

Video Article

In Vitro Analysis of Myd88-mediated Cellular Immune Response to West Nile Virus Mutant Strain Infection

Guorui Xie¹, Melissa C. Whiteman², Jason A. Wicker², Alan D.T. Barrett^{1,2,3}, Tian Wang^{1,2,3}

¹Department of Microbiology & Immunology, The University of Texas Medical Branch

²Department of Pathology, The University of Texas Medical Branch

³Center for Biodefense and Emerging Infectious Diseases, Sealy Center for Vaccine Development, The University of Texas Medical Branch

Correspondence to: Tian Wang at ti1wang@utmb.edu

URL: <https://www.jove.com/video/52121>

DOI: [doi:10.3791/52121](https://doi.org/10.3791/52121)

Keywords: Immunology, Issue 93, West Nile Virus, Dendritic cells, T cells, cytokine, proliferation, *in vitro*

Date Published: 11/27/2014

Citation: Xie, G., Whiteman, M.C., Wicker, J.A., Barrett, A.D., Wang, T. *In Vitro Analysis of Myd88-mediated Cellular Immune Response to West Nile Virus Mutant Strain Infection*. *J. Vis. Exp.* (93), e52121, doi:10.3791/52121 (2014).

Abstract

An attenuated West Nile virus (WNV), a nonstructural (NS) 4B-P38G mutant, induced higher innate cytokine and T cell responses than the wild-type WNV in mice. Recently, myeloid differentiation factor 88 (MyD88) signaling was shown to be important for initial T cell priming and memory T cell development during WNV NS4B-P38G mutant infection. In this study, two flow cytometry-based methods – an *in vitro* T cell priming assay and an intracellular cytokine staining (ICS) – were utilized to assess dendritic cells (DCs) and T cell functions. In the T cell priming assay, cell proliferation was analyzed by flow cytometry following co-culture of DCs from both groups of mice with carboxyfluorescein succinimidyl ester (CFSE) - labeled CD4⁺ T cells of OTII transgenic mice. This approach provided an accurate determination of the percentage of proliferating CD4⁺ T cells with significantly improved overall sensitivity than the traditional assays with radioactive reagents. A microcentrifuge tube system was used in both cell culture and cytokine staining procedures of the ICS protocol. Compared to the traditional tissue culture plate-based system, this modified procedure was easier to perform at biosafety level (BL) 3 facilities. Moreover, WNV- infected cells were treated with paraformaldehyde in both assays, which enabled further analysis outside BL3 facilities. Overall, these *in vitro* immunological assays can be used to efficiently assess cell-mediated immune responses during WNV infection.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52121/>

Introduction

West Nile virus (WNV), a neurotropic, plus-sensed flavivirus, is an emerging public health threat. Currently, no vaccines have been approved for human use¹. An attenuated WNV strain, which has a P38G substitution in the nonstructural (NS)4B protein, is known to induce no lethality in mice but higher innate cytokines and T cell responses in mice than wild-type WNV NY99 strain². Mice immunized with the NS4B-P38G mutant were all protected from a secondary challenge with lethal wild-type WNV. This suggests that the NS4B-P38G mutant has suitable features for an ideal vaccine candidate. The mechanisms by which the NS4B-P38G mutant induces high protective adaptive immunity are not clearly understood yet. Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns, play an essential role in the initiation of innate immunity to viral infection. The core TLR signaling pathway utilizes myeloid differentiation primary response gene 88 (MyD88) as the primary adaptor^{3,4}. In a recent study, MyD88 signaling was shown to play an important role in development of cell mediated immunity during WNV NS4B-P38G mutant infection in mice⁵. Dendritic cells (DCs) are one of the most important antigen-presenting cells exhibiting the unique capacity to initiate primary T cell responses during viral infection^{6,7}. CD4⁺ and CD8⁺ T-cells both contribute to long-lasting protective immunity and are important for host survival following wild-type WNV infection^{8,9}. Two immunological assays were used in this study to assess the functions of these cells in the NS4B-P38G mutant infected mice.

First, an *in vitro* T cell priming assay was utilized to compare the antigen-presenting capability of DCs of WNV-infected wild-type and MyD88^{-/-} mice. To increase sensitivity of the assay, naïve CD4⁺ T cells were isolated from OTII transgenic mice, which express a Vα2/Vβ5 TCR specific for the chicken ovalbumin (OVA) peptide 323 - 339. DCs of WNV infected mice were purified, and co-cultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4⁺ T cells in the presence of OVA peptide. After 5 days of co-culture, cells were harvested and fixed with paraformaldehyde (PFA) and analyzed by flow cytometry. Proliferative assays have been traditionally carried out through incorporation of 5-bromo-2'-deoxyuridine (BrdU) or tritiated thymine deoxyriboside (³HTdr)¹⁰. Nevertheless, these assays are either radioactive and/or in need of special equipments at biosafety level (BL) 3 facilities, where WNV studies are conducted. The flow cytometric analysis of lymphocyte proliferation by serial halving of the fluorescence intensity of the vital dye CFSE has become more commonly used in immunological assays as the dye is more stably and evenly incorporated into cells, detected easily by flow cytometry, and is nonradioactive¹¹. The assay also has the ability to assess the number of cell divisions. One major advantage of using this assay in WNV studies is that fixation of the infected cells with 1-2% PFA could inactivate WNV¹², which will enable sample acquisition with a flow cytometer in a BL2 laboratory.

Next, a modified intracellular cytokine staining (ICS) procedure was used to study the role of MyD88 signaling in regulation of WNV specific T cell responses in NS4B-P38G mutant-infected mice. In this assay, splenocytes isolated from infected mice were treated *in vitro* with WNV specific peptides. Brefeldin A was added to retain the cytokines within the cell. After 5 hr incubation, cells were harvested, washed and stained for T cell subsets. Cells were then fixed in PFA, permeabilized and stained for interferon (IFN)- γ and analyzed by flow cytometry. As with other flow cytometry-based assay, once the cells are treated with fixation and permeabilization buffer containing PFA, infected samples can be transferred to a BL2 laboratory for further processing and analysis. In several published studies, we have used ICS to measure T cell effector functions in WNV-infected mice^{13,14}. Although it's well established, one major drawback of this assay is that the procedure is very lengthy and could be more time consuming when performed inside BL3 facilities. Here, a micro-centrifuge tube-based ICS method was shown to be more feasible, easier to proceed and less time consuming when performed within a BL3 laboratory.

Protocol

All animal experiments were approved by the Animal Care and Use Committee at the University of Texas Medical Branch.

1. Isolation of DCs from Non-infected and WNV-infected Mice

1. Age- and sex- match 6-10 week-old, wild-type C57BL/6 (B6) and MyD88^{-/-} mice. Inoculate intraperitoneally (i.p.) with 500 plaque forming unit (PFU) of WNV NS4B- P38G mutant. At day 3 post-infection, euthanize B6 and MyD88^{-/-} mice with CO₂. Use non-infected mice of both groups as controls. Use three mice from each group.
2. Wet fur on left side of sacrificed mouse using 70% ethanol and cut away the fur along the left side of the mouse, about half-way between the front and back legs. Cut open the body cavity. Remove the spleen by using the forceps. Place the spleen in a Petri dish with RPMI.
3. Isolate splenic DCs by using anti-CD11c magnetic beads according to the manufacturer's instructions.

2. Purification and Labeling of T Cells of OTII Transgenic Mice

1. Harvest one spleen from a naïve OTII mouse as described in **step 1.2** above and homogenize the spleen between the frosted ends of two slides. Transfer the cell suspension to a 15 ml conical tube, add RPMI medium up to 14 ml. Let the suspension sit for 5 min and transfer 13 ml of it to a new 15 ml conical tube.
2. Isolate splenic CD4⁺ T cells by using a CD4⁺ T cell isolation kit according to the manufacturer's instructions.
3. Transfer cells into a 50 ml conical tube, wash twice with PBS. Resuspend cells in PBS with 0.5% bovine serum albumin (BSA) at 1 x 10⁶ cells per ml, and add 0.5 μ mol/ml of CFSE. Incubate at 37 °C for 10 min, protected from light.
4. Add 5 ml cold complete medium (RPMI-1640 with 10% heat-inactivated fetal bovine serum, 1/100 vol antibiotics/antimycotics, 1/100 vol L-glutamine and 1/1,000 vol of 1,000 x 2-mercaptoethanol).
5. Incubate on ice for 5 min. Wash cells once with PBS, fix 0.2 x 10⁶ labeled CD4⁺ T cells in 2% PFA and acquire on a flow cytometer. Use this sample to determine the basal level of CFSE. Re-suspend the rest of labeled cells in complete medium.

3. Co-culture OTII T cells with DCs of WNV-infected Mice

1. Culture 2 x 10⁵ purified CD4⁺ T cells of OTII mice alone or with purified DCs of wild-type or MyD88^{-/-} mice (2 x 10⁴) in a 24 well plate with or without OVA residue 323-339 (1 g/ml) in 1 ml complete medium per well.
2. Incubate cells at 37 °C for 5 days. Harvest cells, wash twice in FACS buffer (PBS with 1% FBS) and fix in 0.25 ml 2% PFA. Vortex immediately.

4. Flow Cytometry Analysis of *In Vitro* T Cell Priming

1. For acquisition, double click the flow cytometry software icon. For a density plot of linear FSC-A vs. linear SSC-A, select channels 0 to 200,000 on FSC-A and 0 to 200,000 on SSC-A. Then create a gate on live cells (P2; see **Figure 1A**).
2. To analyze the fluorescence intensity of CFSE, open the histogram for the FL1 channel and acquire 20,000 events on the gated population. Set up a marker on samples for OTII cells cultured alone (**Figure 1A**).
3. Acquire samples for OTII cells co-cultured with wild-type DCs (**Figure 1B**) or MyD88^{-/-} DCs (**Figure 1C**) in a similar way.

5. Isolation and Stimulation of Splenocytes for Cytokine Assays

1. Infect B6 and MyD88^{-/-} mice with WNV NS4B-P38G mutant using the same procedure as described in **step 1.1**. At day 30 post-infection, re-challenge surviving mice with 2000 PFU of the wild-type WNV strain i.p. At days 8 and 21 post-primary WNV infection or at day 4 following secondary infection, euthanize mice with CO₂ for collection of spleens.
NOTE: We used 2-3 mice per group for each time point.
2. Make a single cell suspension of splenocytes as described above in **step 1.2** & **step 2.2**. Count cells using a hemocytometer and re-suspend them in 10 ml complete medium.
3. Dilute cells with complete medium to 2.5 x 10⁶ cells/ml. Add 1 ml of splenocytes in a 1.5 ml micro-centrifuge tube.
4. To stimulate CD8⁺ T cells, dilute WNV-specific NS4B and E peptides (SSVWNATTA and IALTFLAV) to 1 mg/ml in DMSO. For stimulation of CD4⁺ T cells, dilute WNV-specific NS3 and E peptides (RRWCFDGPRTNTILE and PVGRLVTVPFVSVA) to 1 mg/ml in DMSO. Add 10 μ l of peptides to the cells followed by 1 μ l of Brefeldin A solution. Use cells without peptides treatment as controls. Mix the cells.
5. Punch two holes with an 18 G needle in the cap of the tube. Incubate cells at 37 °C for 5 hr.

6. Intracellular Cytokine Staining

1. Transfer cells to a new micro-centrifuge tube. Spin cells for 5 min at 300-400 x g and pour off supernatant. Add 1 ml FACS buffer, spin 5 min at 300-400 x g and re-suspend cells by pipetting with 120 μ l FACS buffer.
2. Add 2 μ l Fc blocker and incubate cells at room temperature for 10 min. Add 1 ml FACS buffer to each tube. Spin 5 min at 300 - 400 x g.
3. Re-suspend cells in 300 μ l FACS buffer and split them into three tubes (about 0.8×10^6 cells per tube). As shown in **Table 1**, reserve the first tube of each culture condition for rat IgG-PE staining. For the second tube, add 3 μ l of anti-CD4 APC to CD4 peptides-treated cells, 3 μ l of anti-CD8 FITC to CD8 peptides-treated cells or both antibodies to cells without peptides treatment. Use the third tube for compensation staining. For each culture condition, use three tubes of cells stained with APC-conjugated CD4, FITC-conjugated CD8 or PE- labeled CD3 antibodies alone (3 μ l per tube) as compensation controls for FL1, FL2 and FL4 channels.
4. Vortex cells briefly. Leave on ice for 20 min and protect from light. Add 1.4 ml FACS buffer. Spin 5 min at 300-400 x g and pour off supernatant.
5. Agitate to disrupt cell pellet (or briefly vortex). Re-suspend cell pellet in 250 μ l fixation/permeabilization solution by pipetting. Incubate at room temperature for 20 min and protect from light.
6. Add 1.2 ml FACS buffer. Spin 5 min at 300-400 x g and resuspend cells in 300 μ l FACS buffer.
NOTE: At this point, cells can be transferred to a BL2 laboratory for further processing or stored in a refrigerator and protected from light for up to three days.
7. Add 500 μ l FACS buffer. Spin 5 min at 300-400 x g and resuspend cells in 500 μ l of 1x permeabilization/wash buffer. Spin 5 min at 300-400 x g.
8. Re-suspend cells in 100 μ l 1x Perm/Wash, add 3.5 μ l anti-IFN γ -PE to the tube previously added with antibodies for CD4 and/or CD8 T cell markers in **step 6.3** and 3.5 μ l rat IgG-PE in the tube reserved for IgG control for 25 min on ice and protect from light. Wash cells by adding 1.4 ml of 1x permeabilization/wash buffer. Spin 5 min at 300-400 x g. Repeat the washing step by adding 1.4 ml 1x permeabilization/wash buffer.
9. Spin 5 min at 300-400 x g and decant supernatant. Wash cells by adding 1.4 ml of FACS buffer and re-suspend in 400 μ l of FACS buffer for final acquisition.

7. Flow Cytometry Analysis of Intracellular Cytokine Staining

1. Use the same acquisition settings as in **step 4.1**. Create a gate on live cells (P2; see **Figure 2A**). Adjust the voltages using the compensation tubes for each group.
2. Open two new dot plots, one to display data collected for FL1 vs. FL2 (CD8 vs. IFN- γ), and the other one is to display data collected for FL4 vs. FL2 (CD4 vs. IFN- γ). Acquire 50,000 events for the gated population of each sample.

Representative Results

In the T cell priming assay, CFSE labeled CD4⁺ T cells were cultured with purified DCs from the NS4B-P38G mutant-infected wild-type and MyD88^{-/-} mice in the presence or absence of OVA peptides. Labeled T cells cultured alone with or without OVA for 5 days were used as negative controls. As shown in **Figure 1A**, total T cells were gated for analysis of fluorescence intensity on the FL1 channel. The marker was set up based on the freshly labeled CD4⁺ T cells on day 0 to determine the proliferation rate without co-culture of DCs. There was a low level of proliferation rate (1.6%) under this culture condition. The same marker was used to determine the proliferation rate of CD4⁺ T cells co-cultured with DCs at a 10:1 ratio. Due to their high ratio in the co-culture and their proliferative nature, T cells can be gated on the mixed populations without additional phenotypic staining. As shown in **Figure 1B**, there was an 87.0% proliferation rate in wild-type group as shown in one representative of three samples treated under the same conditions. Furthermore, CFSE-labeled T cells co-cultured with DCs of MyD88^{-/-} mice in the presence of OVA had a 74.5% proliferation rate (**Figure 1C**). Thus, the proliferation rate of OT II CD4⁺ T cells co-cultured with DCs of NS4B-P38G mutant-infected MyD88^{-/-} mice was lower than of those co-cultured with DCs from wild-type mice. These results suggest that a deficiency in MyD88- signaling pathway leads to an impaired antigen presenting capacity of DCs during NS4B-P38G mutant infection.

To analyze ICS results, we gated on total splenocytes isolated from wild-type mice at day 8 post-infection (**Figure 2A**), the percentages of double-positive populations (CD4⁺IFN γ ⁺ and CD8⁺IFN γ ⁺) in one representative sample were 0.4% and 1.7% respectively. The percentages of CD4⁺IFN γ ⁺ and CD8⁺IFN γ ⁺ of total splenocytes gated in one representative sample of MyD88^{-/-} mice were 0.2% and 0.6% respectively (**Figure 2B**). No differences were noted in the double-positive populations of the two groups of splenocytes without peptides treatment (data not shown). Similar analyses were performed with splenocytes isolated from non-infected, wild-type and MyD88^{-/-} mice (**Figures 2C & 2D**). The percentages of double-positive populations (CD4⁺IFN γ ⁺ and CD8⁺IFN γ ⁺) between the two groups were not different. Furthermore, In **Figure 2A**, the single positive populations of CD4⁺ and CD8⁺ T cells were 21% and 13.3% of the total gated splenocytes. Whereas, they were shown to be 15.9% and 11.2% of MyD88^{-/-} splenocytes (**Figure 2B**). Compared to the infected groups, the differences of the percentage of single positive populations between the two non-infected wild-type and MyD88^{-/-} mice were much less (19.1% vs. 18.4% for CD4⁺ T cells, 15.7% vs. 13.3% for CD8⁺ T cells). Combined together, both CD4⁺ and CD8⁺ T cell responses were reduced in MyD88^{-/-} mice compared to the wild-type group at day 8 post-NS4B- P38G mutant infection. A similar analysis was performed for samples collected at day 21 post-infection. As shown in **Figure 3A & 3B**, the percentage of CD4⁺IFN γ ⁺ of total splenocytes in MyD88^{-/-} group (0.1%) was slightly lower than the wild-type group (0.2%). The single positive population of CD4⁺ T cells in MyD88^{-/-} group (17%) was also lower than that of wild-type group (20.6%). In comparison, neither the percentage of CD8⁺IFN γ ⁺ nor the percentage of single positive population of CD8⁺ T cells was different between the two groups. At day 4 post-secondary infection, the percentages of double positive populations (CD4⁺IFN γ ⁺ and CD8⁺IFN γ ⁺) in one representative sample of wild-type group were 0.3% and 0.5% respectively (**Figure 4A**). The percentages of CD4⁺IFN γ ⁺ and CD8⁺IFN γ ⁺ of total splenocytes gated in one representative sample of MyD88^{-/-} group were 0.1% and 0.2% (**Figure 4B**). Further, the single positive population of CD4⁺ and CD8⁺ T cells was 18.7% and 15.8% of total splenocytes of wild-type mice. Whereas, this percentage was reduced as 12.1% and 12.3% in MyD88^{-/-} mice (**Figure 4B**). In summary of these results, MyD88 signaling is involved in the initial T cell activation of both populations and contributes to CD4⁺ T cell response at later stage of infection. It is also involved in memory CD4⁺ and CD8⁺ T cell responses.

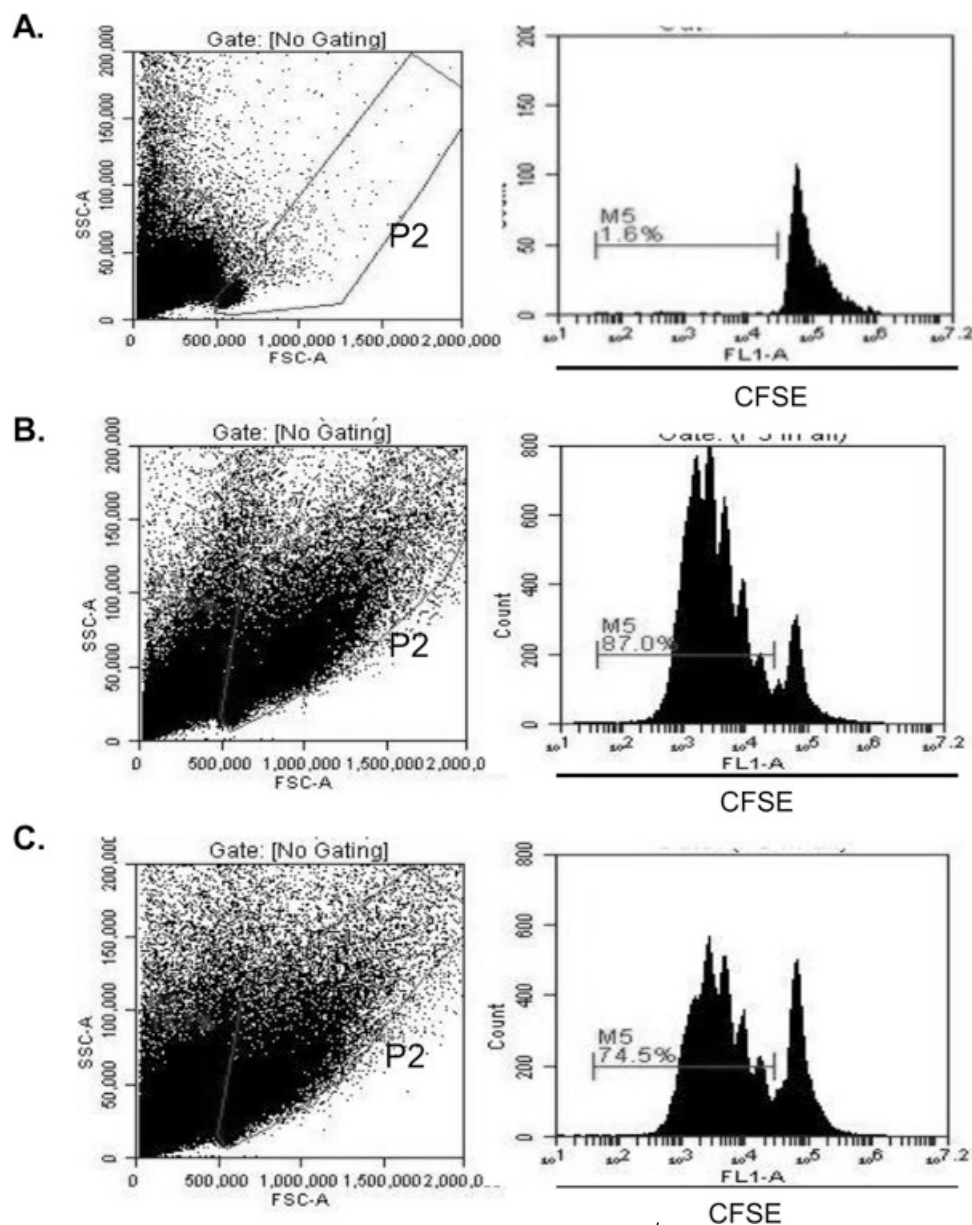


Figure 1: DC antigen-presenting ability in wild-type and *MyD88*^{-/-} mice during NS4B- P38G infection. CFSE labeled CD4⁺ T cells were co-cultured alone (A) or with DCs of WNV-NS4B-P38G mutant- infected wild-type (B) and *MyD88*^{-/-} mice (C) in the presence of OVA 323–339. Cells were harvested at day 5, gated on T cells based on FSC/SSC (left panels) and analyzed for their proliferation rates (right panels). One representative of three samples in each group was shown. [Please click here to view a larger version of this figure.](#)

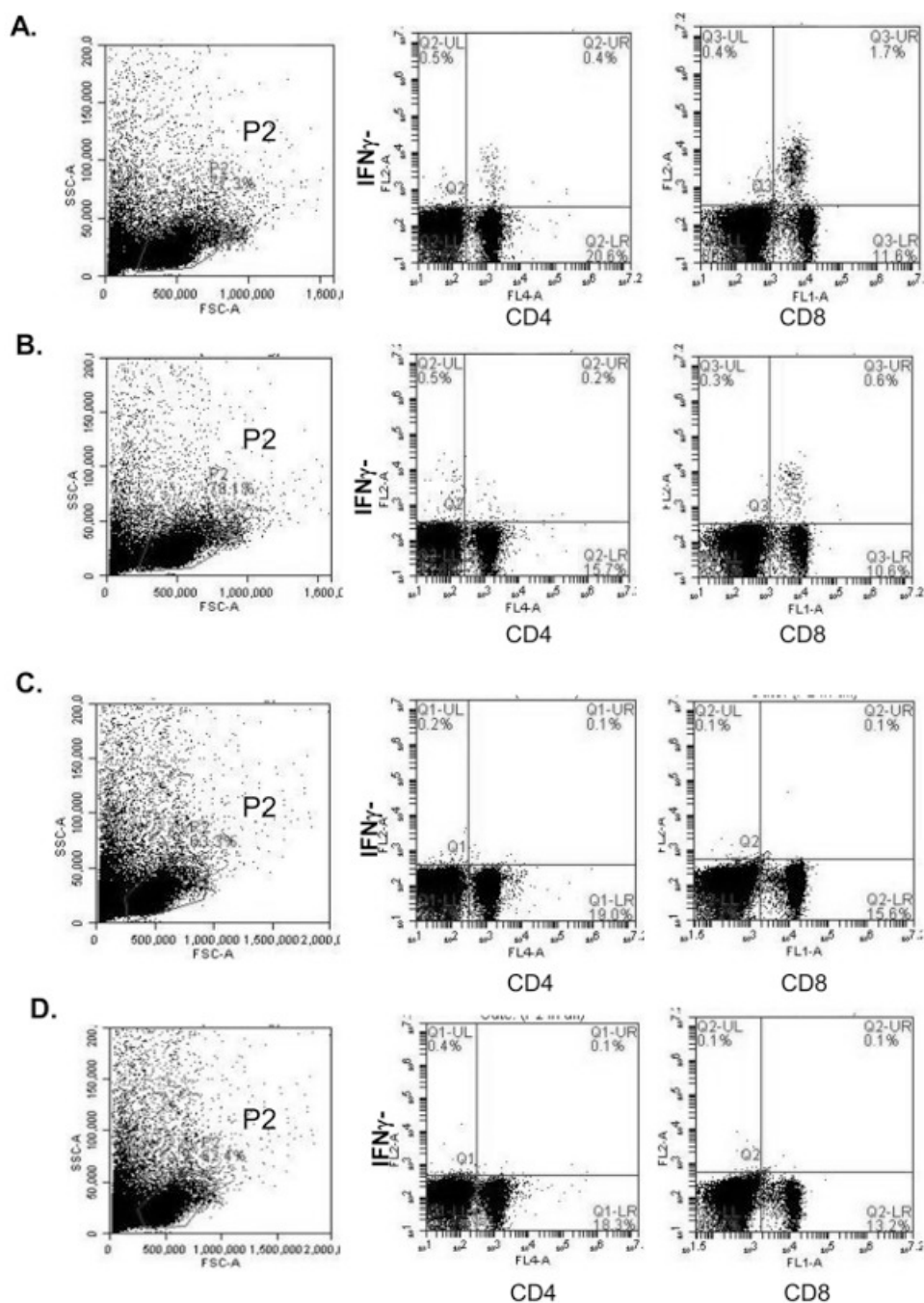


Figure 2: T cell responses at an early stage of WNV NS4B-P38G infection. Splenocytes were isolated from WNV NS4B-P38G mutant-infected wild-type (A) and MyD88^{-/-} mice (B) at day 8. As controls, splenocytes were harvested from non-infected wild-type (C) and MyD88^{-/-} mice (D). Cells were cultured *ex vivo* with WNV peptides for 5 hr, harvested, and stained for IFN γ and CD4 or CD8. Total splenocytes from each group were gated (P2) and analyzed. The dot plots shown are one representative of three samples in each group. [Please click here to view a larger version of this figure.](#)

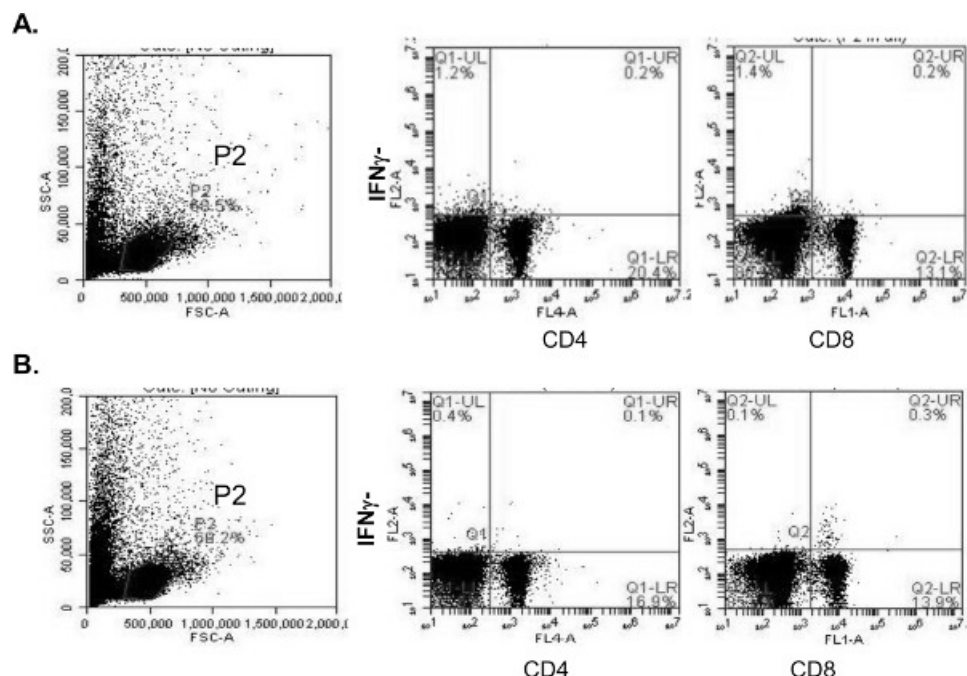


Figure 3: T cell responses at late stage of WNV NS4B-P38G infection. Splenocytes were isolated from WNV NS4B-P38G mutant- infected wild-type (A) and MyD88^{-/-} mice (B) at day 21. Cells were cultured *ex vivo* with WNV peptides for 5 hr, harvested, and stained for IFN- γ and CD4 or CD8. Total splenocytes from each group were gated (P2) and analyzed. One representative of three samples in each group was shown. [Please click here to view a larger version of this figure.](#)

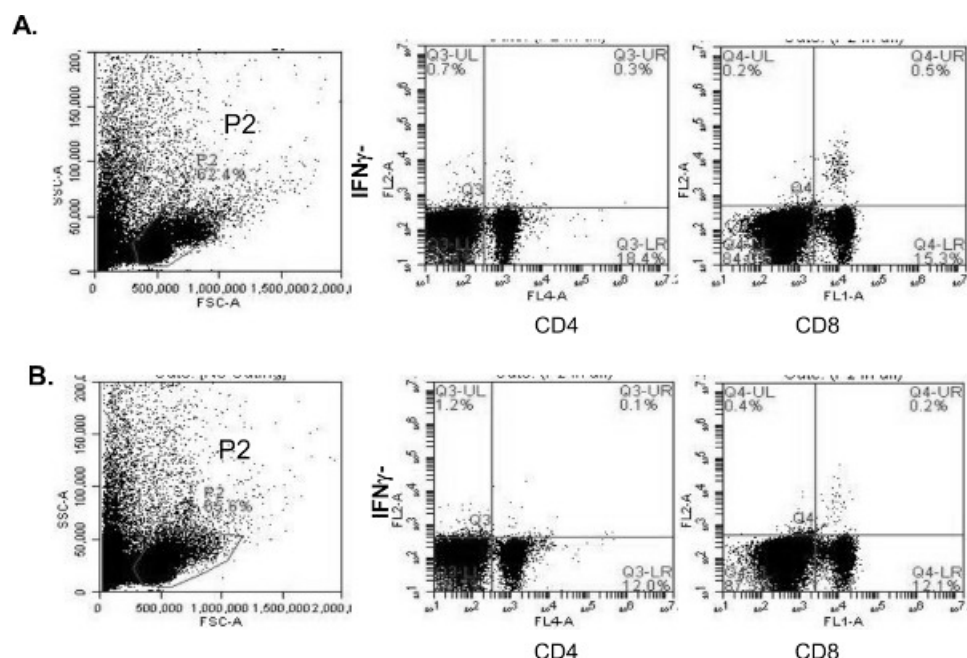


Figure 4: T cell responses during secondary challenge with wild-type WNV. Mice that survived from a primary infection with WNV NS4B-P38G mutant were re-infected with a LD₁₀₀ of wild-type WNV. At day 4 post-secondary infection, splenocytes were isolated from WNV NS4B-P38G mutant- infected wild-type (A) and MyD88^{-/-} mice (B). Cells were cultured *ex vivo* with WNV peptides for 5 hr, harvested, and stained for IFN- γ and CD4 or CD8. Total splenocytes from each group were gated (P2) and analyzed. One representative of three samples in each group was shown. [Please click here to view a larger version of this figure.](#)

Discussion

WNV is a BL3 pathogen. Due to safety regulations, immunological assays with WNV-infected samples are often restricted by the availability of equipment at BL3 facilities or more lengthy and tedious to perform. In a recent study, we used two flow cytometry-based methods to study cell mediated immune responses during WNV infection⁵. In both assays, WNV-infected cells were treated with 1-2% PFA directly or with fixation/permeabilization solution containing 4% PFA. It is known that 1% PFA fixation of virus-infected cells could efficiently reduce the number of

infectious particles below detection limits¹². Therefore, both methods have significantly reduced the performance time inside BL3 facilities. There are many established assays to measure T cell proliferation, including those through incorporation of BrdU or radioactive thymidine, the flow cytometry-based *in vitro* T cell priming assay using CFSE labeled OTII T cells has provided more accurate determination of the percentage of proliferating CD4⁺ T cells with significantly improved overall sensitivity. Here, a 10:1 ratio for DCs and T cells was used to efficiently define antigen presenting capacity during WNV infection. A dose titration is recommended to study DC functions during infection with another pathogen, as this ratio may differ. ICS is a commonly used flow cytometry-based assay to study antigen specific T cell responses. Nevertheless, the procedure is lengthy, and includes cell culture and multiple steps of washing and staining of cells. It is potentially troublesome when performing at BL3 facilities. We have modified the protocol so that cells were initially stimulated with WNV specific peptides in a micro-centrifuge tube instead of a tissue-culture plate. Next, cells were washed and stained within the micro-centrifuge tubes instead of conical tubes. These modifications have enabled the entire process being performed inside a biosafety cabinet by using a micro-centrifuge machine, which have eliminated the disinfecting procedure for centrifuging cells outside the biosafety cabinet. Cells were also acquired in the micro-centrifuge tubes by a flow cytometer. Overall, the micro-centrifuge tube system had saved time and effort (about 2 hr) in ICS compared to the traditional tissue culture plate based assay. Lastly, the microcentrifuge tube method does not have special instrument requirement, which is economic and offers more flexibility in performance. In addition, it was easier to perform and less time consuming, which had ultimately increased cell viability (data not shown). There is one minor safety concern due to the use of 18 G needle in setting up cell culture for ICS.

Investigation of the mechanism by which WNV NS4B-P38G mutant induces higher protective immunity can be utilized as a paradigm to aid in the rational development of other efficacious live attenuated flavivirus vaccines. By using ICS and *in vitro* T cell priming assays, we have shown that MyD88 signaling is important for development of cell-mediated adaptive immunity during WNV NS4B-P38G mutant infection. Neither assay is specific designed for WNV. They can also be used to assess DCs and T cell functions with samples-infected with other BL3 agents.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by NIH grants to T.W. (R01AI072060 and R01AI099123). G. Xie was supported by a Sealy Center for Vaccine Development Predoctoral Fellowship. We thank Dr. Richard Flavell (Howard Hughes Medical Institute, Yale University School of Medicine, New Haven) and Dr. Shizuo Akira (Osaka University, Japan) for providing the MyD88^{-/-} mice and Dr. Y Cong (UTMB, Galveston) for providing OTII transgenic mice.

References

- Campbell, G. L., Marfin, A. A., Lanciotti, R. S., & Gubler, D. J. West Nile virus. *Lancet Infect. Dis.* **2**, 519-529. (2002).
- Welte, T. *et al.* Immune responses to an attenuated West Nile virus NS4B-P38G mutant strain. *Vaccine*. **29**, 4853-4861, doi:S0264-410X(11)00602-5 [pii] 10.1016/j.vaccine.2011.04.057 (2011).
- Iwasaki, A., & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* **5**, 987-995 (2004).
- Akira, S., & Hemmi, H. Recognition of pathogen-associated molecular patterns by TLR family. *Immunol. Lett.* **85**, 85-95 (2003).
- Xie, G. *et al.* A West Nile virus NS4B-P38G mutant strain induces adaptive immunity via TLR7-MyD88-dependent and independent signaling pathways. *Vaccine*. **31** (38), 4143-51 doi:S0264-410X(13)00903-1 [pii] 10.1016/j.vaccine.2013.06.093 (2013).
- Fang, H. *et al.* gammadelta T cells promote the maturation of dendritic cells during West Nile virus infection. *FEMS Immunol. Med. Microbiol.* **59**, 71-80, doi:FIM663 [pii] 10.1111/j.1574-695X.2010.00663.x (2010).
- Silva, M. C., Guerrero-Plata, A., Gilfoy, F. D., Garofalo, R. P., & Mason, P. W. Differential activation of human monocyte-derived and plasmacytoid dendritic cells by West Nile virus generated in different host cells. *J. Virol.* **81**, 13640-13648 (2007).
- Shrestha, B., Samuel, M. A., & Diamond, M. S. CD8+ T Cells Require Perforin To Clear West Nile Virus from Infected Neurons. *J. Virol.* **80**, 119-129 (2006).
- Wang, Y., Lobigs, M., Lee, E., & Mullbacher, A. CD8+ T cells mediate recovery and immunopathology in West Nile virus encephalitis. *J. Virol.* **77**, 13323-13334 (2003).
- Plebanski, M., Katsara, M., Sheng, K. C., Xiang, S. D., & Apostolopoulos, V. Methods to measure T-cell responses. *Expert Rev. Vaccines*. **9**, 595-600, doi:10.1586/erv.10.53 (2010).
- Lyons, A. B. Analysing cell division *in vivo* and *in vitro* using flow cytometric measurement of CFSE dye dilution. *J. Immunol. Methods*. **243**, 147-154, doi:S0022-1759(00)00231-3 [pii] (2000).
- Kraus, A. A., Priemer, C., Heider, H., Kruger, D. H., & Ulrich, R. Inactivation of Hantaan virus-containing samples for subsequent investigations outside biosafety level 3 facilities. *Intervirology*. **48**, 255-261, doi:84603 [pii] 10.1159/000084603 (2005).
- Saxena, V. *et al.* A hamster-derived west nile virus isolate induces persistent renal infection in mice. *PLoS Negl. Trop. Dis.* **7**, e2275, doi:10.1371/journal.pntd.0002275 PNTD-D-13-00050 [pii] (2013).
- Welte, T. *et al.* Vgamma4+ T cells regulate host immune response to West Nile virus infection. *FEMS Immunol. Med. Microbiol.* **63**, 183-192, doi:10.1111/j.1574-695X.2011.00840.x (2011).