IL-6 and IL-18) and Immune Cells in Japanese Encephalitis Patients With Different Progression. *Front Cell Infect Microbiol*, *12*, 826603. https://doi.org/10.3389/fcimb.2022.826603

Zinkernagel, R. M., & Doherty, P. C. (1974). Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature*, *248*(5450), 701-702. https://doi.org/10.1038/248701a0

Zuckermann, F. A., & Husmann, R. J. (1996). Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. (0019-2805 (Print)).

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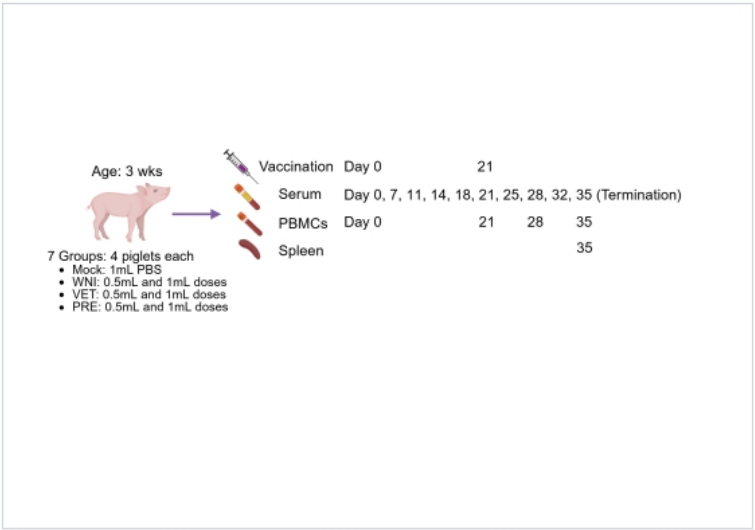

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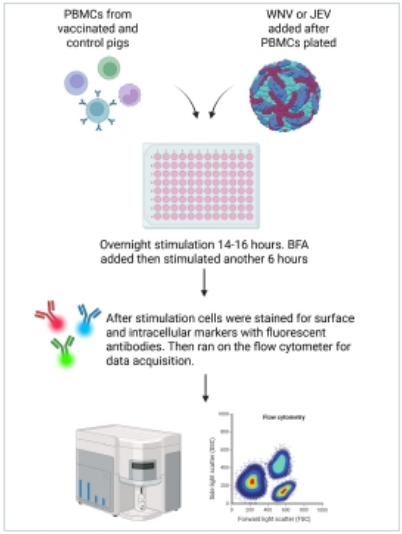

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