Viral pathogenesis in mice is similar for West Nile virus derived from mosquito and mammalian cells

Pei-Yin Lim[a,1], **Karen L. Louie**[a,b,1], **Linda M. Styer**[a], **Pei-Yong Shi**[a,b,c], and **Kristen A. Bernard**[a,b]

[a] Wadsworth Center, New York State Department of Health, P.O. Box 509, Albany, NY 12201

[b] Department of Biomedical Sciences, School of Public Health, University at Albany, Albany, NY 12201

Abstract

West Nile virus (WNV) is a mosquito-borne pathogen. During replication, WNV acquires different carbohydrates and lipid membranes, depending on its mosquito or vertebrate hosts. Consequently, WNV derived from mosquito and vertebrate cell lines differ in their infectivity for dendritic cells (DCs) and induction of type I interferon (IFN-α/β) *in vitro*. We evaluated the pathogenesis of WNV derived from mosquito ($WNV^{C6/36}$) and vertebrate (WNV^{BHK}) cell lines in mice. The tissue tropism, infectivity, clinical disease, and mortality did not differ for mice inoculated with $WNV^{C6/36}$ or WNV^{BHK}, and there were only minor differences in viral load and serum levels of IFN-α/β. The replication kinetics of $WNV^{C6/36}$ and WNV^{BHK} were equivalent in primary DCs and skin cells although primary DCs were more susceptible to $WNV^{C6/36}$ infection than to WNV^{BHK} infection, suggesting that less virus is produced per infected cell for $WNV^{C6/36}$. In conclusion, viral source has minimal effect on WNV pathogenesis *in vivo*.

Keywords

West Nile virus; flavivirus; virus-host interaction; pathogenesis; Type I interferon; dendritic cells; skin; mosquito; mice

Introduction

West Nile virus (WNV), a member of the *Flaviviridae* family, is one of the most widespread arboviruses in the world. The virus is maintained in an enzootic cycle between mosquitoes and avian hosts, and many other animals, including humans and horses, can become infected with WNV and can develop disease (Briese and Bernard, 2005). The WNV genome is a positive-sense RNA, which forms a nucleocapsid with multiple copies of the viral capsid (C) protein. The outermost layer of the virion contains a lipid-bilayer envelope that is derived from the host membrane. Embedded within the envelope are two viral proteins, envelope (E) and membrane

(prM/M) proteins, and both contain N-linked glycosylation sites (Adams et al., 1995; Hanna et al., 2005; Mukhopadhyay et al., 2003).

In nature, WNV is transmitted to its vertebrate hosts through the bite of an infected mosquito. A number of factors may influence this initial interaction of the virus with its target cell, including mosquito saliva, dose of virus, location in skin, and source of the virus. For example, the first round of infection is with virus derived from mosquito cells, and subsequent infections occur with vertebrate-derived virus. The initial target cell for WNV remains unknown; however, based on findings for other flaviviruses (Wu et al., 2000), it is thought that WNV first infects Langerhans cells, the resident dendritic cells (DCs) of the skin, at the site of inoculation, and the Langerhans cells then migrate to local draining lymph nodes (Johnston et al., 2000).

Differences in the source of virus, whether mosquito or vertebrate cell-derived, affects the interaction between the virus and its host cell. Carbohydrate processing in insect cells, particularly N-glycosylation, is markedly different from processing in vertebrate cells. Extensive studies have shown that N-glycans produced in arthropod cells are less complex than those produced in vertebrate cells (reviewed by Altmann et al., 1999). Consequently, viruses generated from these different sources differ in their carbohydrate content; viruses propagated in mosquito cells have high mannose glycans (Hsieh and Robbins, 1984; Lozach et al., 2005). In addition to the glycan structures, the lipid compositions of arthropod and mammalian cells are different (Brotherus and Renkonen, 1977; Mitsuhashi et al., 1983; Silberkang et al., 1983). Thus, the envelopes of viruses generated in different hosts vary in their carbohydrate and lipid compositions.

The impact of viral source on WNV pathogenesis has not been examined *in vivo*; however, *in vitro* studies showed that WNV generated in cells from different hosts influences infectivity and the innate immune response (Davis et al., 2006; Silva et al., 2007). Mosquito cell-derived WNV has greater infectivity for DCs than mammalian cell-derived WNV. This enhancement is mediated through interaction of the virus with DC-SIGNR, a C-type lectin (Davis et al., 2006). Similar results were observed in other arthropod-borne viruses such as the alphaviruses, Sindbis virus (Klimstra et al., 2003) and Ross River virus (Shabman et al., 2007). In addition, viruses derived from different hosts elicit different type I interferon (IFN-α/β) responses *in vitro*. Using primary cell cultures, Silva and coworkers (Silva et al., 2007) showed that mammalian cell-derived WNV induces more IFN-α than does mosquito cell-derived WNV in plasmacytoid DCs. These findings suggest that the source of virus plays an important role in pathogenesis since type I interferons are crucial for controlling WNV replication *in vivo* (Morrey et al., 2004; Samuel and Diamond, 2005).

In this study, we examined the effect of viral source on WNV pathogenesis using a mouse model. To our knowledge, this is the first study conducted *in vivo* to examine the effect of viral source for any arbovirus. We found that WNV derived from mosquito or mammalian cells did not differ substantially with respect to clinical disease, mortality rate, infectivity, tissue tropism, and replication kinetics in adult mice. In general, low levels of IFN-α/β were detected in the serum of mice after WNV inoculation. In contrast to the *in vitro* results by others (Silva et al., 2007), our results from mice suggest that mosquito cell-derived WNV elicits a faster IFN-α/β response than does mammalian cell-derived WNV at low viral doses (10 PFU). Similar to others (Davis et al., 2006), we showed that the mosquito cell-derived WNV infected greater numbers of DCs *in vitro*, confirming that our *in vivo* results were not unique to our virus preparation. The two viruses showed equivalent *in vitro* replication kinetics, suggesting that cells infected with mosquito cell-derived virus produce fewer viruses per infected cell. In summary, we conclude that although viral source differentially modulated WNV infectivity *in vitro*, viral source had minimal effect on WNV pathogenesis in mice. Knowledge obtained

from our studies shed light on the early virus-host interaction for WNV and other arthropod-borne viruses.

Results

Mortality and infectivity of WNV[C6/36] and WNV[BHK] are similar when assessed in adult mice

Viral stocks were derived from different host cells by transfection of *in vitro* transcribed WNV RNA into the mosquito cell line C6/36 (WNV[C6/36]) or the vertebrate cell line BHK (WNV[BHK]). These viruses were subsequently used to study the effect of viral source on WNV pathogenesis *in vivo*. We first determined the mortality rate and infectivity of WNV[C6/36] and WNV[BHK] by inoculating mice with 1 or 10 PFU of virus subcutaneously (SC) in the left-rear footpad. The actual mortality rates (mortality for infected mice) of the two viruses were similar at the 1 PFU dose (WNV[C6/36] and WNV[BHK] = 17%) and also the 10 PFU dose (WNV[C6/36] = 25%; WNV[BHK] =30%) (Table 1). In addition, all infected mice had similar morbidity and day of disease onset (data not shown). Furthermore, the ID_{50} for both viruses was 1 PFU (Table 1). In conclusion, there were no significant differences in the clinical disease, mortality, and infectivity in mice between WNV[C6/36] and WNV[BHK].

Tissue tropism and replication kinetics do not differ substantially between WNV[C6/36] and WNV[BHK] in mice inoculated at several doses of virus

Others have shown that arboviruses derived from mosquito cells have greater infectivity for DCs *in vitro* than viruses derived from mammalian cells (Davis et al., 2006; Klimstra et al., 2003; Shabman et al., 2007); therefore, we predicted that mosquito cell-derived virus would result in higher viral loads than mammalian cell-derived virus *in vivo*. We compared the tissue tropism and viral load for these viruses in mice. Styer and coworkers previously showed that the median dose of WNV inoculated by *Culex tarsalis* mosquitoes is ~10^5 PFU (Styer et al., 2007). Accordingly, we inoculated mice with 10^5 PFU of either WNV[C6/36] or WNV[BHK] and assessed WNV titers in two initial targets of WNV, the skin at the inoculation site (ipsilateral footpad) and the draining popliteal lymph node (Brown et al., 2007). We also assessed viremia and spread to secondary tissue targets, the spleen, brain and spinal cord. The viral loads in the skin were very similar for mice inoculated with either WNV[C6/36] or WNV[BHK] at all time points (Fig 1B). In the other initial target tissue, the draining popliteal lymph node, the viral loads were similar for mice inoculated with either virus except at the earliest time point (Fig 1C). At 6 hour post-inoculation (hpi), significantly higher viral burden (P<0.05) was found in the draining popliteal lymph node of mice inoculated with WNV[BHK] (geometric mean of $10^{3.8}$ PFU/g) than for mice inoculated with WNV[C6/36] (geometric mean of $10^{2.8}$ PFU/g). Also at 6 hpi, five of six mice inoculated with WNV[BHK] had detectable levels of viremia (geometric mean of $10^{2.4}$ PFU/mL; p<0.05), whereas virus was not detected in the serum of mice inoculated with WNV[C6/36] (Fig 1A). After 6 hpi, the viremic levels were not different for mice inoculated with either virus. Since progeny virus is produced after ~8 h for WNV (Shi et al., 2002), virus detected at 6 hpi is likely from the viral inoculum; therefore, higher viremia and viral load in the draining lymph node at 6 hpi are most likely due to more rapid spread of the WNV[BHK] inoculum into the blood and via lymphatics. The very early viremia at 6 hpi in mice inoculated with WNV[BHK] likely resulted in significantly higher viral load (P<0.005) in their spleens at 24 hpi (geometric mean $10^{4.4}$ PFU/g) (Fig 1D). In comparison, only one of the six mice inoculated with WNV[C6/36] had detectable virus in the spleen at this time point ($10^{2.7}$ PFU/g). By 120 hpi, similar amounts of virus were found in the spleen for both viruses (geometric mean of $10^{3.8}$ PFU/g). Viral loads in brains and spinal cords were assessed at 120 hpi, and there were no significant differences between WNV[C6/36]- and WNV[BHK]-inoculated mice (data not shown). In addition, virus was detected in the brains of mice inoculated with either WNV[C6/36] or WNV[BHK] at the same frequency (six of eight mice) (data not shown). Overall, these results suggest that WNV[BHK] spreads more rapidly from the inoculation site to

the draining lymph node and into the blood, resulting in higher viral load in the spleen at 24 hpi. However, this difference in the spread of the virus did not affect the tissue tropism or viral load in other tissues, including the central nervous system.

Our *in vivo* results are in contrast to our prediction based on published *in vitro* studies, in which higher infectivity of mosquito cell-derived WNV had been seen for DCs (Davis et al., 2006). One possible explanation for this discrepancy is that we saturated infection at 10^5 PFU, and thereby masked any subtle differences between the two viruses. Since mosquitoes inoculate a wide range of viral doses into hosts, ranging from 10 to 10^6 PFU for *Cx. tarsalis* mosquitoes (Styer et al., 2007), we determined whether differences between $WNV^{C6/36}$ and WNV^{BHK} *in vivo* could be detected when mice were inoculated with lower doses of virus. Mice were inoculated SC with 10 or 10^3 PFU of virus and sacrificed at various time points, and tissues were harvested. For the initial targets of infection (skin at inoculation site and draining popliteal lymph node), the viral loads were very similar between mice inoculated with either $WNV^{C6/36}$ or WNV^{BHK} at a given viral dose (Fig 2B and 2C) except for a single tissue at a single time point. In the skin at the inoculation site, levels of virus in mice inoculated with 10^3 PFU of $WNV^{C6/36}$ (geometric mean of $10^{4.3}$ PFU/g) were significantly ($P<0.05$) higher than mice inoculated with the same dose of WNV^{BHK} (geometric mean of $10^{3.5}$ PFU/g) at 24 hpi (Fig 2B). In addition, there were no significant differences in viremic levels or viral loads in the spleens between the two viruses for a given dose (Fig 2A and 2D). Although not significantly different from the corresponding WNV^{BHK}-group, one of the $WNV^{C6/36}$ groups had a higher percentage of mice with detectable viremia; viremia was detected in all mice inoculated with 10 PFU of $WNV^{C6/36}$, but in only two of the six mice inoculated with WNV^{BHK} at 48 hpi (Fig 2A). At the 10 and 10^3 PFU doses, the early spread of WNV^{BHK}, which was observed at the 10^5 PFU dose, was not detected and is most likely due to the limit of detection for the assays. Although WNV^{BHK} may spread from the inoculation site more quickly than $WNV^{C6/36}$, the viremia and viral loads were not significantly affected after 24 hpi at any of the doses. In summary, viral loads in the initial and secondary targets of infection and viremic levels did not differ substantially between $WNV^{C6/36}$ and WNV^{BHK} when mice were inoculated with a high, medium, or low viral dose.

Mice inoculated with mosquito cell-derived WNV produce an earlier functional type I interferon response than mice inoculated with mammalian cell-derived WNV

Several laboratories have reported that mammalian cell-derived arboviruses induce higher levels of IFN-α/β than mosquito cell-derived arboviruses *in vitro* (Shabman et al., 2007; Silva et al., 2007); therefore, we predicted that WNV^{BHK} would induce higher levels of type I interferon than $WNV^{C6/36}$ in mice. Accordingly, we assessed the abilities of $WNV^{C6/36}$ and WNV^{BHK} to stimulate functional IFN-α/β *in vivo* at different viral doses. Contrary to our prediction, there was a trend toward earlier induction of IFN-α/β in mice inoculated with $WNV^{C6/36}$ compared to mice inoculated with WNV^{BHK} (Fig 3). A significant difference was only observed between mice inoculated with a single dose at a single time point; for the 10 PFU groups, IFN-α/β levels in sera were significantly higher in $WNV^{C6/36}$-inoculated mice (mean of 211 IU/ml) than in WNV^{BHK}-inoculated mice (mean of 51 IU/mL) ($P<0.05$) (Fig 3B). For the 10^3 PFU groups, IFN-α/β was more frequently detected at 48 hpi in sera of mice inoculated with $WNV^{C6/36}$ than in mice inoculated with WNV^{BHK} (Fig 3B). At 10^5 PFU of virus, IFN-α/β appeared to peak earlier in sera of mice inoculated with $WNV^{C6/36}$ than in mice inoculated with WNV^{BHK}; IFN-α/β was more frequently detected at 24 hpi and less frequently detected at 48 hpi in mice inoculated with $WNV^{C6/36}$ than in mice inoculated with WNV^{BHK} (Fig 3A). In summary, these results contradict our prediction and suggest that $WNV^{C6/36}$ elicits an earlier IFN-α/β response in mice than WNV^{BHK}.

No immature particles were detected in virus prepared from mosquito and mammalian cells

The presence of non-infectious particles and/or immature particles could impact the induction of IFN-α/β *in vivo* and explain our unexpected results. Therefore, we examined the genome copy numbers and the presence of prM proteins in our virus preparations. WNV RNA was extracted from our virus stocks, and the genome copy numbers were determined using an established real-time reverse transcription PCR assay (Lanciotti et al., 2000). The stock of WNV[BHK] had a 2.7-fold higher genome copy-to-PFU ratio and, therefore, more non-infectious virus particles than the stock of WNV[C6/36] (Table 2). In addition, equivalent genome copies of the viruses were analyzed by western blot using an antibody specific for prM/M to determine the presence of immature particles in the virus stocks. M protein was detected in WNV[C6/36] and WNV[BHK], but prM protein was not detected in the virus stocks (Fig 4), demonstrating that neither stock had detectable immature viral particles. These results suggest that the earlier induction of IFN-α/β in mice by WNV[C6/36] was not due to more non-infectious or immature viral particles in the stock of WNV[C6/36].

Primary myeloid dendritic cells are more susceptible to mosquito cell-derived virus than to mammalian cell-derived virus

Mosquito cell-derived arboviruses are more infectious than mammalian cell-derived arboviruses for myeloid DCs (mDCs) *in vitro* (Davis et al., 2006; Klimstra et al., 2003; Shabman et al., 2007); however, we showed that there were minimal differences in the replication kinetics between WNV[C6/36] and WNV[BHK] *in vivo*. We conducted studies to ensure that the disparities between our results and the results of published reports were not due to differences in our virus preparations by examining the infectivity on DCs. We generated immature mDCs from the bone marrow of mice. Approximately 96% of these cells expressed CD11b, a cell surface marker for mDCs, after 7 days in culture (Fig 5A). We subsequently inoculated these cells with WNV[C6/36] or WNV[BHK], and the presence of WNV nonstructural protein 3 (NS3) was measured as an indicator of infection at 48 hpi. As shown in Figure 5B and 5C, WNV[C6/36] infected approximately five-fold more mDCs than did WNV[BHK] ($16 \pm 2\%$ vs. $3 \pm 1\%$), which is consistent with results in published reports for WNV (Davis et al., 2006). In addition, WNV[C6/36] stimulated approximately three-fold more IFN-α/β (23,000 $\pm 1,000$ IU/mL) than did WNV[BHK] (8,000$\pm$1,000 IU/mL) in DCs (Fig 6), which is consistent with the findings of Silva and co-workers for mDCs (Silva et al., 2007) and supports our *in vivo* results (Fig 3). Furthermore, these results confirm that the viral stocks that were used in our *in vivo* studies generated similar data in primary mDCs as published studies.

During our flow cytometric analysis, we also examined the expression of CD11b and CD11c, surface markers for mDCs. The expression of both markers was decreased following inoculation with WNV[C6/36], but not after inoculation with WNV[BHK] or mock-inoculation (Fig 7). For the DCs inoculated with WNV[C6/36], the surface expression of CD11c decreased from a mean fluorescent intensity (MFI) of 34.7$\pm$1.2 (mock) to 24.8$\pm$1.3 (Fig 7A and 7B), and the surface expression of CD11b decreased from a MFI of 878$\pm$14 (mock) to 662$\pm$39 (Fig 7C and 7D). These results suggest that viruses generated from different host cells differentially affect the phenotype of mDCs. This effect was not due to more non-infectious or immature particles in our stock of WNV[C6/36] (Table 2 and Fig 4).

Replication kinetics of WNV[C6/36] and WNV[BHK] are indistinguishable in primary mDCs and primary skin cells

The DCs of the skin, Langerhans cells, are postulated to be an initial cell target for WNV, which subsequently migrate to the draining lymph node upon infection (Johnston et al., 2000). Since WNV[C6/36] infected more DCs than did WNV[BHK] (Fig 5), we predicted higher viral loads in the draining lymph nodes for WNV[C6/36] during early viral replication in mice; however, we did not observe any significant differences after 6 hpi (Fig 1 and 2). Thus, we

examined viral replication kinetics in primary DCs, using several multiplicities of infection (MOI), to address these discrepancies between our *in vitro* and *in vivo* studies. Growth curves for WNV$^{C6/36}$ and WNV^{BHK} were very similar in primary DCs at all MOIs (Fig 8). These data, together with the data on infectivity for DCs (Fig 5), indicate that DCs infected with WNV$^{C6/36}$ produced two- to five-fold fewer viruses per infected cell than did DCs infected with WNV^{BHK} (i.e., 200–700 PFU/cell and 1,000–1500 PFU/cell for WNV$^{C6/36}$ and WNV^{BHK}, respectively). These results may explain why there were no significant differences in viral load due to replication (after 6 hpi) in draining lymph nodes of mice inoculated with WNV$^{C6/36}$ or WNV^{BHK}. In addition to Langerhans cells, other possible initial cell targets reside in the skin (Brown et al., 2007). Thus, we also investigated the growth of WNV$^{C6/36}$ and WNV^{BHK} in primary skin cells. As shown in figure 9, titers of WNV$^{C6/36}$ in primary skin cells were identical to the titer of WNV^{BHK} at all MOIs and were consistent with the similar results that we observed for the two viruses in the skin of mice (Fig 1B and 2B). In conclusion, replication kinetics of WNV$^{C6/36}$ and WNV^{BHK} were identical in primary DCs and primary skin cells, supporting our *in vivo* results.

Discussion

The effect of viral source, whether the virus is derived from mammalian or mosquito cells, on arbovirus-host interactions has been studied by several investigators using *in vitro* models (Davis et al., 2006; Klimstra et al., 2003; Shabman et al., 2007). In general, mosquito cell-derived virus results in more infected dendritic cells and lower levels of type I interferon than mammalian cell-derived virus. The assumption in the arbovirus field is that viral source would similarly affect the pathogenesis *in vivo*.

The goal of the present study was to test this assumption for WNV, and we demonstrated that the viral source has minimal effect on WNV pathogenesis in mice. These results are very robust since three doses, 10, 10^3 and 10^5 PFU, were studied, covering the lowest to median dose inoculated by *Cx. tarsalis* mosquitoes (Styer et al., 2007). The clinical disease, mortality rates, ID$_{50}$, tissue tropism, and neuroinvasion were equivalent for mice inoculated with mosquito cell-derived WNV and mammalian cell-derived WNV. In addition, the viral loads in initial and secondary target tissues and viremic levels were similar after the initiation of viral replication (>6 hpi) with only two exceptions, and after 24 hpi, there were no significant differences in viremia or viral load of any tissue tested for a particular viral dose. There was a trend for an earlier induction of IFN-α/β in mice inoculated with WNV$^{C6/36}$ compared to mice inoculated with WNV^{BHK}, but a significant difference in serum levels of IFN-α/β was only observed at low virus dose at 48 hpi. In conclusion, although the source of WNV had minor effects on viral loads and IFN-α/β production in mice at early time points, it did not significantly affect WNV pathogenesis *in vivo*.

Our results contradict the current thinking in the arbovirus field that mosquito cell-derived virus induces less type I interferon *in vivo* as it does *in vitro* (Shabman et al., 2007; Silva et al., 2007). Plasmacytoid DCs (pDCs) inoculated with mammalian cell-derived WNV produce over 1,000-fold higher levels of IFN-α than cells inoculated with mosquito cell-derived WNV (Shabman et al., 2007; Silva et al., 2007). Thus, we expected higher levels of IFN-α/β in the mice inoculated with WNV^{BHK}, but we actually observed a trend toward earlier and in one instance higher IFN-α/β in the mice inoculated with WNV$^{C6/36}$. A possible explanation is that pDCs are not initial targets of WNV infection *in vivo*. pDCs are rare in normal, non-inflamed skin and are present in low numbers in quiescent lymph nodes, migrating into these tissues during inflammation (reviewed in Villadangos and Young, 2008). Thus, pDCs may not play a role until after initiation of WNV infection when the cells are exposed to viral progeny of vertebrate origin. On the other hand, conventional mDCs, present in relatively high numbers in skin and lymph nodes, are exposed to the viral inoculum and are assumed to participate in

the first round of infection (Johnston et al., 2000). In our *in vitro* studies, mDCs that were infected with WNV[C6/36] produced three-fold more IFN-α/β than cells infected with WNV[BHK], and these results are consistent with those of Silva and co-workers (Silva et al., 2007). Furthermore, our results with primary mDCs infected with WNV in culture are consistent with our *in vivo* results; WNV[C6/36] tended to induce an earlier type I interferon response in mice than WNV[BHK].

Several other factors may contribute to the apparent contradiction of our *in vivo* results with published *in vitro* results, including the particle-to-PFU ratios of the viruses, the infected cell type, and sensitivity of our methods. UV-inactivated WNV triggers IFN-α release by pDCs (Silva et al., 2007), and defective interfering particles of Sindbis virus stimulate type I IFN production in primary cells (Fuller and Marcus, 1980). These studies suggest that the particle-to-PFU ratio of the virus is a determinant of IFN-α/β production. Thus, we determined the genome copy-to-PFU ratios for our stocks of WNV[C6/36] and WNV[BHK], and the latter had approximately three-fold higher ratio. These results indicate that our stock of WNV[BHK] contains slightly more non-infectious particles, arguing against the possibility that WNV[C6/36] has more non-infectious particles, inducing a greater IFN-α/β response *in vivo*. In addition, we did not detect immature viral particles in either viral stock, providing additional support that our data was not due to an artifact of our virus preparation. Another explanation is that cells that do not belong to the DC lineage may be infected initially. Cells in the skin support WNV replication at the inoculation site and distal sites (Brown et al., 2007), suggesting that non-migrating cells in the skin are infected. These cells may produce similar amounts of type I interferon in response to WNV derived from mosquito and mammalian cells. An additional possibility is that we measured IFN-α/β in the serum, which reflects production from all infected tissues. This method may not be sensitive enough to pick up any differences early during infection.

The similar viremic titers and viral loads in mice inoculated with WNV[C6/36] or WNV[BHK] after the initiation of viral replication (> 6 hpi) were unexpected since previous reports demonstrated that mosquito cell-derived WNV is more infectious for DCs than mammalian cell-derived virus (Davis et al., 2006). We confirmed that our viral stocks were not unique and had similar infectivity as reported by Davis and co-workers; WNV[C6/36] was approximately five-fold more infectious for primary mDCs than WNV[BHK]. We examined why this increase in infectivity did not result in greater viral loads for WNV[C6/36] by conducting growth curves. The multi-step and one-step growth curves on primary mDCs were equivalent for the two different viruses at a specific MOI. Assuming that all infected cells produced progeny virions, we estimate that WNV[C6/36]-infected DCs produced two- to five-fold fewer viruses per cell than did WNV[BHK]-infected DCs. This may explain the lower than expected viral loads for WNV[C6/36]-inoculated mice. Another explanation is that DCs may not be the only initial target cell, and again, cells in the skin are likely initial targets, contributing to early viral production in the animal. Furthermore, multi-step and one-step growth curves on primary skin cultures were equivalent for the two different viruses at a specific MOI.

There are several possible mechanisms for the lower viral yield in mDCs infected with WNV[C6/36] compared to cells infected with WNV[BHK]. One possible explanation is that infection by WNV[C6/36] was aborted in a subset of DCs through some unknown mechanism(s). Alternatively, the greater IFN-α/β response elicited by WNV[C6/36] may subsequently lead to lower amounts of progeny virus production. A third possibility is an unknown effect of the virus on DC function, resulting in lower viral yield or abortive infection. Several viruses inhibit DC function upon infection, including herpes simplex virus, hepatitis C virus, and lymphocytic choriomeningitis virus (Eksioglu et al., 2009; Kruse et al., 2000; Pollara et al., 2003; Sevilla et al., 2004). Our results suggest that WNV generated from mosquito cells alters the function of mDCs; incubation of DCs with mosquito cell-derived WNV, but not mammalian cell-

derived WNV, resulted in down-regulation of CD11b and CD11c on mDCs. This finding was previously reported for human cytomegalovirus-infected macrophages (Gafa et al., 2005). To our knowledge, we are the first to report lower expression of these integrins on DCs due to viral infection. In addition, down-modulation of CD11c and CD11b may not require infection by WNV because cells that were negative for WNV antigen also had lower levels of CD11c and CD11b following WNV inoculation (data not shown). CD11c and CD11b are alpha integrin chains, which form heterodimers with the beta integrin chain, CD18. These complexes have many functions and play a role in phagocytosis, opsonization, cell migration, and antigen presentation. One ligand of CD11b/CD18 and CD11c/CD18 is iC3b, an opsonin produced during activation of the complement system (reviewed by (Bajtay et al., 2006). Although a direct role has not been documented for these receptor-ligand interactions during WNV infection, complement activation is crucial for controlling the virus, given that mice lacking the third complement component are more susceptible to lethal WNV infection (Mehlhop et al., 2005). Our results suggest that WNV derived from mosquito cells potentially inhibits the functions of DCs; however, any effect *in vivo* would be limited to the first round of infection, which most likely does not have any lasting effects on viral immunity since viral source did not affect infectivity, morbidity, or mortality in mice.

Although our mouse studies revealed similar viremic titers and viral loads in mice inoculated with $WNV^{C6/36}$ or WNV^{BHK} after the initiation of viral replication, there were differences between mice inoculated with either of the viruses at the earliest time point (6 hpi) at the 10^5 PFU dose. Virus detected at 6 hpi represents extracellular virus from the initial viral inocula, rather than progeny viruses, which are not detected until at least 8 hpi (Shi et al., 2002). We propose the following model to explain differences in spread of the $WNV^{C6/36}$ or WNV^{BHK} inocula and subsequent viral replication. WNV derived from mosquito cells binds cells and/ or the extracellular matrix in the skin more tightly than does WNV derived from mammalian cells. Thus, upon subcutaneous inoculation, mosquito cell-derived WNV from the initial viral inoculum is more likely to be trapped at the site, resulting in minimal spread of the inoculum to the blood and draining lymph node at 6 hpi and potentially higher levels of infectious virus in the skin at the inoculation site at 24 hpi. In contrast, WNV generated from mammalian cells may have weaker binding to cells and/or the extracellular matrix in the skin, allowing more rapid dissemination of the viral inoculum to other tissues through the blood and/or lymphatics. This model is supported by the higher viremia and viral burden in the draining lymph node at 6 hpi for WNV^{BHK}-inoculated mice. In addition, we propose that the more rapid spread of WNV^{BHK} resulted in higher viral load in the spleen at 24 hpi. It is important to note that these observations were dose dependent. Higher viral load in the skin of $WNV^{C6/36}$-inoculated mice was only observed at the medium dose of 10^3 PFU, and difference in spread of the viral inocula was only observed at the high dose of 10^5 PFU. Despite this early difference in spread, inoculation of the very low doses (1 PFU and 10 PFU) resulted in equivalent ID^{50} for both $WNV^{C6/36}$ and WNV^{BHK}, indicating that both viruses were equally infectious for mice. Furthermore, these differences at early time points did not affect the viral burdens after 24 hpi, neuroinvasion, or clinical outcome.

Our model that viral source affects binding *in vivo* is consistent with the results of *in vitro* studies. $WNV^{C6/36}$ infects a greater number of DCs in culture, as shown by our present results and in a previous report (Davis et al., 2006). The presence of high mannose glycans on mosquito cell-derived viruses (Hsieh and Robbins, 1984; Hsieh et al., 1983) could facilitate virus attachment to cell surface receptors on skin cells. Interactions between C-type lectins and mosquito cell-derived viruses contribute to enhanced infectivity *in vitro* for several mosquito-borne viruses, such as WNV (Davis et al., 2006), dengue virus (Navarro-Sanchez et al., 2003), and alphaviruses (Klimstra et al., 2003; Shabman et al., 2007).

To our knowledge, this is the first study to examine the effect of viral source on viral pathogenesis *in vivo*. Contrary to the accepted assumption of the arbovirus field, we demonstrated that the source of virus did not significantly impact WNV pathogenesis, using a mouse model that closely resembles WN disease in humans (Brown et al., 2007; Petersen et al., 2002), and our results can likely be extended to other flaviviruses. The outcome for alphaviruses, however, may differ since there is a more pronounced effect of viral source on type I interferon induction in conventional mDCs for alphaviruses (Klimstra et al., 2003; Shabman et al., 2007) than is observed for WNV. Thus, further studies are warranted to understand the effect of mosquito cell- and mammalian cell-derived viruses for other arbovirus systems. In addition, viral source is only one factor in the early arbovirus-host interaction. In nature, WNV is transmitted via the bite of WNV-infected mosquitoes, and several studies have shown that mosquito transmission enhances WNV infection in hosts (Schneider et al., 2006; Styer et al., 2006). Future studies will be conducted to determine how mosquito saliva impacts the early virus-host interaction and affects the pathogenesis of arboviruses.

Materials and methods

Cells

African green monkey kidney cells (Vero; ATCC #CCL-81) and baby hamster kidney cells (BHK-21; ATCC #CCL-10) were maintained in minimal essential medium (MEM, Gibco Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 100 U/mL of penicillin, and 100 μg/mL of streptomycin]. Mouse fibroblast cells (L929; ATCC #CCL-1) were maintained in MEM supplemented with 10% HI-FBS, 2 mM L-glutamine, and 0.6 g/L sodium bicarbonate (L929 medium). *Aedes albopictus* mosquito cells (C6/36; ATCC # CRL-1660) were maintained in MEM supplemented with 10% HI-FBS, 1.1 g/L sodium bicarbonate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were incubated in 5% CO_2 at 37°C (Vero, BHK-21, and L929) or 28°C (C6/36).

Viruses

WNV was produced from a full-length cDNA clone of a 2000 New York strain as previously described (Shi et al., 2002). Briefly, *in vitro* transcribed RNA from the full length cDNA clone was electroporated into BHK-21 cells and into C6/36 cells, to produce WNV[BHK] and WNV[C6/36], respectively. For virus stocks, supernatants were harvested on day 3 (BHK-21) or day 4 (C6/36) after electroporation when cytopathic effect was observed. Supernatants were clarified by centrifugation at 10,000 x g for 20 min at 4°C and stored in aliquots at −80°C. Viral titers were determined by plaque assays on Vero cell monolayers. Encephalomyocarditis virus (EMCV) was a gift from Dr. Laura White at University of North Carolina at Chapel Hill. EMCV was produced in BHK-21 cells, and viral titers were determined by plaque assays on L929 cells.

Viral RNA was isolated in triplicate from virus stocks using Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). A real-time RT-PCR assay for WNV RNA was performed as described previously (Lanciotti et al., 2000).

Mice

We purchased five-week-old female C57BL/6 mice from Taconic (Germantown, NY). Mice were acclimated in the biosafety-level-3 animal facility for at least 1 week and were provided with food and water ad libitum. All studies were approved by Institutional Animal Care and Use Committee of the Wadsworth Center and followed criteria established by the National Institutes of Health.

Mortality and infectivity of WNV-inoculated mice

Six-week-old mice were inoculated SC in the left rear footpad with 10 μL of $WNV^{C6/36}$ or WNV^{BHK} at 1 or 10 plaque forming unit (PFU) (n=11 to 12 mice per group) as described previously (Brown et al., 2007). Virus was diluted in low endotoxin PBS (tissue culture grade; Invitrogen) with 1% HI-FBS. All mice were observed for clinical signs, which included ruffled fur, ataxia, weakness, and weight loss, at least once a day during the entire study. They were weighed daily for at least 14 days post-inoculation (p.i.) and three times weekly during the rest of the study. Mice with severe disease were euthanized. On day 28, all surviving mice were bled, and virus infection was confirmed in WNV-inoculated mice by the presence of WNV-specific antibodies using an ELISA as previously described (Ebel et al., 2002).

WNV replication *in vivo*

Six-week-old mice were inoculated with virus as described above, at a dose of 10, 10^3, or 10^5 PFU of WNV. At various times after inoculation, mice (n=6–8) were euthanized, and the following tissues were harvested: blood, ipsilateral footpad (inoculation site), draining popliteal lymph node, spleen, spinal cord, and brain. For the 24 and 48 hour time points, mice were sacrificed and perfused with PBS with 1% HI-FBS at 6 mL per min for 10 minutes prior to harvesting tissues. Blood was processed to collect serum, which was frozen at −80°C. All other tissues were processed as previously described (Brown et al., 2007). Briefly, buffer was added to make a 20% homogenate for central nervous system tissues and a 10% homogenate for peripheral tissues, and samples were stored at −80°C until testing by plaque assays on Vero cells.

Interferon α/β bioassay

The presence of functional type I interferon in the serum was determined as described previously (Trgovcich et al., 1996). Briefly, L929 cells were seeded in 96-well plates (4×10^4 cells/well) and incubated at 37°C overnight. Serum samples and recombinant mouse interferon β (standards; Chemicon International, Temecula, CA) were diluted in L929 medium, acidified to pH 2 with 2 N HCl, and incubated at 4°C overnight. Samples were neutralized to pH 7 with 2 N NaOH. Samples and standards were added to triplicate wells of 96-well plates of L929 cells and serially diluted two-fold. Plates were incubated at 37°C overnight. EMCV (2×10^5 PFU) was added to all wells containing standards and samples. After 24 hour at 37°C, cell viability was assessed using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI), as per manufacturer's instruction. The amounts of IFN-α/β in the serum were interpolated from the standard curve. The values reported are averages from triplicate wells.

Western blot analysis

Virus was mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and then incubated at 95°C for 10 min. Equivalent genome copies of the virus were electrophoresed on a 12% Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA). Supernatants from mock-electroporated cells were used as negative controls, and lysates of Vero cells inoculated with WNV^{BHK} or mock-inoculated were used as controls for the detection of prM. Following transfer to PVDF membranes (Bio-Rad Laboratories, Hercules, CA), prM and M proteins were detected with antibodies against WNV M protein (1:200 dilution; Imgenex, San Diego, CA), followed by incubation with horseradish peroxidase conjugated goat-anti-rabbit (1:1000 dilution; Vector, Burlingame, CA). Protein bands were visualized using Immun-Star WesternC Chemiluminescent Kit (Bio-Rad Laboratories, Hercules, CA).

Isolation of dendritic cells

Primary mDCs were isolated from bone marrow of adult C57BL/6 mice and cultured as previously described (Serody et al., 2000; Shabman et al., 2007). Briefly, cells were isolated from bone marrow by flushing the femur and tibia with RPMI 1640 medium (Invitrogen; Carlsbad, CA), supplemented with 10% HI-FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/mL Penicillin, and 100 μg/mL streptomycin sulfate (R10F medium). Red blood cells were lysed using ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4), and cells were cultured in ultra-low binding plates (Corning, Lowell, MA) in the presence of 20 ng/mL of recombinant granulocyte-macrophage colony stimulating factor (GM-CSF; PeproTech Inc, Rocky Hill, NJ). Cells were grown in the presence of GM-CSF and recombinant interleukin-4 (IL-4; PeproTech Inc, Rocky Hill, NJ) at 10 ng/mL (days 3–4) and then at 5 ng/mL (days 5–7) to generate immature mDCs. After 7 days in culture, cells were used for flow cytometric analysis or were infected with virus.

WNV infection of immature dendritic cells

On day 7, immature mDCs were harvested and infected with WNV prepared in RPMI-1649, supplemented with 1% HI-FBS and 10 mM HEPES (RPMI-1H) in triplicate for 2 h at 37°C. Cells were washed three times to remove extracellular viruses, then cultured in RPMI-1H, supplemented with 5 ng/mL of GM-CSF and IL-4 at 37°C. At various times post-inoculation, an aliquot of the supernatant was harvested and stored at −80°C for plaque assay analysis.

Cell surface and intracellular staining for flow cytometry

Flow cytometric analysis was performed to characterize the immature mDCs and to determine the infectivity of WNV in mDCs. Cells were harvested, washed with cold staining buffer (PBS supplemented with 1% HI-FBS) by centrifugation at 400xg for 5 min, and resuspended in staining buffer. Samples were incubated with Fc block (BD Pharmingen) at room temperature for 10 min. FITC-conjugated antibodies against CD11c (1:100 dilution; clone N418; eBioscience, San Diego, CA) and PE-conjugated antibodies against CD11b (1:100 dilution; clone M1/70; BD Pharmingen) were added to the cells and incubated for 20 min at 4°C. Samples were washed 3 times with staining buffer and were then fixed with 2% paraformaldehyde (PFA) for 20 min at 4°C. Cells were washed once with permeabilization buffer (PBS containing 0.1% (w/v) saponin, 0.09% sodium azide, and 0.5% bovine serum albumin), incubated in permeabilization buffer for 10 min at 4°C, and washed. Cells were resuspended with Alexa674-conjugated monoclonal antibodies against WNV NS3 protein (provided by Dr. David Lawrence, Wadsworth Center, Albany, NY) and Fc block, and incubated at 4°C for 20 min. Samples were washed twice with permeabilization buffer and once with staining buffer. Finally, cells were resuspended in 2% PFA and stored at 4°C overnight. Data were collected using a FACScalibur flow cytometer (BD Pharmingen) and were analyzed using FLOWJO flow cytometric analysis software (Tree Star, Inc). Cells were gated based on forward and side scatter plot, and 10,000 cells were collected for each sample. During flow cytometry analysis, isotype controls were used to set the background for expression of CD11c and CD11b, and mock-inoculated cells that had been incubated with antibodies against NS3 were used to set the background for WNV infection. Expression of CD11c was presented as mean fluorescent intensity (MFI).

Isolation and infection of primary skin cells

We adapted a previously described method to isolate primary skin cells from ears of adult mice (Johnston et al., 2000). Mice were euthanized, and ears were removed and placed in PBS with 10% HI-FBS. Ears were rinsed with PBS, split into dorsal and ventral halves, and placed in PBS containing 0.03% trypsin (Invitrogen; Carlsbad, CA), 0.01% DNase I (Promega, Madison, WI),100 U/mL of penicillin, 100 μg/mL of streptomycin, and 4 μg/mL of amphotericin B at

4°C overnight. On the next day, the epidermal layer was separated from the dermal layer, and both layers were incubated in PBS with 0.03% trypsin at 37°C for 20 min. Following incubation, tissues were cut into smaller pieces, DMEM supplemented with 10% HI-FBS, 25 mM HEPES, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 4 µg/mL of amphotericin B (DMEM) was added, and tissues were pipetted vigorously for 10 min. Cell suspensions were strained through a 100 µm cell strainer and centrifuged at 460 x g for 10 min at 4°C. Cells were washed and cultured in DMEM at 37°C overnight to allow for the recovery of cell surface protein. On the next day, cells were inoculated with virus for 1 h at 37°C, washed three times, and cultured in DMEM at 37°C, 5% CO_2. At various times post-inoculation, an aliquot of the medium was removed from the cells and stored at −80°C for later titration.

Statistical analysis

The Mann-Whitney test (GraphPad, San Diego, CA) was used to assess the difference between the two viruses for all the data. A P-value of <0.05 was considered statistically significant.

Acknowledgments

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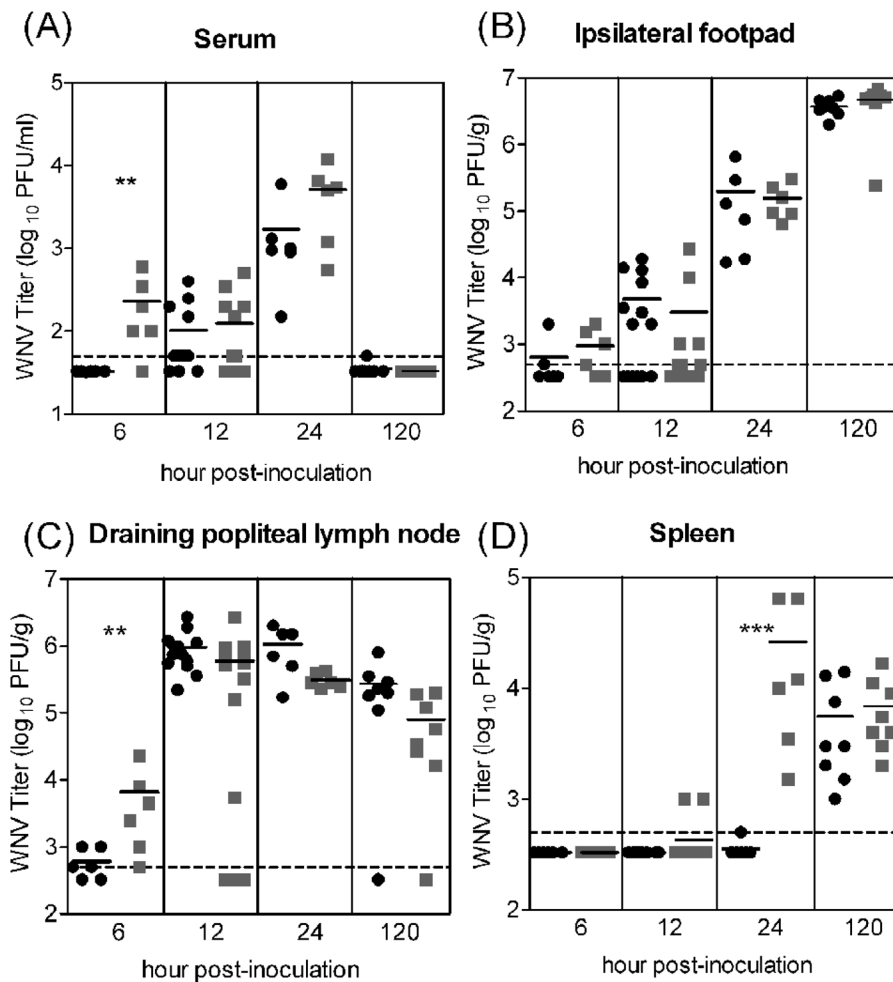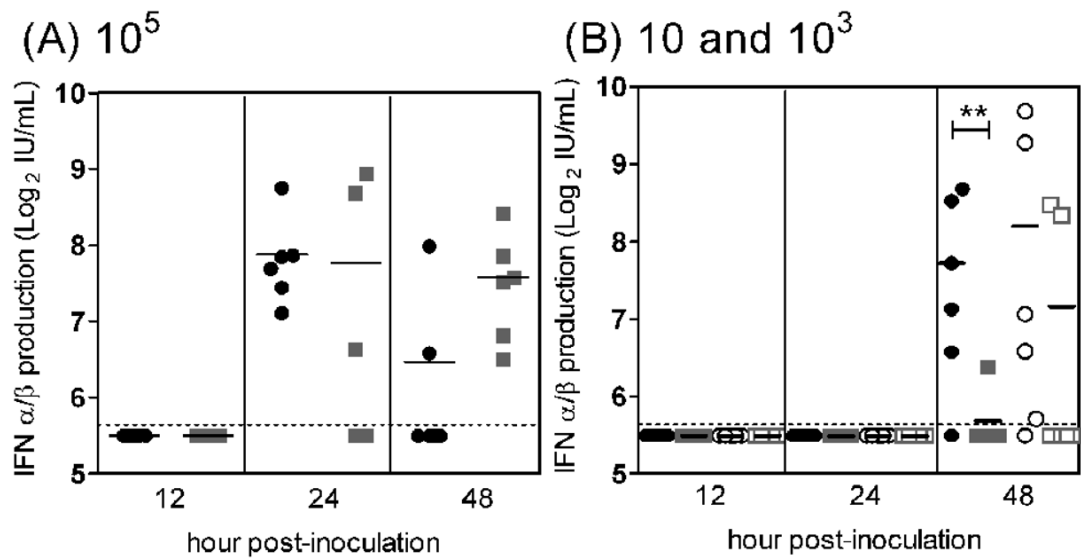
Figure 1.
WNV titers did not differ substantially in mice inoculated with 10^5 PFU of $WNV^{C6/36}$ or WNV^{BHK} except at the earliest time points. Mice were inoculated subcutaneously with 10^5 PFU of $WNV^{C6/36}$ (●) or WNV^{BHK} (■) in the left rear footpad. Tissues were harvested at various times post-inoculation, and plaque assays were performed to determine the viral load in (A) serum, (B) ipsilateral footpad (inoculation site), (C) draining popliteal lymph node, and (D) spleen. Data from two independent experiments are shown. Solid lines indicate the geometric mean for each group of 6–12 mice. Dashed lines represent the limit of detection (LOD), and data points below the line are < LOD. Asterisks denote significant difference between groups (Mann-Whitney test: ** $p < 0.05$; ***$p < 0.005$).
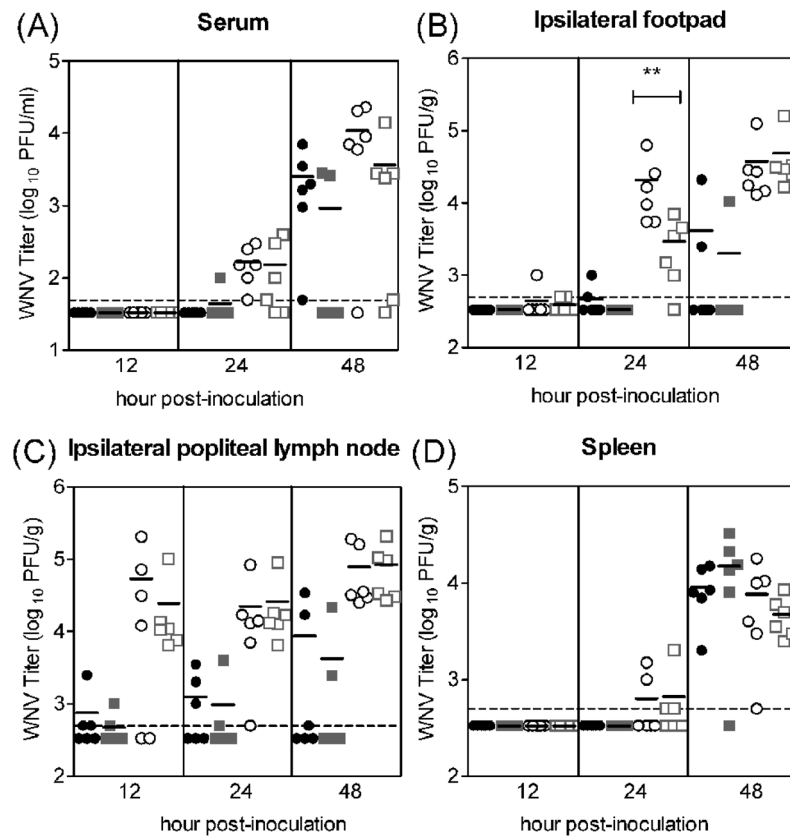
Figure 2.
WNV titers were similar between $WNV^{C6/36}$ and WNV^{BHK} in mice inoculated with low or medium viral dose. Mice (n=6) were inoculated subcutaneously with 10 PFU of $WNV^{C6/36}$ (●) or WNV^{BHK} (■) or 10^3 PFU of $WNV^{C6/36}$ (○) or WNV^{BHK} (□) in the left rear footpad. Tissues were harvested at various times post-inoculation, and plaque assays were performed to determine the viral load in (A) serum, (B) ipsilateral footpad (inoculation site), (C) draining popliteal lymph node, and (D) spleen. Data from one experiment is shown. Solid lines indicate the geometric mean for each group of 6 mice. Dashed lines represent the limit of detection (LOD), and data points below the line are < LOD. Asterisks show significant difference between groups (Mann-Whitney test: ** p <0.05).

Figure 3.
Mice inoculated with WNV$^{C6/36}$ produced IFN-α/β earlier than mice inoculated with WNV^{BHK}. Mice were inoculated subcutaneously with (A) 10^5 PFU of WNV$^{C6/36}$ ($\bullet$) or WNV^{BHK} ($\blacksquare$), (B) 10 PFU of WNV$^{C6/36}$ ($\bullet$) or WNV^{BHK} ($\blacksquare$) or 10^3 PFU of WNV$^{C6/36}$ ($\circ$) or WNV^{BHK} ($\square$) in the left rear footpad. Sera were harvested at different times post-inoculation, and IFN bioassays were performed on L929 cell monolayer. Data from two independent experiments are shown. Solid lines indicate the geometric mean of 6 mice per group. Dashed lines represent the limit of detection (LOD), and data points below the line are < LOD. Asterisks show significant difference between groups (Mann-Whitney test: ** p <0.05).
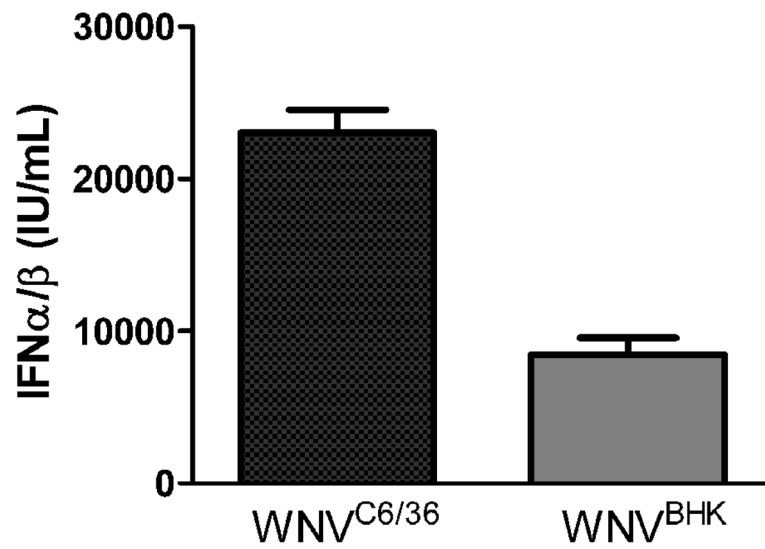
Figure 4.
Immature particles were not detected in virus preparations. Equivalent genome copies of WNV$^{C6/36}$ and WNV^{BHK} were electrophoresed on a 12% Tris-HCl gel under denaturing conditions. Following transfer to PVDF membrane, membranes were incubated with antibodies against M, followed by horseradish peroxidase conjugated-goat-anti-rabbit. Protein bands were visualized using chemiluminescent detection kit. Supernatants from mock-electroporated cells were negative controls, and a lysate from Vero cells infected with WNV^{BHK} was a positive control for the detection of prM and M proteins. Three independent experiments were performed, and a representative western blot is shown.
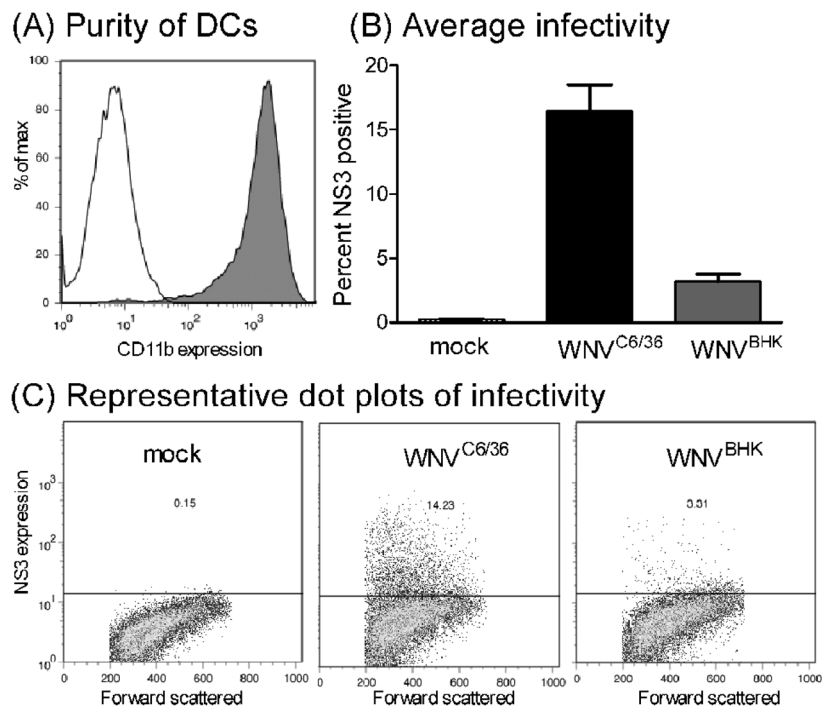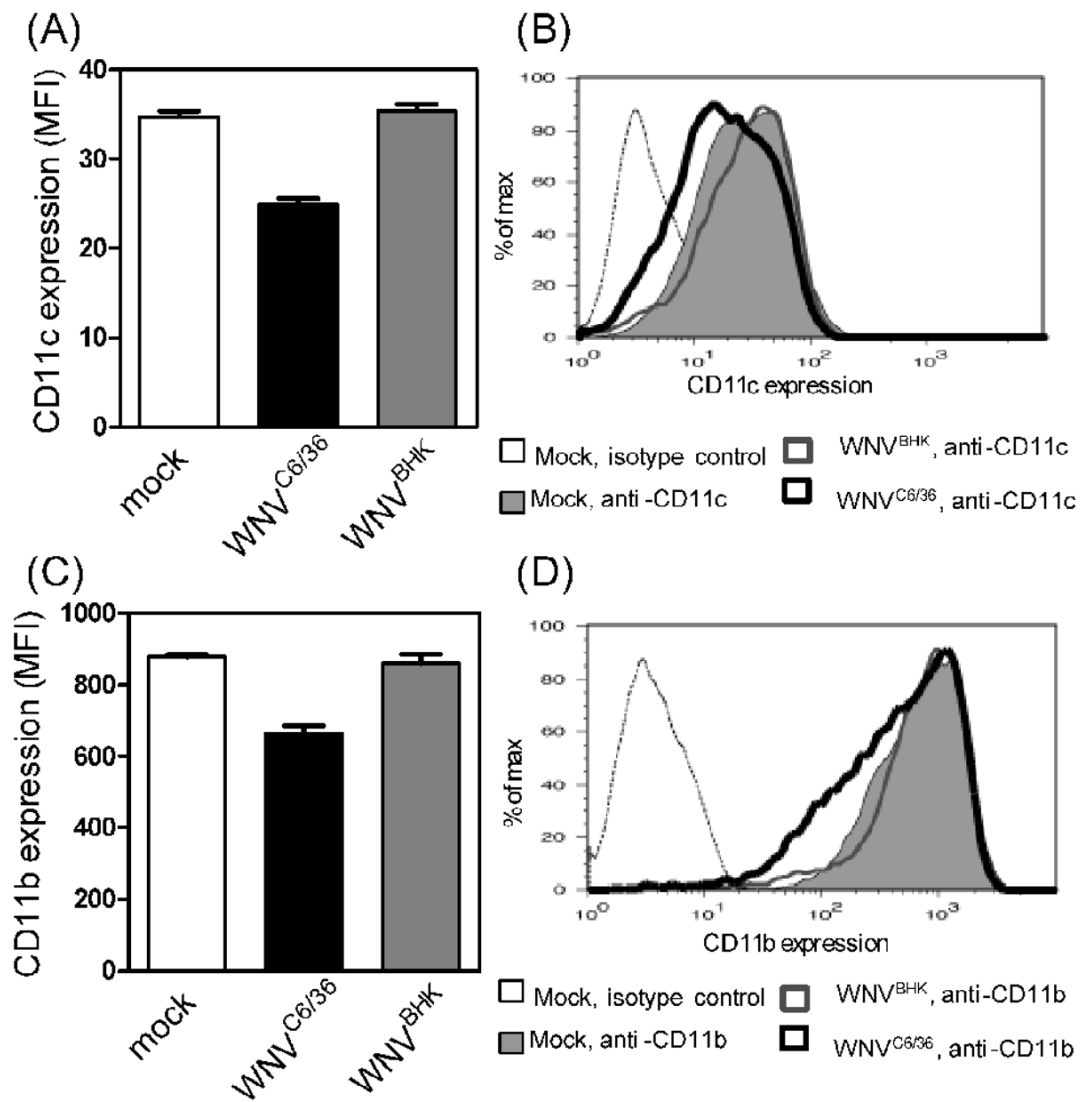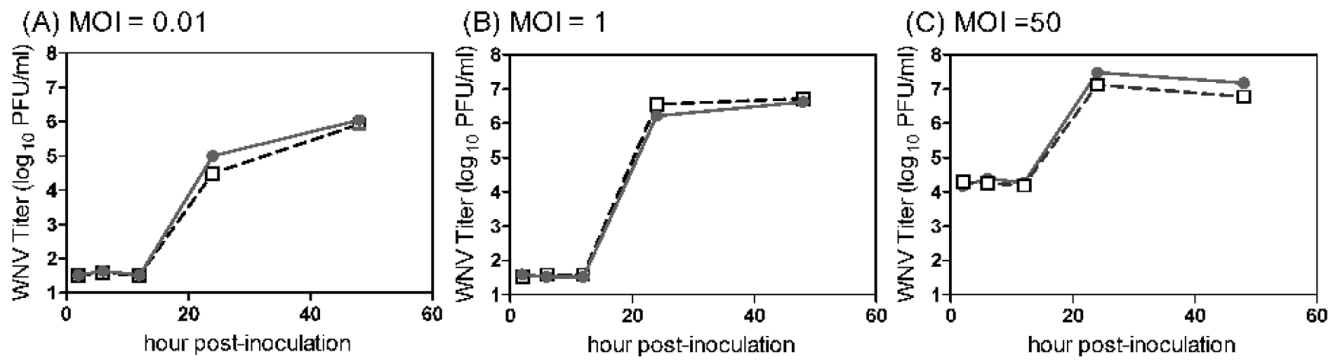
Figure 5.
The percentage of primary dendritic cells infected by WNV[C6/36] was greater than the percentage infected by WNV[BHK]. (A) After 7 days in culture, primary immature DCs were incubated with PE-conjugated anti-CD11b (grey, filled) or isotype control (black, unfilled) prior to infection with WNV. (B and C) Primary DCs were inoculated with diluent (mock) or with WNV[C6/36] or WNV[BHK] at MOI of 50. Cells were harvested at 48 hpi, and incubated with Alexa fluor 647-conjugated antibodies against NS3. All samples were fixed overnight at 4°C and then analyzed by flow cytometry. (B) Average ± standard error from 2 independent experiments and (C) representative dot plots are shown.

Figure 6.
WNV$^{C6/36}$ induced greater IFN α/β production in primary myeloid dendritic cells than WNV^{BHK}. Primary DCs were inoculated with diluent (mock) or with WNV at MOI of 50. Culture supernatants were harvested at 48 hpi, and IFN bioassays were performed as described in legend of figure 3. Two independent experiments were performed, and the averages ± standard deviations from one experiment, performed in triplicates, are shown.
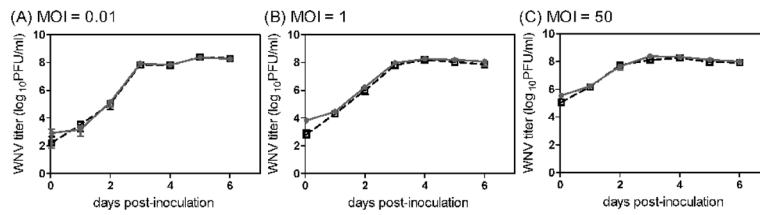
Figure 7.
WNV$^{C6/36}$, but not WNV^{BHK}, down-regulated expression of CD11c and CD11b on primary dendritic cells. Primary DCs were inoculated with diluent (mock) or with WNV$^{C6/36}$ or WNV^{BHK} at MOI of 50. Cells were harvested at 48 hpi and incubated with (A and B) FITC-conjugated anti-CD11c and (C and D) PE-conjugated anti-CD11b. Samples were fixed overnight at 4°C and then analyzed by flow cytometry. Mean fluorescent intensities (MFI) ± standard deviations from triplicates are reported in (A and C). Two independent experiments were conducted, and representative histograms are shown in B and D.

Figure 8.
The replication kinetics for WNV$^{C6/36}$ in primary dendritic cells was similar to the kinetics for WNV^{BHK}. Primary DCs were inoculated with WNV$^{C6/36}$ (●) or WNV^{BHK} (□) at MOI of (A) 0.01, (B) 1, or (C) 50. At various times post-inoculation, medium was harvested and stored at −70°C. WNV titer in the medium was determined by plaque assays on Vero cell monolayers. The averages ± standard deviations from triplicate wells are shown. The experiment was repeated at least three times at each MOI, and representative results are shown.

Figure 9.
The replication kinetics for WNV$^{C6/36}$ and WNV^{BHK} was nearly identical in primary skin cells. Primary skin cells were inoculated with WNVC6/36 (●) or WNV^{BHK} (□) at MOI of (A) 0.01, (B) 1, or (C) 50. At various times post-inoculation, medium was harvested and stored at −70°C. WNV titer in the medium was determined by plaque assays on Vero cell monolayers. The averages ± standard deviations from triplicate wells are shown.

Table 1

Mortality and infectivity of WNV^C6/36 and WNV^BHK were indistinguishable in mice.

Inoculum	Virus dose (PFU)	No. mice per group	Mortality (no. dead/total)	^{a} Seroconversion (no. seropositive/survivors)	^{b} Infection (no. infected/total)	Actual mortality (no. dead/no. infected)
WNV^C6/36	1	12	1/12 (8%)	5/11 (45%)	6/12 (50%)	1/6 (17%)
WNV^C6/36	10	12	3/12 (25%)	9/9 (100%)	12/12 (100%)	3/12 (25%)
WNV^BHK	1	11	1/11 (9%)	5/10 (50%)	6/11 (55%)	1/6 (17%)
WNV^BHK	10	11	3/11 (27%)	7/8 (88%)	10/11 (91%)	3/10 (30%)

Adult, female C57BL/6 mice were inoculated SC with 1 or 10 PFU of WNV^C6/36 or WNV^BHK in the left rear footpad. They were observed for clinical signs and weighed daily.

^{a}Sera from mice that survived infection were tested for antibodies against WNV proteins by an ELISA.

^{b}Number of infected mice equals mice that died or were euthanized due to WN disease and surviving mice that seroconverted.

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Table 2

WNV prepared from mammalian cells contains more noninfectious particles than WNV prepared from mosquito cells.

Virus	(genome copies)/mL[a]	PFU/mL	(genome copies)/PFU
WNV$^{C6/36}$	$(5.7 \pm 1.0) \times 10^{10}$	1.1×10^9	51
WNV^{BHK}	$(2.8 \pm 0.3) \times 10^{11}$	2.0×10^9	138

[a]Viral RNA was extracted, and then real-time RT-PCR assays were performed. Average ± standard deviations from triplicates are shown