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In vitro analysis of MyD88-mediated cellular immune response to West Nile virus mutant strain infection

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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 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.
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Title: *In vitro* analysis of MyD88-mediated cellular immune response to West Nile virus mutant strain infection

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

Short Abstract:
Two flow cytometry-based methods – an *in vitro* T cell priming assay and intracellular cytokine staining were utilized to measure antigen presenting capacity of dendritic cells and antigen-specific T cell responses to a West Nile virus mutant infection in mice.

Long 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 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.

Introduction:

West Nile virus (WNV), a neurotropic, plus-sensed flavivirus, is an emerging public health threat. Currently, no vaccines have been approved for human use [1]. 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 strain [2]. 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 mice [5]. Dentritic 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 easter (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 (^{3}HTdr)[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 nonradioactive [11]. 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 WNV [12], 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:

Ethics Statement:

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

1.1) 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 CO_2. Use non-infected mice of both groups as controls. Use three mice from each group.

1.2) 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.

1.3) 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

2.1) Harvest one spleen from a naïve OTII mouse as described in #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.

2.2) Isolate splenic CD4$^+$ T cells by using a CD4$^+$ T cell isolation kit according to the manufacturer's instructions.

2.3) 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 x10^6 cells per ml, and add 0.5 µmol/ml of CFSE. Incubate at 37 ^{0}C for 10 min, protected from light.

2.4) 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 1/1000 vol of 1000 x 2-mercaptoethanol).

2.5) Incubate on ice for 5 min. Wash cells once with PBS, fix 0. 2 x10^6 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

3.1) Culture 2 x10^5 purified $CD4^+$ T cells of OTII mice alone or with purified DCs of wild-type or MyD88$^{-/-}$ mice (2 x 10^4) in a 24-well plate with or without OVA residue 323-339 (1 μg/ml) in 1 ml complete medium per well.

3.2) 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

4.1) 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 2000, 000 on FSC-A and 0 to 200, 000 on SSC-A. Then create a gate on live cells (P2) (**see Figure 1A**).

4.2) 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**).

4.3) 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

5.1) Infect B6 and MyD88$^{-/-}$ mice with WNV NS4B-P38G mutant using the same procedure as described in # 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 CO_2 for collection of spleens. Note: We used 2-3 mice per group for each time point.

5.2) Make a single cell suspension of splenocytes as described above in # 1.2. & # 2.2. Count cells using a hemocytometer and re-suspend them in 10 ml complete medium.

5.3) Dilute cells with complete medium to 2.5 x10^6 cells/ml. Add 1ml of splenocytes in a 1.5 ml micro-centrifuge tube.

5.4) To simulate $CD8^+$ 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 PVGRLVTVNPFVSVA) to 1 mg/ml in DMSO. Add 10 μl of peptides to the cells followed by 1 μl of Brefeldin A solution. Use cells without peptides treatment as controls. Mix the cells.

5.5) Punch two holes with an 18-gauge needle in the cap of the tube. Incubate cells at 37 °C for 5 hr.

6. Intracellular cytokine staining

6.1) Transfer cells to a new micro-centrifuge tube. Spin cells for 5 min at 300-400x*g* and pour off supernatant. Add 1 ml FACS buffer, spin 5 min at 300-400x*g* and re-suspend cells by pipetting with 120 μl FACS buffer.

6.2) Add 2 μl Fc blocker and incubate cells at room temperature for 10 min. Add 1 ml FACS buffer to each tube. Spin 5 min at 300-400x*g*.

6.3) Re-suspend cells in 300 μl FACS buffer and split them into three tubes (about 0.8 $x10^6$ 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 μl of anti-CD4 APC to CD4 peptides- treated cells, 3 μl 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 μl per tube) as compensation controls for FL1, FL2 and FL4 channels.

6.4) Vortex cells briefly. Leave on ice for 20 min and protect from light. Add 1.4 ml FACS buffer. Spin 5 min at 300-400x*g* and pour off supernatant.

6.5) Agitate to disrupt cell pellet (or briefly vortex). Re-suspend cell pellet in 250 μl fixation/permeabilization solution by pipetting. Incubate at room temperature for 20 min and protect from light.

6.6) Add 1.2 ml FACS buffer. Spin 5 min at 300-400x*g* and resuspend cells in 300 μl 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.

6.7) Add 500 μl FACS buffer. Spin 5 min at 300-400x*g* and resuspend cells in 500 μl of 1x permeabilization/wash buffer. Spin 5 min at 300-400x*g*.

6.8) Re-suspend cells in 100 μl 1x Perm/Wash, add 3.5 μl anti-IFNγ-PE to the tube previously added with antibodies for CD4 and/or CD8 T cell markers in # 6.3 and 3.5 μl 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 300-400x*g*. Repeat the washing step by adding 1.4 ml 1x permeabilization/wash buffer.

6.9) Spin 5 min at 300-400x*g* and decant supernatant. Wash cells by adding 1.4 ml of FACS buffer and re-suspend in 400 μl of FACS buffer for final acquisition.

7. Flow cytometry analysis of intracellular cytokine staining

7.1) 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.

7.2) 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 ($CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$) in one representative sample were 0.4% and 1.7% respectively. The percentages of $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ 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 ($CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$) 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^{+}IFNγ^{+} 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^{+}IFNγ^{+} 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^{+}IFNγ^{+} and CD8^{+}IFNγ^{+}) in one representative sample of wild-type group were 0.3% and 0.5% respectively **(Figure 4A**). The percentages of CD4^{+}IFNγ^{+} and CD8^{+}IFNγ^{+} 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 Legends:

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.

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 h, 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.

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.

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

LD_{100} 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.

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 infection [5]. 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 limits [12]. 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-gauge 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.

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Figure 1
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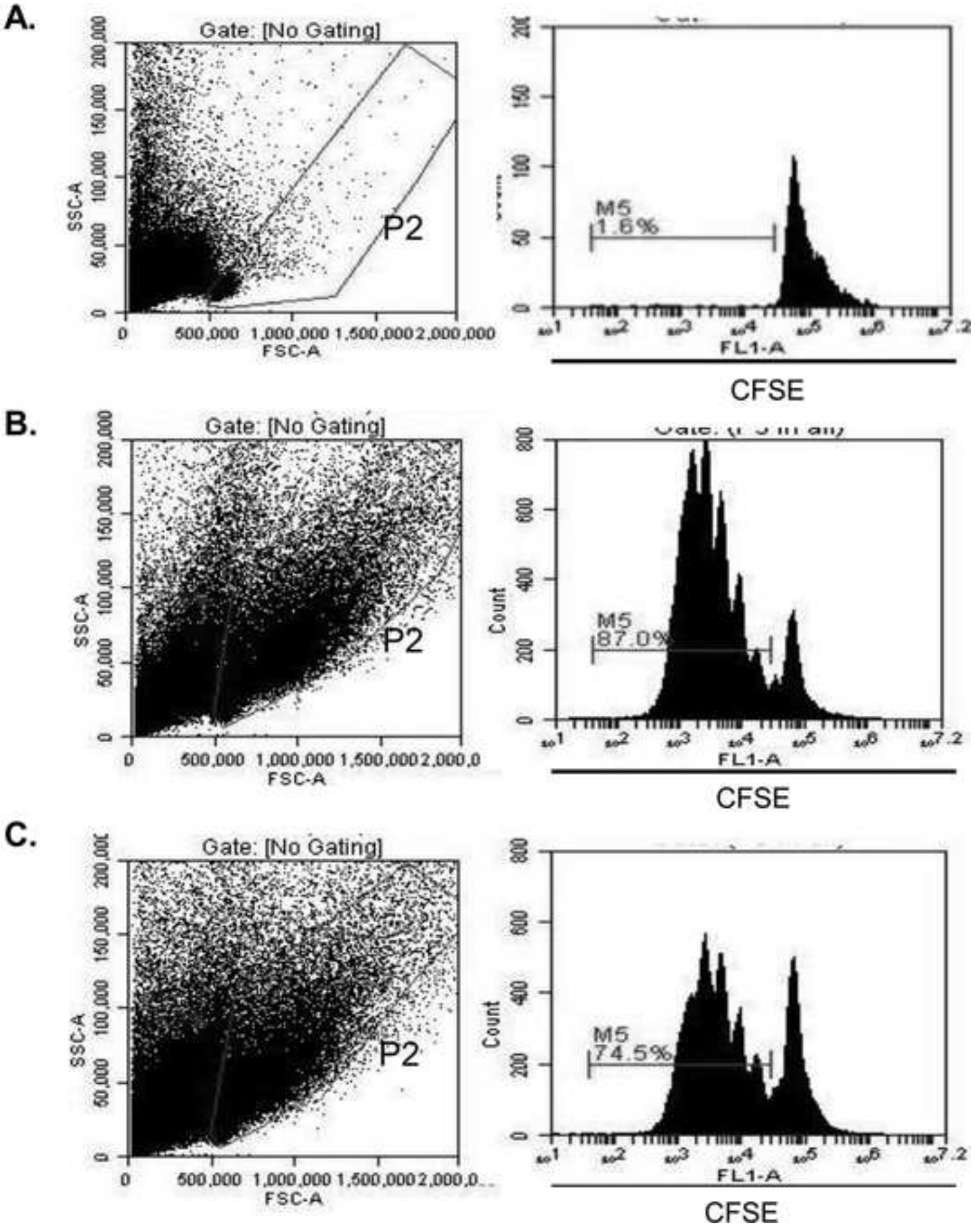

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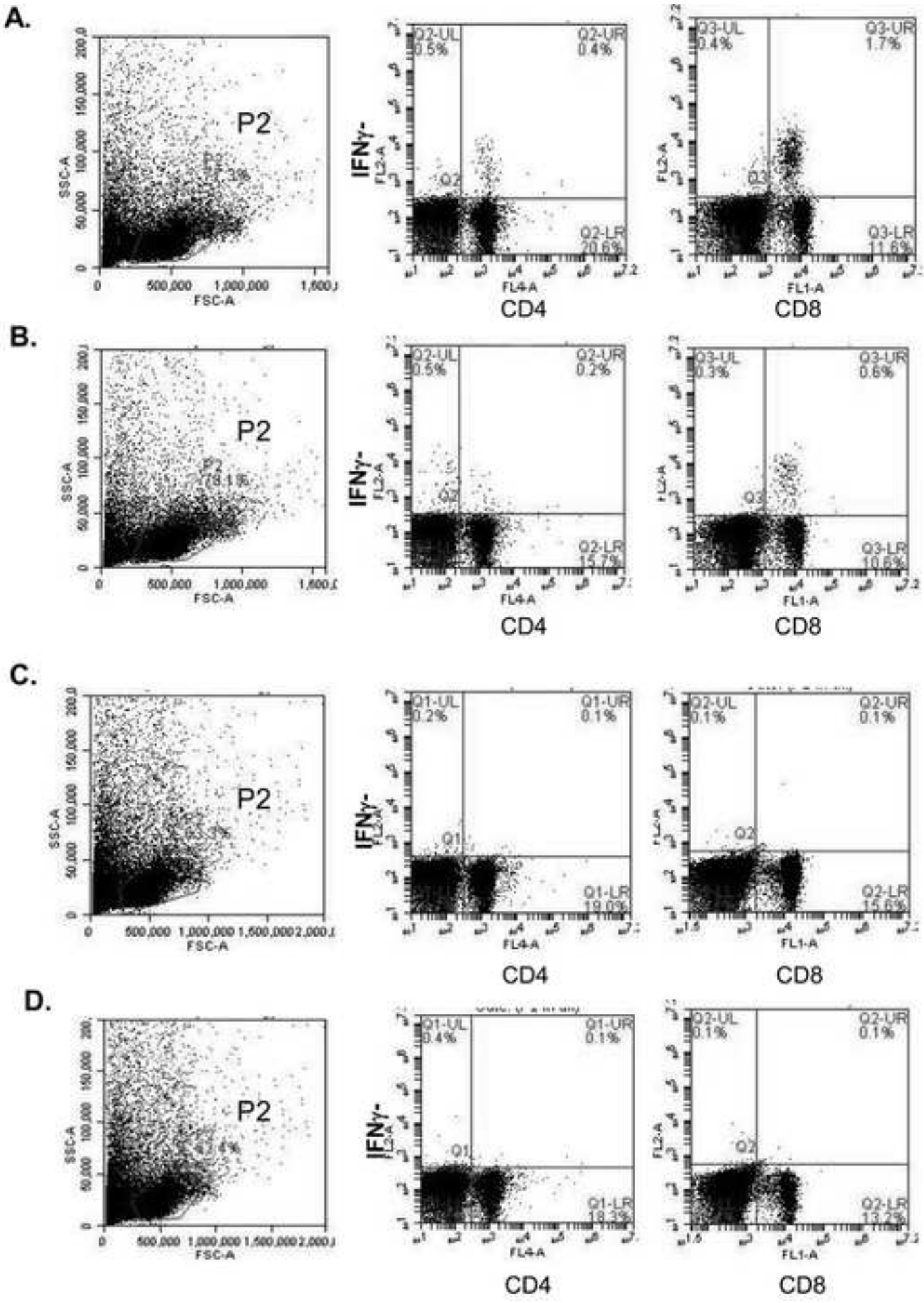

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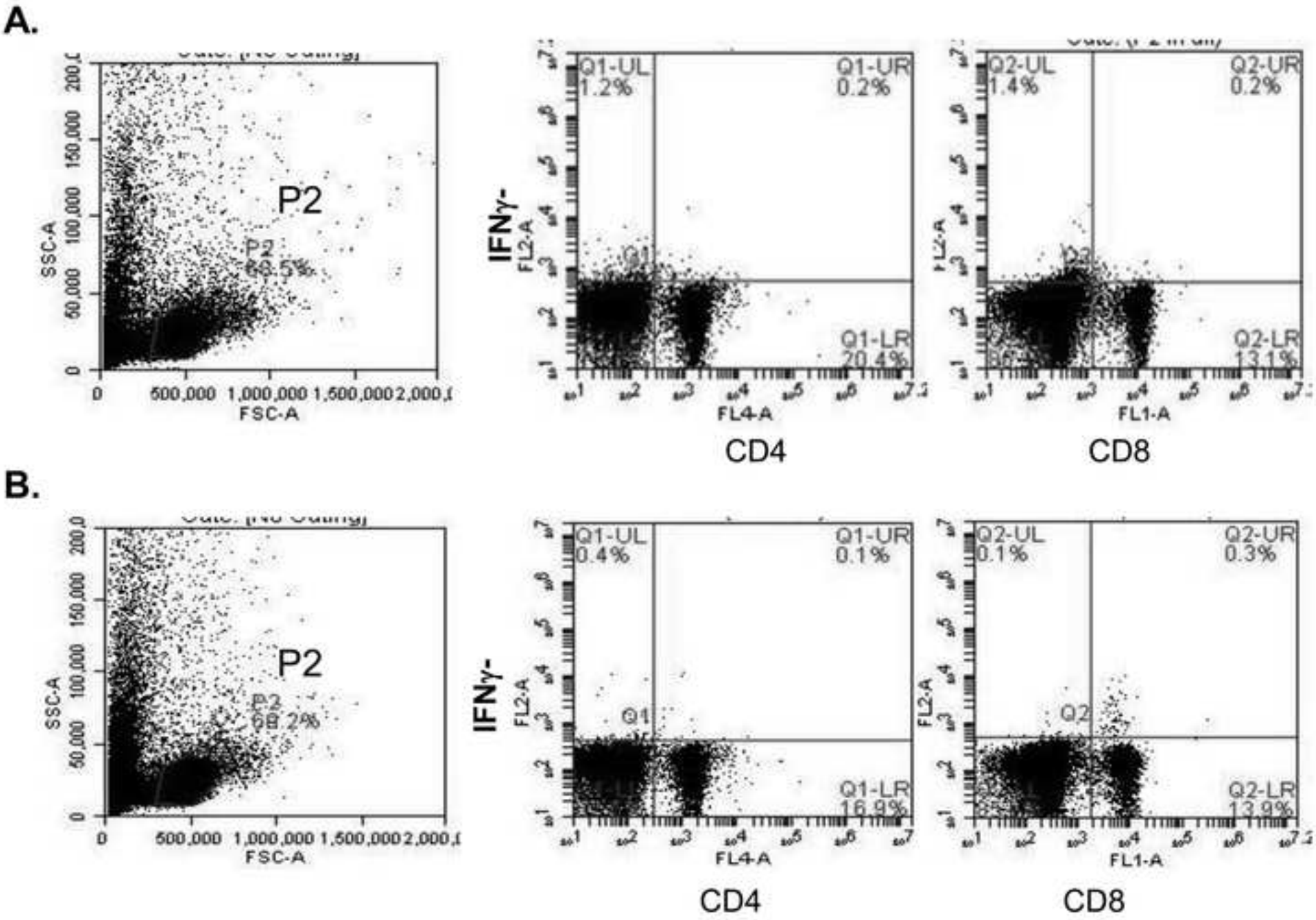

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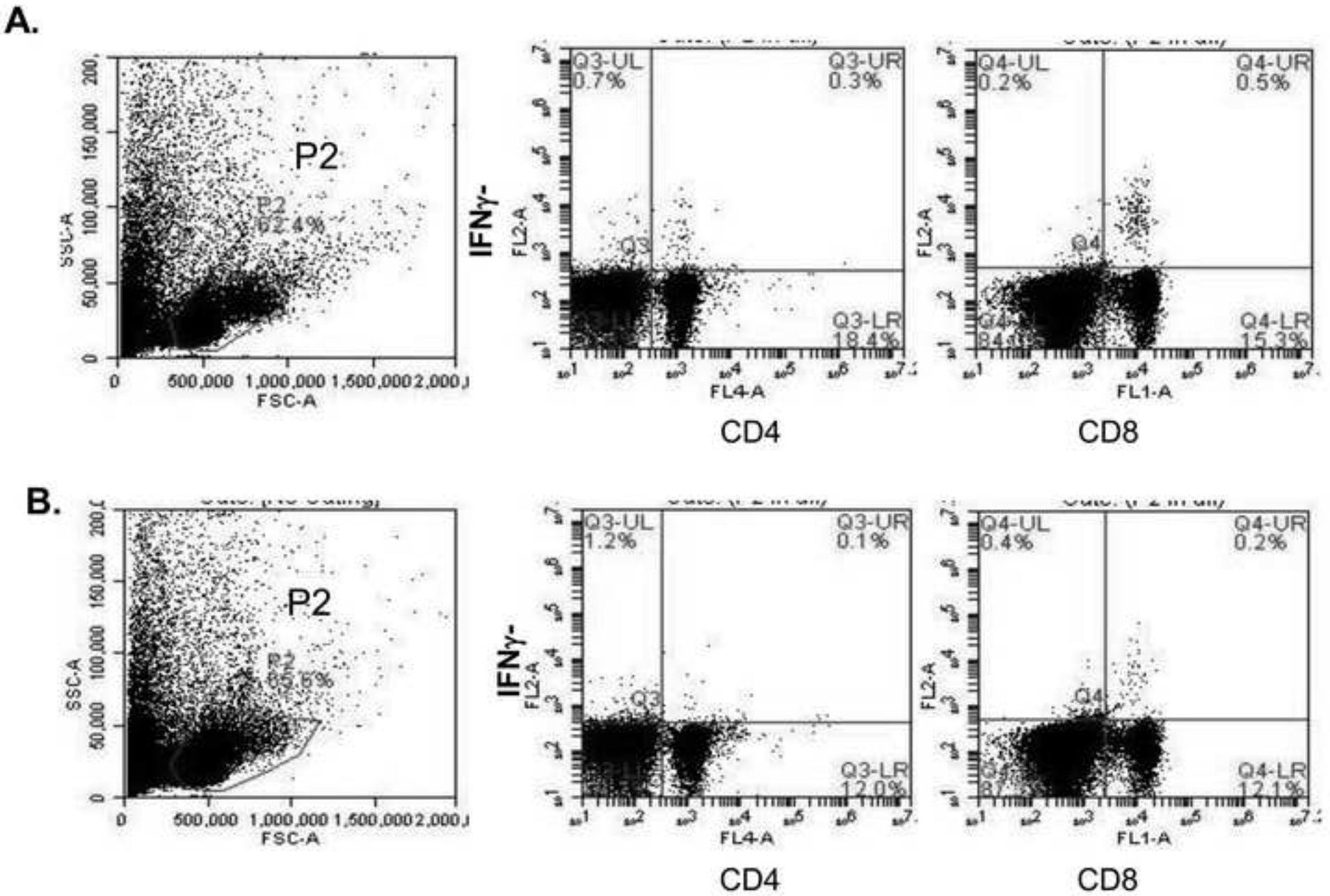

Table 1
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Table 1. Tube list for intracellular cytokine staining

Treatment	Wild-type	MyD88$^{-/-}$	
No Peptides	1. RatIgG-PE	1. RatIgG-PE	1. RatIgG-F
	2. CD4-APC/CD8-FITC/IFNγ-PE	2. CD4-APC/CD8-FITC/IFNγ-PE	2. CD4-APC
	3. CD4-APC	3. CD3-PE	3. CD8-FIT
CD4 Peptides treated	1. RatIgG-PE	1. RatIgG-PE	1. RatIgG-F
	2. CD4-APC/ IFNγ-PE	2. CD4-APC/ IFNγ-PE	2. CD4-APC
	3. CD4-APC	3. CD3-PE	3. CD8-FIT
CD8 peptides- treated	1. RatIgG-PE	1. RatIgG-PE	1. RatIgG-F
	2. CD8-FITC/ IFNγ-PE	2. CD8-FITC/ IFNγ-PE	2. CD8-FIT
	3. CD8-FITC	3. CD3-PE	3. CD4-APC

TLR7$^{-/-}$

PE
C/CD8-FITC/IFNγ-PE
C

PE
C/ IFNγ-PE
C

PE
C/ IFNγ-PE
C

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
RPMI 1640	Invitrogen	11875	warm up at 37C
anti-CD11c magnetic beads	Miltenyi Bio	130-052-001	follow the manufacturer's instructions
anti-CD4 magnetic beads	Miltenyi Bio	130-095-248	follow the manufacturer's instructions
CFSE	Invitrogen	C34554	
OVA residue 323-339	Genscript C	RP10610	
Peptides	Proimmune	PC0AD-D	
Brefeldin A solution	BD Bioscience	555029	
Mouse Fc Blocker	e-Bioscience	14-0161-85	
APC-conjugated CD4	e-Bioscience	17-0041-81	
FITC-conjugated CD8	e-bioscience	11-0081-82	
Fixation/Permeabilization Solutic	BD-Bioscience	554722	
Permeabilization/wash buffer	BD-Bioscience	554723	
anti-IFNg-PE	e-Bioscience	12-7311-82	
Accuri flow cytometer	BD Bioscience		

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Author(s): Guonui Xie, Melissa C Whiteman, Jason A Wicker, Alan D. T. Barrett and Tian Wang

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Department: Microbiology / Immunology

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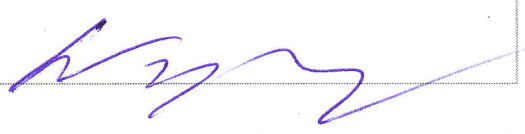
Article Title: In vitro analysis of MyD88-mediated cellular immune response to West Nile virus Mutant strain infection

Signature: 

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May 27th, 2014

Sephorah Zaman, Ph.D.
Science Editor
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Tel: 16176741888

Dear Dr. Zaman:

Thank you for the comments on our manuscript (JoVE52121R1) "In vitro analysis of MyD88-mediated cellular immune response to West Nile virus mutant strain infection". We now respond, point by point, to the comments. All changes in the text are identified using the track-changes function.

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"Perm/wash buffer" has been replaced by "permeabilization/wash buffer" in the text.

2) "*Prior to peer review, the protocol length is exactly at our 3 page limit. If, in response to peer review, additional details are added to the protocol, please use yellow highlighting to identify a total of 2.75 pages of protocol text (which includes headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification and remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader*"

Yes, we have kept the protocol length within the 3- page limit.

3) "*Please reduce use of the pronoun "we" in the Introduction*"

The introduction has been revised to meet this requirement.

4) "*Please ensure that your Discussion covers the critical steps in the protocol and limitations of your protocol compared to comparable techniques*".

The discussion has been revised to further address these points.

5) "*Please disregard the comment below if all of your figures are original*."

All Figures presented in this manuscript are original.

6) "The Novelty of the two assays in this study" addressed by Reviewers #1, 2 & 3

In this manuscript, we described two immunological assays that were utilized in a recent publication to study cell mediated immune response in West Nile virus- infected mice. These assays are not "novel" by themselves, as they are well documented in literature in many areas of research. However, the goal of this manuscript is NOT to emphasize the novelty of these methods, but to introduce the available immunological assays for researchers working with BL3 pathogens, like us. Due to safety regulations, there is an increasing need in demonstration of feasible approaches/methods for immunological studies performed at high biocontainment. Furthermore, we have used a microcentrifuge-tube system in the intracellular cytokine staining procedure. Here, we have demonstrated that this modification provides many advantages over the traditional ICS method, including saving time/efforts, better cell viability, feasibility, economic and more flexibility in performance. In summary, we think the above two points represent the novelty of this study.

Reviewer #2:

7) "*The method for the intracellular cytokine staining is outdated as the entire assay can be completed in a 96 well plate without the need for tube transfer*"

We agree that a 96-well plate can be used to perform intracellular cytokine staining. Nevertheless, we prefer the microcentrifuge tube system due to the following reasons: 1) it does not require additional instrument requirements, such as a plate adaptor for the microcentrifuge machine or accessory to assist plate reading by a flow cytometer. 2) For the microcentrifuge system, 2.5 x10^6 cells were cultured in each tube- this would allow analysis of 2-3 parameters using the same cells. The same number of cells will be cultured in 2-3 wells in a 96-well plate, which could potentially increase sample variability. Overall, the microcentrifuge tube system is economic, efficient to use and very flexible with instrument requirement.

7) "*The analysis of the CFSE examines total proliferation but fails to mention the ability to also assess number of cell divisions, which can provide additional information to the experiment*"

We have edited the text to include this information (Introduction section, line 115).

8) **"***0.5% BSA in a CFSE assay is excessive. The CD4 T cell labeling can be completed in PBS alone*"

The manufacturer, from where we purchased CFSE recommends use PBS/0.1% BSA for labeling cells. We have increased it to 0.5% BSA to improve the viability of labeled cells.

9) "*The CD8a chain antibody also will stain a subset of DCs (CD8α DCs). An anti-CD8β or co-stain with anti-CD3ε antibody is needed to distinguish T cell populations*"

We agree. In most assays, we have used both antibodies for CD3+ and CD8a to study CD8+ T cells. This was not done in this study.

10) "*No experimental evidence was given of enhanced cell viability using this method as opposed to previous methods (Line 384-385)"*

In our initial studies of using the microcentrifuge tube system in ICS assay, we compared cells recovered from both methods by trypan blue staining. The viability of cells recovered

by the traditional method was at least 15% higher. We have added "data not shown" in the text.

11) "*Positive selection with anti-CD4 beads should not be used if the CD4 T cells are to be used in a functional assay. Negative selection is preferable, possibly followed by sorting using a flow cytometer*"

We have made a correction in the protocol. In this study, we used "CD4+ T cell isolation Kit" which is a negative selection method to isolate CD4+ T cells.

12) " *Viability should not be concluded from FSC and SSC as debris can be misinterpreted as dead cells (Lines 297-299)*"

We agree. FSC/SSC is only a rough estimate of the viability of cells. Trypan blue and propidium iodide staining can provide more accurate information on cell viability. To avoid confusion, we have now removed this sentence.

Reviewer #3:

13. "*The authors note the use of microcentrifuge tubes for culture of T cells stimulated with WNV peptides as an important advantage. (The advantage of microcentrifuge tubes during the staining steps is easily appreciated.) However, they used 24-well plates for co-culture of DCs and OTII T cells, which appears to contradict their assessment of the advantage of avoiding use of tissue culture plates in BSL3. Also, the use of an 18-gauge needle to puncture the cap of the microcentrifuge tube containing potentially infectious material would seem to be highly undesirable in a BSL3 laboratory and introduce unnecessary risk to personnel*"

The microcentrifuge tube system was used for ICS assay in order to reduce the time spent on harvesting and decontamination procedure during multiple steps of staining and washing. The T cell priming assay performed in this study is a simple procedure, in which cells were harvested, washed and ready for acquisition. Further, 0.5 x10^6 cells T cells/ condition was sufficient for performing T cell priming assay in a 24-well plate. This number of cells will not be enough to allow sufficient cell to cell contact to stimulate T cells if culturing in the microcentrifuge tube. Thus, the 24-well plate is preferable for T cell priming assay. Although we have attempted to reduce any potential risk when handling infectious materials at BL3 facility, the many advantages of using the microcentrifuge system for ICS assay could overcome this minor safety concern.

14. "*Both of these assays are well established in the immunology literature, and detailed methods have been published by other groups. The authors should be more complete in citing other sources for these methods, and should focus their discussion more on the particular adjustments made for application in BSL3 laboratories. The authors could provide more detail about the time savings of their method compared to alternatives*"

This has been addressed in #6 above.

15. "*Figures- Labels should be added for each panel to enhance clarify. The exclusion of the high-FSC-A/high-SSC-A cells in Figure 1 panels B and C is problematic as proliferating T cells may have been excluded. Negative controls (no peptide) should be included in the intracellular cytokine staining assay*"

We have edited the figures as suggested. Negative controls (no peptide) were indeed used in this study. This information is now added in the text and Table 1.

16. "*A more specific title would be more informative for literature searches*"

This has been addressed in #6 above.

17. "*For a methodological report, there is not a strong rationale to include Figures 3 and 4, which follow the same assay method as Figure 2 but do not include useful controls*"

We agree. Figures 1 and 2 were sufficient to support the goal of this study. Results from Figures 3 & 4 will only provide additional information in cellular immunity studies in WNV infected mice. We now list these two figures as supplementary information.

18. "*The manuscript contains several typographical errors of technical importance. For example: In steps 4.1 and 7.1, the settings being recommended for FSC-A should presumably be either "2,000,000" or "200,000." In step 5.4, the text should read "… stock concentrations of 10 mg/ml in DMSO) to 1 mg/ml*"

For 4.1 & 7.1, we chose the channels for setting FSCA-A and SSC-A as recommended by the flow cytometer instrument manual. We have edited the text in step #5.4.

19. "*The text is confusing in explaining the purpose and treatment of the different tubes in sections 5 and 6. A table listing all experimental samples might be helpful*"

As suggested, Table 1 has been added to provide more detailed information of samples used in sections 5 & 6.

20. "*It would be helpful to the less experienced investigator to explain the use of OTII T cells for co-culture with DCs*"

This information is now included in the Introduction section.

Thank you for the constructive comments to improve the manuscript.

Sincerely,

Tian Wang, PhD.

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June 1st, 2014

Sephorah Zaman, Ph.D.
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Tel: 16176741888

Dear Dr. Zaman:

Thank you for the comments on our manuscript (JoVE52121R2) "In vitro analysis of MyD88-mediated cellular immune response to West Nile virus mutant strain infection". We now respond, point by point, to the comments. All changes in the text are identified using the track-changes function.

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The goal of this manuscript is to introduce two immunological assays that can be used to evaluate cellular immunity at different stages (initial T cell priming, primary and secondary T cell responses) following infection of West Nile virus (WNV) – a biosafty level 3 pathogen. The two supplementary figures provide information for primary and secondary T cell responses to WNV infection. They have been changed to regular figures.

Thank You.

Sincerely,

Tian Wang, PhD.