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A West Nile virus CD4 T cell epitope improves the immunogenicity of dengue virus serotype 2 vaccines

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ABSTRACT

Flaviviruses, such as dengue virus (DENV) and West Nile virus (WNV), are among the most prevalent human disease-causing arboviruses world-wide. As they continue to expand their geographic range, multivalent flavivirus vaccines may become an important public health tool. Here we describe the immune kinetics of WNV DNA vaccination and the identification of a CD4 epitope that increases heterologous flavivirus vaccine immunogenicity. Lethal WNV challenge two days post-vaccination resulted in 90% protection with complete protection by four days, and was temporally associated with a rapid influx of activated CD4 T cells. CD4 T cells from WNV vaccinated mice could be stimulated from epitopic regions in the envelope protein transmembrane domain. Incorporation of this WNV epitope into DENV-2 DNA and virus-like particle vaccines significantly increased neutralizing antibody titers. Incorporating such potent epitopes into multivalent flavivirus vaccines could improve their immunogenicity and may help alleviate concerns of imbalanced immunity in multivalent vaccine approaches.

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Introduction

Recent history has seen a global resurgence of arboviral diseases once thought to be under control or those which have expanded their geographic distribution and disease severity such as Japanese encephalitis, tick-borne encephalitis and yellow fever, or West Nile fever and dengue, respectively (Gubler, 2002). The recent autochthonous transmission of DENV in Key West, Florida (CDC, 2010), a region that has not seen local dengue transmission in the past 60 years, raises awareness of the need for appropriate control measures for these viruses. DENV, viscerotropic/hemorrhagic viruses that have completely evolved to human hosts, exist as four closely related serotypes (DENV-1, to -4), and WNV, an encephalitic virus maintained in an enzootic mosquito–bird transmission cycle, are both single-stranded RNA viruses in the *Flavivirus* genus. DENV disease is one of the world's most important arboviral infections producing either a mild dengue fever or severe, life-threatening dengue hemorrhagic fever and shock syndrome (DHF/DSS). Severe DENV disease frequently occurs in children residing in hyperendemic countries and is strongly associated with secondary heterotypic infections (Sangkawibha et al., 1984). WNV infection is often asymptomatic with approximately 24% of patients developing West Nile fever, and 1 in 150 symptomatic patients developing meningitis or encephalitis (Ferguson et al., 2005). With no DENV or WNV vaccines yet available on the market, vector control and behavioral education programs are the only control methods available to reduce their public health impact.

DNA vaccination is an active field in vaccine technology following the first reports of plasmid DNA inducing an immune response to plasmid-encoded antigens (Tang et al., 1992). Although DNA vaccines are considered an important discovery in the field of vaccinology (Mor, 1998), they have been hampered by low immunogenicity and efficacy. Thus, various strategies to improve the immune response by DNA vaccination have been developed. Earlier attempts to increase DNA vaccine immunogenicity have included optimization of route, dosage, and timing of administration; DNA encoded or exogenously administered co-stimulatory molecules and cytokines; and differential prime-boost regimens (Leitner et al., 1999).

We have previously described the development and protective efficacy of a WNV DNA vaccine, pVWNi, directing the expression of premembrane and envelope (prM/E) proteins that assemble to form non-infectious viral-like particles (VLP) in vivo (Davis et al., 2001). This DNA vaccine is highly immunogenic, a single intramuscular (i.m.) injection of pVWNi protected mice and horses from WNV challenge (Davis et al., 2001). Additionally, pVWNi confers protection in endangered California Condors (Chang et al., 2007) and is safe and immunogenic in humans (Martin et al., 2007). In this report, we describe the temporal kinetics of pVWNi immune protection resulting in 90–100% protection from lethal challenge in mice by 2–4 days post vaccination, respectively. In an attempt to understand this rapid protection and possibly improve the immunogenicity of DENV vaccine candidates, we screened an overlapping peptide library of the prM/E proteins of each vaccine to look for

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epitopes that differed between WNV and DENV-2 and identified a potent and promiscuous WNV CD4+ T cell epitope in the transmembrane domain of the WNV E protein. We introduce this T cell epitope into DENV-2 DNA and VLP vaccines and demonstrate improved immunogenicity of both vaccines through significant increases in neutralizing antibody responses. Thus, we have identified a novel, promiscuous CD4+ T cell epitope from WNV and present a strategy to introduce this epitope into heterologous flavivirus vaccines to increase their immunogenicity, an approach which may have applications for multivalent flavivirus vaccine development.

Results

pVWNi vaccination elicits rapid protection from virus challenge

Previous studies have shown three weeks post vaccination (WPV) with a single 100 μg injection of pVWNi, mice elicited high levels of WNV neutralizing antibodies which protected 100% of mice from WNV challenge (Davis et al., 2001). To understand the temporal kinetics of the immune response to pVWNi plasmid immunization and to estimate the potential of using this vaccine as an emergency counter measurement to reduce the impact of an ongoing epidemic, C57BL/6 J mice were vaccinated with a single i.m. injection of 100 μg of DNA, and challenged with 100,000 LD_{50} WNV (NY-99) at 1, 2, 4, 7, 14, or 21 days post vaccination (DPV) and compared to 1 and 21 days naïve age-matched controls. Surprisingly, 2 DPV 90% of mice survived challenge ($p<0.0001$). All mice survived challenge by 4 DPV and beyond ($p<0.0001$) (Fig. 1), with the exception of the 7 DPV group ($p=0.0004$) where 2 mice succumbed to lethal disease. These data suggest the pVWNi vaccine induces a rapid, protective immune response.

Vaccination induces a rapid cellular influx and activation

To investigate the causal nature of the rapid immune response and protection elicited by pVWNi vaccination, we investigated the participation of cellular immunity in the early immune response. C57BL/6J mice were vaccinated i.m. with pVWNi, pVax (vector only) and PBS as controls. Five mice per group were sacrificed at 2, 4, 7 and 14 DPV for histology and flow cytometry. Muscle from the vaccination site 4 and 7 DPV was dissected and histology performed (Fig. 2A). Four DPV with pVWNi, hematoxylin and eosin staining revealed a marked infiltration of cells with lymphocytic and monocytic morphology around the injection site and 7 DPV the infiltrate had mostly subsided. Mock vaccination with PBS or pVax vector control did not show any cellular infiltration suggesting pVWNi induced a transgene driven, transient cellular infiltration at the injection site. Flow cytometric analysis of single cell

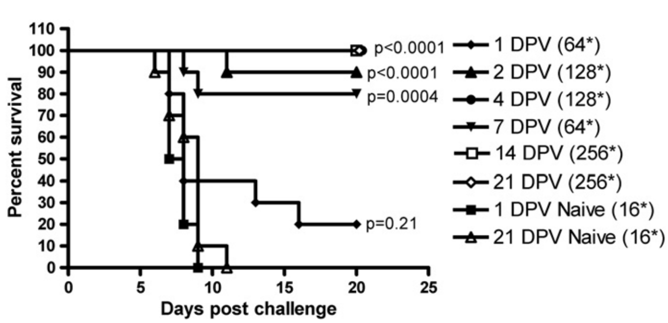

Fig. 1. pVWNi elicits rapid protection from WNV challenge. Kaplan–Meier survival of C57BL/6 vaccinated mice ($n=10$) challenged at 1 (closed diamond), 2 (closed triangle upright), 4 (closed circle), 7 (closed downward triangle), 14 (open square), or 21 (open diamond) days post vaccination i.p. with 100,000LD_{50} WNV NY99 compared to the naïve 1 day post vaccination (closed square) and 21 days post vaccination (open triangle) age-matched controls. *FRμNT_{50} values of pooled group sera.

preparations from muscle tissue demonstrated a large proportion of the cellular infiltrate 4 DPV with pVWNi was composed of F4/80+ antigen presenting cells (APC), B220+ B cells, CD4+ and CD8+ T cells (Fig. 2B). Antigen driven CD4+ T cells accounted for significantly higher proportions of cellular infiltrate to the injection site than did CD8+ T cells ($p=0.008$). Moreover, there was a larger proportion of activated T cells (Fig. 2C) and APC (Fig. 2D) compared to the pVax control animals ($p<0.0001$) suggesting the cellular activation was transgene specific. Cytokine secretion by activated CD4+ T cells indicated Th1 and Th2 cytokine production as early as 2 DPV for IL-2, IFNγ, TNFα, and IL-4, and by 4 DPV for IL-5 (Fig. 3). IL-2 and TNFα remained elevated through the entirety of the experiment while IFNγ and IL-4 subsided by 14 DPV. These data suggest pVWNi vaccination induces both early Th1 and Th2 cytokine profiles. Taken together, these data indicate the important role of antigen activated CD4+ T cells and F4/80+ APC in the establishment of the immune response to this vaccine.

CD4 positive epitope present in transmembrane domain of WNV envelope protein

The rapid immune response and protection elicited by the pVWNi vaccine led us to ask if there might be differential T cell epitopes that could explain some of the immunogenicity differences between the pVWNi and our previously described DENV-2 DNA vaccine that expresses DENV-2 prM, the 80% ectodomain of DENV-2 E, and the 20% stem-anchor region of JEV E (Chang et al., 2003). The 20% JEV region was shown to enhance VLP secretion and vaccine immunogenicity compared to 100% DENV-2 E (Chang et al., 2003). Unlike the pVWNi plasmid which only requires one 100 μg vaccination to protect 90% of mice 2 DPV (Fig. 1), two vaccinations of 100 μg of DENV-2 plasmid 4 weeks apart were required to elicit sufficient neutralizing antibody to passively protect neonatal mice from DENV-2 challenge (Chang et al., 2003). Although these viruses are very different in terms of their pathogenesis, growth kinetics and immunogenicity, the differences in immunogenicity between these vaccines is still remarkable despite both containing identical transcriptional enhancer and promoter, translational control element and JEV signal sequences for optimal prM translocation (Chang et al., 2003). Based upon the above observations of the rapid cellular immune response toward pVWNi, we hypothesized that differential CD4 T cell antigenic determinants might be responsible for some of the immunogenicity differences between the WNV and DENV-2 DNA vaccines (pVD2i).

To differentiate the cellular immune responses between pVWNi and pVD2i, we constructed an overlapping peptide library covering the entire prM and E protein coding sequences of each vaccine. We initially screened the library using 23 pools of 10 15mer peptides. C57BL/6 J mice ($n=5$) immunized with 100 μg of pVWNi or pVD2i were boosted at three weeks and sacrificed at 6 weeks. Spleens were homogenized, pooled, and the cells used to determine the positive peptide pools by fluorescence activated cell sorting analysis by intracellular cytokine staining. Positive peptide pools were deconvoluted and individual peptides were further tested in two independent experiments. Splenocytes from vaccinated mice were incubated with 2 μg of each individual peptide and stained for CD4 or CD8 and IFNγ. Positive peptides were compared to identify peptides unique to pVWNi or significantly different from pVD2i. Interestingly, a strong CD4+ epitopic region was identified in WNV E between amino acids 466–495 which was present, but much weaker in the 20% JEV corresponding region of pVD2i (Fig. 4). Positive peptides in pVWNi: TQGLLGALLLWMGIN, GALLLWMGINARDRS, WMGINARDRSIALTF, and ARDRSIALTFLAVGG elicited 1.1, 2.5, 38.6, and 1.35% CD4+/IFNγ+ T cells, respectively. While the similar peptide region in pVD2i: QGLMGALLLWMGVNA, ALLLWMGVNARDRSI, MGVNARDRSIALAFL, and RDRSIALAFLATGGV elicited 1.59, 3.17, 19.9, and 1.0% CD4+/IFNγ+ T cells, respectively. Although other peptides identified E protein epitopes with similar immunogenicity for both pVWNi and pVD2i, this

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