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A West Nile virus NS4B-P38G mutant strain induces adaptive immunity via TLR7-MyD88-dependent and independent signaling pathways

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Abstract

Prior work shows that an attenuated West Nile virus (WNV), the nonstructural (NS)4B-P38G mutant infection in mice induced strong immune responses and protected host from subsequent lethal wild-type WNV infection. Here, we investigated NS4B-P38G mutant infection in myeloid differentiation factor 88-deficient (MyD88^{-/-}) and Toll-like receptor 7-deficient (TLR7^{-/-}) mice and found they had enhanced susceptibility compared to wild-type mice. Both groups had lower WNV-specific IgM response and reduced effector T cell functions. Dendritic cells (DCs) also exhibited a reduced maturation and impaired antigen-presenting functions compared to wild-type DCs. Moreover, infection with NS4B-P38G mutant in TLR7^{-/-} and MyD88^{-/-} mice provided full and partial protection respectively from subsequent challenge with lethal wild-type WNV. There were reduced T cell responses in MyD88^{-/-} and interleukin-1 receptor deficient (IL-1R^{-/-}) mice during secondary challenge with wild-type WNV. In contrast, TLR7^{-/-} mice displayed normal T cell functions. Collectively, these results suggest that TLR7-dependent MyD88 signaling is required for T cell priming during NS4B-P38G mutant infection, whereas the TLR7-independent MyD88 signaling pathways are involved in memory T cell development, which may contribute to host protection during secondary challenge with wild-type WNV

Keywords

West Nile virus; NS4B protein; Immune response; T cell

1. Introduction

West Nile virus (WNV), a mosquito-borne flavivirus with a positive-sense, single-stranded RNA genome has caused annual outbreaks of viral encephalitis in North America since 1999 [1]. Infection of the central nervous system (CNS) commonly presents as encephalitis,

meningitis or acute flaccid paralysis. Fatality has been recorded in 10% of WNV neuroinvasive disease-confirmed cases with a higher frequency in the elderly and immunocompromised. 20–50% of WNV convalescent patients have significant long-term morbidity [2–4]. Multiple approaches have been taken to develop WNV vaccines, including recombinant DNA [5], recombinant protein [6,7] and chimeric live attenuated vaccines [8]. Nevertheless, no vaccines have been approved for human use.

The flavivirus genome encodes ten proteins – three structural proteins (envelope (E), membrane, nucleocapsid) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [9,10]. The NS4B protein is a 27-kDa, non-glycosylated, hydrophobic protein. Mutation studies in the NS4B protein have suggested that it is associated with both viral replication and evasion of host immunity [11–13]. The NS4B P38 residue is conserved in the majority of mosquito- and tick-borne flaviviruses. By utilizing site-directed mutagenesis of a WNV New York 1999 strain (NY99) infectious clone, we have identified an attenuated mutation with a P38G substitution in NS4B protein. This P38G mutant has significantly reduced neuroinvasiveness in NIH Swiss outbred mice as compared to the parental wild-type WNV. Full genome sequencing of the mutant revealed two compensatory mutations – NS4B-T116I and NS3-N480H [14]. The NS4B-P38G mutant also induced a greater innate cytokine production and stronger T cell responses in C57BL/6 (B6) mice than wild-type WNV. Mice immunized with the NS4B-P38G mutant were protected from subsequent lethal wild-type WNV infection, which suggests that the NS4B-P38G mutant has suitable features for an ideal vaccine candidate [15]. The underlying immune mechanisms are not fully understood. Toll like receptor (TLR) s play an important role in the regulation of innate and adaptive immunity to viral infection. The core TLR signaling pathway utilizes myeloid differentiation factor 88 (MyD88) as the primary adaptor [16,17], except TLR3. TLR7 expression has been shown to be important for protection of the host against wild-type WNV infection [18,19]. Here, we investigated the role of the TLR7-MyD88 signaling pathway in regulating protective immunity to WNV NS4B-P38G mutant infection in mice.

2. Materials and methods

2.1. Mice

6–10-Week-old OT-II, interleukin-1 receptor (IL-1R)^{-/-} and control B6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). MyD88^{-/-} and TLR7^{-/-} mice (B6 × 129 F₂ background, Regeneron Inc., Tarrytown, NY) were bred to the B6 background by backcrossing for 7–10 successive generations [18]. Groups were age and sex-matched and housed under identical conditions. All animal experiments were approved by the Animal Care and Use Committee at the University of Texas Medical Branch.

2.2. WNV infection

WNV NY99- or NS4B-P38G mutant infectious clone-derived virus was each passaged once in Vero cells to make a virus stock for cell culture. Mice were inoculated intraperitoneally (i.p.) with 500 plaque forming unit (PFU) of WNV NS4B-P38G mutant [14]. In some experiments, surviving mice were re-challenged with a lethal dose (LD₁₀₀, 2000 PFU) of wild-type WNV at day 30 post-primary infection. Infected mice were monitored twice daily for signs of morbidity. Bone-marrow (BM)-derived DCs were generated as described previously [20]. Briefly, BM cells were cultured for 6 days in RPMI-1640 supplemented with granulocyte-macrophage-colony stimulating factor, and interleukin-4 (Peprotech) to generate myeloid DCs and were infected with WNV with a multiplicity of infection (MOI) of 0.2. Supernatants and cells were collected at 96 h post-infection to measure viral load and cytokine production.

2.3. Plaque assay

Vero cells were seeded in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) and incubated with mouse sera for 1 h. 2× DMEM containing 1% low-melting-point agarose was added and incubated for 3 days. A second overlay of 1% agarose-medium containing 0.01% neutral red was added to visualize plaques. Virus concentrations were calculated as PFU/ml.

2.4. Quantitative PCR (Q-PCR) for viral load and cytokine production

WNV-infected samples were resuspended in Trizol (Invitrogen) for RNA extraction. Complementary (c)DNA was synthesized by using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). The sequences of the primer-probe sets for WNV envelope (*WNVE*) and cytokine gene cDNA and PCR reaction conditions were described previously [21,22]. The assay was performed in an iCycler (Bio-Rad, Hercules, CA). To normalize the samples, the same amount of cDNA was used in a Q-PCR for *β-actin*. The ratio of the amount of amplified gene compared with the amount of *β-actin* cDNA represented the relative levels in each sample.

2.5. ELISA

Microtiter plates were coated with recombinant WNV-E protein [23] overnight at 4 °C at 100 ng/well. Sera were diluted 1/30 in PBS with 2% BSA, and incubated for 1 h at room temperature. Alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Sigma–Aldrich) at a dilution of 1/1000 in PBS–Tween was then added for 1 h. Color was developed with *p*-nitrophenyl phosphate (Sigma–Aldrich) and intensity determined at an absorbance of 405 nm by using a spectrophotometer.

2.6. Flow cytometry

Splenocytes were stained with antibodies for CD11c, CD80, CD86, CD4 or CD8 (e-Biosciences). To measure intracellular cytokine production, splenocytes were stimulated with WNV-specific NS3 and E peptides (RRWCFDGPRTNTILE and PVGRLVTNPFVSVA, respectively [24]) for CD4 T cells or WNV specific NS4B and E peptides (SSVWNATTA and IALTFLAV, respectively [25,26]) for CD8 T cells for 5 h at 37 °C. Golgi-plug (BD Biosciences) was added at the beginning of stimulation. Cells were stained with antibodies for CD4 or CD8, fixed in 2% paraformaldehyde, permeabilized with 0.5% saponin before adding PE-conjugated anti-IFN γ , or control rat IgG1 (e-Biosciences) and examined by using a C6 Flow Cytometer (Accuri cytometers, Ann Arbor, MI).

2.7. In vitro T cell priming assays

CD4⁺ T Cells and DCs were purified from splenocytes of naïve OT II transgenic mice and WNV-infected mice respectively by using anti-CD11c, and anti-CD4 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). CD4⁺ T cells (2×10^6 cells) were labeled with 0.5 μ mol/L carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) and mixed with DCs at a 10:1 ratio with or without OVA residue 323–339 (1 μ g/ml, Gen-script Corporation, Piscataway, NJ) for 5 days. Cells were harvested and CD4⁺ T cells were gated for analysis of proliferation. Supernatant was collected at day 3 for analysis of cytokine production by using a Bio-Plex Pro Mouse Cytokine Assay (Biorad).

2.8. Statistical analysis

Data analysis was performed by using Prism software (Graph-Pad) statistical analysis. Survival curve comparisons were performed using the log rank test (equivalent to the Mantel–Haenszel test). Values for phenotype analysis, viral burden, and cytokine production

experiments were presented as means $\pm$ SEM. The *P* values were calculated with a non-paired Student's *t* test.

3. Results

3.1. TLR7-MyD88 signaling is involved in host protection during NS4B-P38G mutant infection

To determine the role of the TLR7-induced immune response during WNV NS4B-P38G mutant infection, we compared the survival rates of wild-type, MyD88^{-/-} and TLR7^{-/-} mice following i.p. inoculation of 500 PFU of WNV. All wild-type mice (100%) survived a four-week infection period compared to 61% and 71% survival in MyD88^{-/-} and TLR7^{-/-} mice respectively (Fig. 1A). We next measured viral load by a plaque assay. At day 3 post-infection, viremia in MyD88^{-/-} and TLR7^{-/-} mice was about 8- to 20-fold higher than that of wild-type mice (Fig. 1B). Infection was cleared at day 5 in wild-type mice, but was continuously detectable in MyD88^{-/-} and TLR7^{-/-} mice (Fig. 1B). Thus, both MyD88^{-/-} and TLR7^{-/-} mice were more susceptible to NS4B-P38G mutant infection than wild-type mice.

3.2. Innate cytokine and humoral responses in NS4B-P38G mutant-infected mice

Innate cytokine production was studied to further investigate the role of TLR7 and MyD88 during NS4B-P38G mutant infection. On day 3 post-infection, type 1 IFNs and proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) were induced in all groups of mice (Fig. 2A–D and F). IL-1 β gene expression was lower in MyD88^{-/-}, but not in TLR7^{-/-} mice compared to wild-type mice (Fig. 2C). IL-12 gene expression was decreased in both NS4B-P38G mutant-infected MyD88^{-/-} and TLR7^{-/-} mice (Fig. 2E). B cell-mediated humoral immune responses are critical for the host defense against disseminated infection by wild-type WNV [27,28]. We found sera IgM responses were reduced in MyD88^{-/-} and TLR7^{-/-} mice at days 3, 8 and 21 post-infection (Fig. 2G). No differences were observed in sera IgG responses in these mice (Fig. 2H). These results indicate TLR7-MyD88 signaling is involved in induction of IL-12 expression and WNV-specific IgM response following NS4B-P38G infection.

3.3. T cell responses during NS4B-P38G mutant infection

CD4⁺ and CD8⁺ T-cells are important for host survival following wild-type WNV infection and contribute to long-lasting protective immunity [29,30]. Here, we studied T cell responses in NS4B-P38G mutant-infected mice. On day 8 post-infection, the percentages of CD4⁺IFN γ ⁺ and CD8⁺IFN γ ⁺ splenocytes in both MyD88^{-/-} and TLR7^{-/-} mice were lower than in wild-type mice upon *ex vivo* stimulation with WNV peptides (Fig. 3A and B). The absolute numbers of splenic CD4⁺ and CD8⁺ T cells in MyD88^{-/-} or TLR7^{-/-} mice were reduced by 30–40% and 11–15% respectively (Fig. 3C). At the later stage of infection (day 21), there was a decrease in the percentage of CD4⁺IFN γ ⁺, but not of CD8⁺IFN γ ⁺ splenocytes in MyD88^{-/-} mice (Fig. 3D and E). There were also significantly less splenic CD4⁺ and CD8⁺ T cells in MyD88^{-/-} mice compared to wild-type mice (Fig. 3F). In contrast, neither the percentages of CD4⁺IFN γ ⁺ and CD8⁺IFN γ ⁺ splenocytes nor the number of splenic CD4⁺ and CD8⁺ T cells were different between TLR7^{-/-} and wild-type mice (Fig. 3D–F). Thus, TLR7/MyD88 signaling is involved in initial T cell activation; TLR7-independent MyD88 signaling may be involved in T cell response at later stage of infection.

3.4. Antigen presenting functions of NS4B-P38G mutant-infected DCs

DCs represent the most important antigen-presenting cells exhibiting the unique capacity to initiate primary T cell responses and are permissive to WNV infection. We previously

demonstrated that NS4B-P38G mutant induced a MyD88-dependent proinflammatory cytokine response in DCs [15]. Here, we studied NS4B-P38G infection in TLR7^{-/-} DCs. Viral load in NS4B-P38G mutant infected-TLR7^{-/-} DCs was greatly enhanced at day 4 post-infection compared to wild-type DCs (Fig. 4A). No differences in the levels of IFN- α , IFN- β , IL-1 β and IL-12 were noted between the two groups (Fig. 4B–D and F). However, IL-6, and TNF- α genes expression was significantly lower in TLR7^{-/-} DCs (Fig. 4E and G). We next assessed splenic DCs phenotype in NS4B-P38G mutant infected mice. On day 3 post-infection, there was about 30–40% decrease in the total number of CD11c⁺CD80⁺ splenocytes in MyD88^{-/-} mice when compared to the wild-type group (Fig. 5A). There was no difference in the percentage of CD11c⁺CD80⁺ splenocytes between the two groups (Fig. 5B). Neither the number nor the percentage of CD11c⁺CD80⁺ splenocytes was different between TLR7^{-/-} and wild-type mice (Fig. 5A and B). Nevertheless, we noted that the total number and the percentage of CD11c⁺CD86⁺ splenocytes were both decreased in MyD88^{-/-} (32–42%) and TLR7^{-/-} (30–32%) mice (Fig. 5C and D). Finally, we tested the capability of DCs from WNV-infected wild-type, MyD88^{-/-} and TLR7^{-/-} mice to activate naïve CD4⁺ T cells *in vitro*. As shown in Fig. 5E and F, the proliferation rates and IL-2 production of OT II CD4⁺ T cells co-cultured with DCs of NS4B-P38G mutant-infected MyD88^{-/-} and TLR7^{-/-} mice were similar and were lower than of those co-cultured with DCs from wild-type mice. Overall, a deficiency in TLR7-MyD88-signaling pathway leads to an impaired antigen presenting capacity of DCs during NS4B-P38G mutant infection.

3.5. T cell responses during secondary challenge of wild-type WNV in NS4B-P38G mutant-immunized mice

To further investigate the role of TLR7-MyD88 signaling in adaptive immunity, we assessed the survival rate of NS4B-P38G mutant-immunized wild-type, MyD88^{-/-} and TLR7^{-/-} mice during secondary challenge of a LD₁₀₀ of wild-type WNV. Following one-month interval, immunized wild-type and TLR7^{-/-} mice were all (100%) protected from a LD₁₀₀ wild-type WNV infection (Fig. 6A). In comparison, only 72.7% of immunized MyD88^{-/-} mice survived (Fig. 6A). Furthermore, at day 4 post-secondary infection, WNV-specific IgM response was decreased in both MyD88^{-/-} and TLR7^{-/-} mice (Fig. 6B), while the IgG response (Fig. 6C) in both groups was at levels similar to those in wild-type mice. There were lower percentages of CD4⁺IFN γ ⁺ and CD8⁺IFN γ ⁺ splenocytes and significantly less splenic CD4⁺ and CD8⁺ T cells in MyD88^{-/-} group compared to wild-type mice (Fig. 6D–F). In contrast, neither the percentage of CD4⁺IFN γ ⁺ and CD8⁺IFN γ ⁺ splenocytes nor the number of splenic CD4⁺ and CD8⁺ T cells were different between TLR7^{-/-} and wild-type mice (Fig. 6D–F). The MyD88 adapter molecule is required for both TLR and the IL-1 superfamily signaling pathways [31,32]. We next examined T cell responses in NS4B-P38G mutant-immunized IL-1R^{-/-} mice. At day 4 post-secondary infection, we found a lower percentage of CD8⁺IFN γ ⁺ splenocytes (Suppl. Fig. 1A and B) and about 11% less splenic CD4⁺ T cells in IL-1R^{-/-} mice compared to those of wild-type group (Suppl. Fig. 1C). These results suggest that the IL-1R-MyD88 signaling is involved in memory T cell response; TLR7 expression is dispensable for memory T cell development and host protection during secondary challenge by wild-type WNV.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2013.06.093>.

4. Discussion

WNV NS4B-P38G mutant was highly attenuated in neuroinvasiveness and caused no lethality in B6 mice following systemic infection [15]. Here, our results show that MyD88^{-/-} and TLR7^{-/-} mice were more susceptible to NS4B-P38G mutant infection compared to wild-type mice. There was reduced WNV-specific IgM and effector T cell

responses in both groups at early stage of infection. Induction of WNV-specific IgM and T cell responses limits viremia and dissemination into the CNS and protects the host against lethal wild-type WNV infection [33–35]. Thus, TLR7-MyD88-signaling is involved in protective immunity against NS4B-P38G mutant infection. DC maturation is an innate response that leads to T cell mediated immunity to foreign antigens [36,37]. DCs can directly sense pathogen components *via* TLRs, up-regulate surface co-stimulatory molecules, secrete proinflammatory cytokines and enhance antigen presentation [38–40]. Our previous [15] and present studies suggest that NS4B-P38G mutant induced a TLR7-MyD88-dependent proinflammatory cytokine expression in DCs. Further, MyD88^{-/-} and TLR7^{-/-} DCs showed a reduced maturation and impaired antigen presenting functions compared to wild-type DCs, which together suggests that the TLR7-MyD88-signaling pathway is involved in DC maturation and T cell priming during NS4B-P38G mutant infection. The reduction on WNV specific IgM production observed in MyD88^{-/-} and TLR7^{-/-} mice may be the direct outcome of an impaired CD4⁺ T cell response in the absence of MyD88 and TLR7 expression. Alternatively, lack of TLR7 ligation in B cells could lead to a defective IgM response as reported by others [41,42].

We also found that NS4B-P38G mutant-immunized TLR7^{-/-} mice were fully protected from subsequent lethal wild-type WNV infection with a lower IgM response, but normal T cell functions. In contrast, NS4B-P38G mutant-immunized MyD88^{-/-} mice displayed lower IgM response and impaired memory T cell functions and were partially protected from secondary challenge with wild-type WNV. These results indicate that a TLR7-independent, MyD88-dependent memory T cell response is important for host protection during secondary challenge with wild-type WNV in NS4B-P38G mutant-infected mice.

Distinct inflammatory cytokines act directly on naïve T cells to provide a third signal, synergize with signals from the TCR and co-stimulatory receptors, to optimally activate differentiation and clonal expansion. IL-1 was shown to increase the proliferation of CD4⁺ T cells in response to antigen and IL-2, which is consistent with effects on *in vivo* priming of CD4⁺ T cells [43]. IL-12 is one main signal 3 cytokines produced for CD8⁺ T cell in response to intracellular pathogens [44,45]. NS4B-P38G mutant induced lower levels of expression of IL-1 β , IL-12, TNF- α and IL-6 in MyD88^{-/-} BM-DCs compared to wild-type DCs [15]. Here, we noted NS4B-P38G mutant-infected TLR7^{-/-} DCs only had a decreased IL-6 and TNF- α expression. Further, serum IL-12 gene expression was reduced in both MyD88^{-/-} and TLR7^{-/-} mice. This indicates that immune cells other than DCs in blood produced IL-12 in a TLR7-MyD88-dependent manner upon NS4B-P38G mutant infection. MyD88^{-/-} mice also had a lower IL-1 β expression, and this was not observed in TLR7^{-/-} mice. The requirement for MyD88 usually represents the involvement of either a single or multiple TLRs or cytokine signaling *via* IL-1R or IL-18R [31,32]. It is likely that the engagement of other MyD88-dependent TLRs during WNV NS4B-P38G mutant infection contribute to IL-1 β and/or IL-12 production, which may play an important role in CD4⁺ and CD8⁺ memory T cell development, though an early study [18] suggests that the other MyD88-dependent TLRs were dispensable for the induction of protective immunity during wild-type WNV infection. Moreover, other cytokine receptor signaling pathways, such as those of IL-1R and IL-18R, which rely on MyD88 expression could be involved in memory T cell development. T cell responses during a recombinant fowlpox virus infection are mediated *via* the IL-18R-MyD88 signaling pathway [46]. Here, we found IL-1R signaling is involved in memory T cell response during secondary challenge with wild-type WNV. Future studies will continuously be focused on the role of these receptors in memory T cell development during NS4B-P38G mutant infection.

Live attenuated vaccines, which induce durable protective immunity, have been very successful at controlling flavivirus diseases. Our previous studies indicate that the NS4B-

P38G mutant has the suitable features for an ideal vaccine candidate – a greatly reduced potential for neurovirulence and neuroinvasiveness, restricted virus replication, and the ability to induce a robust protective immune response [15]. A major risk factor for fatality of WNV infection in humans is age [1,47]. The alteration of the innate immune response, in particular that of PRR signaling pathways has been reported in the elderly population [48,49]. Treatment with agonists for TLRs or cytokines is one of the important strategies to boost vaccine efficacy [50]. Thus, the investigation of the role of TLR7-MyD88-mediated-signaling pathways in induction of protective adaptive immune responses during WNV NS4B-P38G mutant infection, can be utilized as a paradigm to aid in the rational development of efficacious live attenuated flavivirus vaccines for this highly risk population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

B6	C57BL/6
BM	bone marrow
CNS	central nervous system
DCs	dendritic cells
E	envelope
IFN	interferon
i.p	intraperitoneally
LD	lethal dose
MyD88	myeloid differentiation factor 88
NS	nonstructural
Q-PCR	quantitative PCR
TLR	Toll-like receptor
WNV	West Nile virus

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Fig. 1.

TLR7-MyD88 signaling pathway is required for protecting host from WNV NS4B-P38G mutant infection. (A) Mice were injected with WNV NS4B-P38G mutant and monitored twice daily for morbidity. Data shown are pooled from three independent experiments. $n = 12$ for wild-type (WT) mice. $n = 13$ and $n = 17$ for MyD88^{-/-} and TLR7^{-/-} mice respectively. (B) Viral load in serum was determined by using plaque assay at the indicated time points post-infection. ND: not detected. Data are presented as means $\pm$ SEM. * $P < 0.05$ or ** $P < 0.01$ compared to WT mice.

Fig. 2.

Innate cytokine and antibody production in WNV P38G-NS4B mutant-infected mice. (A–F) Cytokine levels in blood at day 3 post-infection were determined by using Q-PCR. The fold of increase compared to that of the mock group is shown. Data are presented as means $\pm$ SEM, $n = 4–5$. (G and H) Humoral response during primary infection with WNV NS4B-P38G mutant. Sera were collected from non-infected (NF) or mice infected with NS4B-P38G mutant at days 3, 8 and 21 post-infection. The development of WNV specific IgM (G) or IgG (H) antibodies was determined by ELISA. Data are presented as means $\pm$ SEM, $n = 4–7$ pooled from 3 separate experiments. * $P < 0.05$ or ** $P < 0.01$ compared to wild-type mice.

Fig. 3.

T cell responses during primary infection of WNV NS4B-P38G mutant. Mice were infected with WNV NS4B-P38G mutant, at day 8 (A–C) and day 21 (D–F) post-infection, splenocytes were cultured *ex vivo* with WNV peptides for 5hr. Cells were harvested, counted and stained for IFN γ and CD4 or CD8. (A, B and D, E) CD4⁺ or CD8⁺ T cells were gated, percent of IFN γ ⁺ are shown. (C and F) Total number of T cell subsets per spleen is shown. Data are presented as means $\pm$ SEM, $n = 2-4$ per group. * $P < 0.05$ or ** $P < 0.01$ compared to WT mice.

Fig. 4.

WNV P38G NS4B mutant infection in BM-DCs. (A) Viral load was measured at day 4 post-infection. NF: non-infected. IF: NS4B-P38G mutant-infected cells. (B–G) Cytokine expression was determined using Q-PCR. Data are presented as the fold of increase compared to the mock infected. Results are representative of three experiments. $n = 3$. ** $P < 0.01$ compared to wild-type DCs.

Fig. 5. DC maturation and antigen-presenting ability was reduced in MyD88^{-/-} and TLR7^{-/-} mice. At day 3 post-infection, splenocytes were isolated and stained for CD11c and CD80 or CD86. The absolute number or the percentages of CD80⁺ CD11c⁺ (A and B) and CD86⁺CD11c⁺ (C and D) in splenocytes of non-infected (NF) and WNV-infected (IF) mice were shown. *n* = 4 per group. (E and F) *In vitro* T cell priming assay. CFSE labeled CD4⁺ T cells from naïve OT II transgenic mice were co-cultured with DCs from WNV-NS4B-P38G mutant infected wild-type, MyD88^{-/-} and TLR7^{-/-} mice in the presence or absence of OVA 323–339. (E) Cells were harvested at day 5 and analyzed for T cell proliferation. (F) Supernatant was harvested at day 3 and measured for IL-2 production. The fold of increase

compared to those co-cultured with wild-type DCs was shown. $n = 3-6$ pooled from 2 separate experiments. $*P < 0.05$ or $**P < 0.01$ compared to wild-type mice.

Fig. 6.

Adaptive immunity during secondary challenge of wild-type WNV. Mice that survived from a primary infection with NS4B-P38G mutant were re-infected with a LD₁₀₀ of wild-type WNV. (A) Survival rate. Mice were monitored twice daily. $n = 13$ for wild-type mice. $n = 11$ for MyD88^{-/-} mice. $n = 6$ for TLR7^{-/-} mice. (B and C) Sera were collected from mice at day 4 post-secondary infection with wild-type WNV NY99 (R-D4). The development of WNV specific IgM (B) or IgG (C) antibodies was determined by ELISA. Data are presented as means $\pm$ SEM, $n = 4-6$. (D-F) At day 4 post-secondary infection, splenocytes were isolated and cultured *ex vivo* with WNV peptides for 5 h. Cells were stained for IFN γ , and CD4 or CD8. (D and E) CD4⁺ or CD8⁺ T cells were gated, percent of IFN γ ⁺ are shown. (F) Total number of T cell subsets per spleen is shown. Data are presented as means $\pm$ SEM, $n = 2-4$ per group. * $P < 0.05$ or ** $P < 0.01$ compared to wild-type mice.