Final Report

Project Title Effect of CD137 Receptor/Ligand Interaction in Dengue Virus-Mediated Apoptosis And Its Application

By Asst.Prof.Dr.Janjuree Netsawang

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Abstract

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Abstract:

Dengue virus infection has become one of the leading causes of pediatric morbidity and mortality worldwide. CD137 is the member of tumor necrosis factor receptor super family 9 (TNFRSF 9) and influences not only the activation state of T cells but also the activation, proliferation, survival, apoptosis of several immune and nonimmune cells. Our group previously demonstrated the up-regulation of CD137 mRNA and surface CD137 receptor expression in DENV-infected HepG2 cells, which correlated to the increasing of hepatocellular apoptosis. According to the crucial role of monocytes, we firstly reported the upregulation of CD137 ligand both mRNA and surface protein levels corresponding to the increasing of apoptosis upon DENV-infected monocytes, U937 cell line. Moreover, prior treatment with blocking CD137 ligand peptide, not antagonist anti-CD137 ligand antibody, prevented CD137 receptor/ligand interaction between monocyte and hepatocyte, leading to reduction of apoptotic event in monocytes. Furthermore, the increasing dose of blocking peptide stabilized its activity longer. It might be possible that either monocyte or hepatocyte can transmit apoptotic-inducing signal passing through its targets by using the cross-linking of its ligand. The prevention of those interactions might lead to alleviating DENV-mediated tissue injury and disease severity during DENV infection.

Keywords : CD137 receptor/ligand interaction, apoptosis, monocyte, hepatocyte, blocking peptide

บทคัดย่อ

รหัสโครงการ: TRG5780299

ชื่อโครงการ: ผลของปฏิสัมพันธ์ระหว่าง CD137 Receptor และ CD137 Ligand ที่เกี่ยวข้องในการตายแบบอะพอพโทสิดของการติดเชื้อไวรัสเดงกี่ และการประยุกต์ใช้

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ระยะเวลาโครงการ: 5 ปี

บทคัดย่อ:

การติดเชื้อไวรัสไข้เลือดออกเดงกี่จัดเป็นสาเหตุหลักที่ทำให้เกิดการเจ็บป่วย และตายของเด็กทั่วโลก CD137 จัดเป็นสมาชิกของตระกูล tumor necrosis factor receptor super family 9 (TNFRSF 9) มีอิทธิพลไม่เพียงแต่การทำงานของเซลล์เม็ดเลือดขาวชนิด T แต่เกี่ยวข้องกับการกระตุ้น การแบ่งตัวเพิ่มจำนวน การรอดชีวิต และการตายแบบอะพอพโทสิดของเซลล์ในกลุ่มภูมิคุ้มกัน และไม่ใช่ภูมิคุ้มกัน กลุ่มวิจัยของเราได้ค้นพบการแสดงออกที่เพิ่มขึ้นของ mRNA และโปรตีนที่แสดงออกบนผิวเซลล์ของ CD137 ระหว่างที่มีการติดเชื้อไวรัสไข้เลือดออกเดงกี่ในเซลล์มะเร็งตับ (HepG2) ซึ่งสัมพันธ์กับการเพิ่มขึ้นของการตายแบบอะพอพโทสิด เมื่อกล่าวถึงบทบาทที่สำคัญของ monocyte กลุ่มวิจัยของเราได้รายงานครั้งแรกของการแสดงออกที่เพิ่มขึ้นของ mRNA และโปรตีนที่แสดงออกบนผิวเซลล์ของ CD137 ligand ระหว่างที่มีการติดเชื้อไวรัสไข้เลือดออกเดงกี่ในเซลล์มะเร็งเม็ดเลือดขาวชนิด monocyte (U937) นอกจากนั้นการใช้ blocking CD137 ligand peptide ไม่ใช่ antagonist anti-CD137 ligand antibody ช่วยป้องกันปฏิสัมพันธ์ระหว่าง CD137 receptor/ligand ในเซลล์เม็ดเลือดขาวชนิด monocyte และเซลล์ตับ นำมาซึ่งการลดอัตราการตายแบบอะพอพโทสิดในเซลล์เม็ดเลือดขาวชนิด monocyte มากไปกว่านั้นการเพิ่มปริมาณของ blocking CD137 ligand peptide ช่วยทำให้ประสิทธิภาพของการทำงานยาวนานขึ้น สิ่งที่น่าจะเป็นไปได้คือ เซลล์เม็ดเลือดขาวชนิด monocyte หรือเซลล์ตับสามารถส่งสัญญานการตายแบบอะพอพโทสิดสู่เซลล์เป้าหมายโดยผ่านทางการจับกับ ligand ของมัน การป้องกันปฏิสัมพันธ์ดังกล่าวอาจจะนำมาซึ่งการลดการทำลายเนื้อเยื่อ และความรุนแรงของโรคระหว่างการติดเชื้อไวรัสไข้เลือดออกเดงกี่

คำหลัก : ปฏิสัมพันธ์ระหว่าง CD137 receptor/ligand, การตายแบบอะพอพโทสิด, เซลล์เม็ดเลือดขาวชนิด monocyte, เซลล์ตับ, blocking peptide

1. Executive summary

1.1 Introduction to Research

Dengue virus (DENV), a positive-strand RNA virus of the *Flavivirus* family with 4 different serotypes (DENV-1, -2, -3, and -4), is transmitted to human by mosquito *Aedes aegypti* (1). The effect of DENV infection happens worldwide, predominantly in subtropical and tropical regions. All four serotypes of DENV cause human disease with varied degrees of severity. Some patients have asymptomatic infection. Most of infected patients develop dengue fever (DF). Unfortunately, some patients reach to severe forms, which are classified into dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (2).

Monocytes have long been presented as main targets for DENV infection and confer the severity of disease through its interaction with other immune and non-immune cells and cytokine production (3, 4). Nevertheless, hepatic injury is one of the most clinical significances, which lead to the severity of the disease. The cellular apoptosis in hepatic cells convinces most of the researchers to study its molecular mechanism and its association with disease severity. Up to now, there are several evidences revealing the implication of liver involvement during DENV infection. Both in *vitro* and in *vivo* studies demonstrated that hepatocytes are one of the major sites of DENV replication in the liver and induced apoptosis, which might contribute to the pathogenesis (5, 6).

CD137 is the member of tumor necrosis factor receptor super family 9 (TNFRSF 9) and influences not only the activation state of T cells but also activation, proliferation, survival, apoptosis, and differentiation of several immune and nonimmune cells and the course of immune responses (7-9). Our previous reports demonstrated the upregulation of CD137 upon DENV infected-hepatocyte cells line as well as the increasing of apoptosis and cytokine production (10, 11). Monocytes are well known in CD137 signaling, which induces the production of proinflammatory cytokine and apoptosis. Bidirectional signaling regulates the induction of apoptosis between immune and nonimmune cells, especially hepatocytes. However, little is known about the apoptosis-mediated by CD137 signaling in monocytes and CD137/CD137 ligand interaction between hepatocytes and monocytes during DENV infection.

The significance of this research is to identify the role of CD137 signaling in DENV –mediated apoptosis in monocytes and the CD137/ CD137 ligand interaction between hepatocytes and monocytes, which contributed to apoptosis in hepatic cell line, thereby leading to disease severity. This knowledge will lead us to better understanding

in the pathogenesis of DENV infected hepatic cells and be applied in the utilization of specific drug to block those interactions to minimize the disease severity upon DENV infection.

1.2 Literature review

Mononuclear phagocytes are considered the main targets for viral replication (12). Cells such as B lymphocytes, monocytes, hepatocytes and dendritic cells are described as potential virus targets and may undergo programmed cell death when infected in culture. Monocytes/macrophages and dendritic cells are responsible for the dissemination of the virus after its entrance into the dermis by mosquito bite (13). The TNF superfamily cytokines mediate their effects by activating transcription factors, including NF-ĸB, which culminate in apoptotic processes and/or cellular proliferation (14). A few years ago, *In vitro* infection model in which primary human monocytes are infected with DENV-2, produce TNF- α, IL-10 and are expressing apoptotic markers such as phosphatidylserine exposition and Fas upregulation, indicating that apoptotic processes are occurring (15). During the past decade, peripheral blood mononuclear cells (PBMC), B and T lymphocytes, macrophages, and endothelial cells are believed to be main targets for DENV infection (3, 4). Nevertheless, the several attentions have been drawn into liver cells. Many researchers were interested in the phenomenon occurred in liver upon DENV infection. As mention earlier, the apoptosis has been shown in hepatocyte and may correlate with disease severity, particularly hepatic injury and hepatomegaly. In the beginning with report of hepatitis in a fatal case of DENV infection in 1999, viral hepatocytes were clearly demonstrated the DENV antigen in the area with surrounding with the necrotic foci by immunohistochemistry. The *in situ* detection of DNA fragmentation revealed that apoptotic hepatocytes were found in the same area with DENV-infected hepatocytes (16). These findings suggest that hepatocytes are one of the major sites of DENV replication in the liver and DENV induced apoptosis of hepatocytes *in vivo*. Furthermore, the appearance of councilman bodies, which are believed to be cells undergoing apoptosis, was detected by using histological examination of the livers of fatal cases of DENV infection (5). More recently, evidences were accumulated from fatal DHF/DSS during a Cuban dengue epidemic. TUNEL positive in liver cells were observed in two from six cases(6). Overall evidences demonstrated that liver apoptosis is one of the clinical finding in severity cases and might contribute to the pathogenesis. Besides *in vivo* assay, most of *in vitro* studies widely used human hepatoma cell lines (HepG2), which were undergone apoptosis like the situation in DENV-infected liver cells in human. Consequently, HepG2 represents as

a model for studying DENV-induced apoptosis in hepatocytes for the better understanding of the pathogenesis of dengue disease severity.

Our previous studies demonstrated the crucial role of CD137 signaling upon DENV infection. Since 2007, we firstly reported the interaction between dengue virus capsid protein (DENV C) and human Daxx, especially in nucleus, where determined the apoptosis situation in HepG2 cells (17, 18). Therefore, we interested in the role of nuclear DENV C in regulation the apoptosis. A few years ago, wild type (WT DENV C) and mutant DENV C (R85A/R86A), which locates in cytoplasm only, stably-transfected HepG2 cells were examined to cell death expression profiling by using real-time PCR array containing death pathway targets (11). There were several cell death target gene expressions, for example, TNF-α, TRAIL, and CD137, which were up-regulated wild-type DENV C in comparison with mutants DENV C (11). CD137 was selected to study in details because its role in DENV infection has not been investigated. The quantitative real-time PCR was employed to confirm the mRNA expression level. Up to 6.5 fold and 2 fold of CD137 mRNA expression level in wild-type DENV C was clearly detected in comparison with those of control cell and mutant DENV C, respectively. Moreover, CD137 mRNA expression level was also increased in DENV-infected HepG2 cells (11). The up-regulation of CD137 mRNA expression was also confirmed by the surface CD137 protein level as well as the increasing of apoptotic cells. These data implied that CD137 receptor can be inducible upon DENV infection to trigger apoptosis and demonstrated the significant role of CD137 expression in the induction of apoptosis both DENV infection and DENV C transfection model. According to the apoptotic-induced role of CD137, we discovered that almost two-fold increasing of apoptosis was clearly demonstrated in anti-CD137, agonist monoclonal antibody to CD137, treated wild-type DENV C compared to untreated one (11). More importantly, anti-CD137 treatment augmented DENV-induced apoptosis in HepG2 cells (11). Both of these phenomenons obviously indicated the crucial role of CD137-triggered apoptosis upon DENV infection. In 2014, our research team firstly demonstrated that DENV infection resulted in DENV-C translocates to nucleus and interacts with C-terminal part of Daxx, thereby releasing of NF-*k*B to the promoter of CD137 gene and increasing of CD137 gene expression, suggesting that CD137 receptor can be inducible upon cellular stress. Subsequently, those inducible receptors will be engaged with CD137 ligand, leading to trigger downstream signaling and induction of apoptosis in hepatic cells.

CD137 is the member of Tumor necrosis factor receptor super family 9 (TNFRSF 9), also known as ILA, CD137, CDw137, MGC2172, and FLJ43501. CD137

receptor is mostly expressed in immune cells, activated T lymphocyte, and some in monocytes and inducible primary cells (8). Conversely, naïve T cell lacks of CD137 receptor expression. Its ligand is constitutively presented in antigen-presenting cells (APC), B cell, dendritic cells (DC), and macrophage (monocyte) (19). CD137 genome resides on chromosome 1p36 and this region was obviously associated with mutations in several malignancies (20). Its soluble form generated by different spicing, were increased upon certain diseases, such as rheumatoid arthritis, however, the association with disease has not been elucidated (21). The best characteristic function of CD137 is T cell co-stimulation. Accordingly, CD137 influences not only the activation state of T cells but activation, proliferation, survival, apoptosis, and differentiation of several immune and nonimmune cells and the course of immune responses (9). In general, ligand can crosslink its own receptor, thereby leading to trigger only the cells, which contain those receptors. Unlike others, CD137 ligand can transmit the signal transduction in bidirectional, both cells containing receptor and ligand. The reverse signaling can be generated in those cells, which express CD137 ligand (9).

1.3 Objective

1.3.1 To determine whether CD137 signaling triggers apoptosis in DENV-infected monocytes

1.3.2 To determine whether CD137 receptor/CD137 ligand interaction between hepatocytes and monocytes potentiates DENV –mediated apoptosis

1.3.3 To determine how to block CD137 signaling passing through the interaction between monocytes and its targets for reduction of apoptosis induction

1.4 Research methodology

1.4.1 Cell lines, viral stain and reagents

Human monocyte cell line (U937) and Human hepatocellular carcinoma (HepG2) were employed to study in these experiments. Dengue virus serotype 2 (DENV-2) strain 16681 was isolated from clinical samples, provided, maintained in our lab, and was propagated for study in this experiment. DENV-2 E Monoclonal antibody, 4G2, were kindly provided from Dr. Chunya Puttikhunt (22). Anti-CD137L (sc-11817), anti-CD137 receptor antibody (SC-58947) and CD137L peptide (sc-11817P) were purchased from Santa Cruz Biotechnology. Annexin V kits were purchased from BD Bioscience. CD45 conjugated APC and its isotype control were purchased from Immunotool.

1.4.2 Optimized condition for DENV2-infected human monocyte cells line, U937

Up to 3 x 10^5 cells of U937 cells line were grown in 12-well plate and pretreated with 200, 400, and 800 ng of purified anti-E(4G2) antibody, then infected with DENV serotype 2 at moi 1, 5, and 10. These cells were harvested at 24, 48, and 72 hours post infection followed by fixed and permeabilized for staining with anti-E or 4G2 to perform intracellular staining of DENV E protein and finally analyzed the percentage of infection by using flow cytometry. In order to get more than 50% infection, we attempted to optimize the condition by varying level of 4G2 ranging from 50-200 ng, and time post infection from 24-72 hours at MOI of 10.

1.4.3 Characterization of CD137 signaling in DENV –mediated apoptosis in monocytes

Up to 6 x 10^5 U937 cells line were plated into 6-well plate. Both non-infected, mock, and infected cells were prior incubated with 50 ng of anti-E (4G2) in antibody-dependent enhancement model. DENV2 were added the at MOI of 10 and performing the kinetic time points in every 12 hours post infection starting from 24- 72 hours (24, 36, 48, 60, and 72). Each 1 x 10^6 of DENV2-infected U937 cells and mock, were harvested on ice and then, examined as following;

1.4.3.1 quantitative of CD137 receptor and ligand mRNA expression

Briefly, 1 x 10^6 of DENV2-infected U937 cells and mock were extracted for mRNA by using High pure RNA isolation kit (Roche) and 5 µg of total RNA were converted into cDNA by using RT-PCR from RT2 First strand Kit (Qiagen). The real time PCR were performed by mixing those diluted synthesized cDNA with master mix containing SYBR green according the protocol provided by the manufacture and finally, analyzed by using a Roche Light Cycler 480 machine. The Ct of each mRNA and GAPDH control was measured and the difference between their ∇Ct was calculated. The relative expression values ($2^{-\nabla\nabla}$Ct) was then determined. Results were obtained from three independent experiments. Unpaired t-test was used for statistical analysis by StatView program and p value less than 0.05 was considered significant.

1.4.3.2 quantitative for surface of CD137 receptor and ligand expression

Briefly, 1 x 10^6 of DENV2-infected U937 cells and mock were collected on ice and blocked with 0.1% BSA. Subsequently, those blocking lived-cells were immunostained with either anti-CD137 receptor antibody (SC-58947) or anti-CD137 ligand antibody (SC-11817), following with anti-Mouse and anti-goat secondary antibody conjugating with FITC and Alexa488, respectively. Finally, the CD137 positive cells were quantitated by using Flow cytometer analysis on fluorescence activated cell

sorting (FACS) with CellQuest data acquisition and analysis software. Unpaired t-test was used for statistical analysis by StatView program and p value less than 0.05 was considered significant.

1.4.3.3 quantitative of apoptotic cells

Briefly, 1 x 10^6 of mock and DENV2-infected U937 cells were collected, including life and dead, then, subjected to measure apoptosis by using AnnexinV/PI staining and flow cytometry. All three independent experiments were included and analyzed the statistical significance by using StatView program.

1.4.3.4 quantitative of DENV2 infectivity

Briefly, 1 x 10^6 of DENV2-infected U937 cells and mock were collected, fixed, and permeabilized with 0.2% Triton-100. Subsequently, those cells were immunostained with anti-E antibody or 4G2, following with anti-mouse secondary antibody conjugating with FITC. Finally, the DENV2-positive cells were quantitated by using Flow cytometer analysis on fluorescence activated cell sorting (FACS) with CellQuest data acquisition and analysis software. All three independent experiments were performed.

1.4.4 CD137 receptor/ligand interaction between hepatocytes and monocytes in DENV –mediated apoptosis

1.4.4.1 Evaluation of specific surface CD marker for U937

Up 1 x 10^6 of both HepG2 and U937 cells were collected and washed with PBS, then blocked with 2%FBS for 30 min on ice. After that, several CD markers such as CD14, CD80, CD45, CD11, and CD40, and their isotype control antibodies, which were conjugated with fluorescent dye, were subjected to stain these cells and analyzed by Flow cytometry.

1.4.4.2 Examination the effect of CD137 receptor/ligand interaction between hepatocytes and monocytes in DENV –mediated apoptosis

Co-culture experiment was employed in this experiment. Up to 3 x 10^5 cells of HepG2 cells line were plated in 12-well plate in Day0. The day after, 6 x 10^5 cells of U937 cell line were split and separated infected with DENV serotype 2 as previously described at moi of 10 and moi of 5 for HepG2. Two hours post infection, DENV-infected HepG2 were overlaid by DENV-infected U937 in 2%RPMI media. These cells line was collected at 48- and 60-hours post infection to test further for apoptosis, as previously described for each condition containing HepG2 Mock and U937 Mock, HepG2 Mock with DENV-infected U937, U937 Mock with DENV-infected HepG2, and

DENV-infected HepG2 and U937. In order to ascertain U937, we employed CD45-conjugated with APC as specific marker for differentiate U937 from HepG2.

1.4.5 The effect of specific inhibitors targeting CD137 receptor/ligand interaction between monocyte and its targets for reduction of apoptosis induction

1.4.5.1 The effect of specific inhibitors targeting CD137 receptor/ligand interaction upon DENV-infected monocyte for reduction of apoptosis induction

Up to 3 x 10^5 U937 cells line were plated into 12-well plate. Both non-infected, mock, and infected cells were prior incubated with 50 ng of anti-E (4G2) in antibody-dependent enhancement model with MOI of 10. Twenty-four hours post infection, either 0.5 µg/mL of blocking CD137L peptide or 5 µg/mL of antagonist anti-CD137L antibody were treated to both non infected- and infected- U937 cell line. These cells were harvested by performing the kinetic time points in every 12 hours (36, 48, and 60) post infection and examined for apoptosis, as previously described. The activity of blocking Peptide was no longer than 12 hours. Then, we collected only at 36 hours post infection. Briefly, 1 x 10^6 of mock and DENV2-infected U937 cells were collected, including life and dead, then, subjected to measure apoptosis by using AnnexinV/PI staining and test infectivity of DENV infection, as previously described, and flow cytometry. All three independent experiments were included and analyzed the statistical significance by using StatView program. In order to obtain the increasing time for blocking activity, we tried to increase dose up to 2 ug of blocking CD137L peptide and then harvested cells at 48 hours post infection and measured apoptosis as mention before.

1.4.5.2 The effect of specific inhibitors targeting CD137 receptor/ligand interaction between monocyte and hepatocyte for reduction of apoptosis induction

Co-culture experiment was employed in this experiment. Up to 3 x 10^5 cells of HepG2 cells line were plated in 12-well plate in Day0. The day after, 6 x 10^5 cells of U937 cell line were split and separated infected with DENV serotype 2 as previously described at moi of 10 and moi of 5 for HepG2. Two hours post infection, DENV-infected HepG2 were overlaid by DENV-infected U937 in 2%RPMI media. Twenty-four hours post infection, 2 µg/mL of blocking CD137L peptide was treated to these cells line, and then collected at 60-hours post infection to test further for apoptosis, as previously described for each condition containing HepG2 Mock and U937 Mock, HepG2 Mock with DENV-infected U937, U937 Mock with DENV-infected HepG2, and DENV-infected HepG2 and U937. In order to ascertain U937, we employed CD45-conjugated with APC as specific marker for differentiate U937 from HepG2.

2. Result

2.1 To determine whether CD137 signaling triggers apoptosis in DENV-infected monocytes

2.1.1 Optimized condition for DENV2-infected human monocyte cells line, U937

We employed U937 monocyte cell line as a target to be infected with dengue virus serotype 2 by using ADE to increase the yield of infection. Previous reports demonstrated that the suboptimum level of antibody to help DENV2 infection to U937 cells was 200 ng of anti-E, 4G2 or 3H5 with high Multiplicity of Infection (MOI) of 10. These condition can increase the percentage of infection from 0.1+ 0.09 up to 23+ 8 (23) at 72 hours post infection. We tested those condition by varying viral dose from (MOI) from 1 to 10, level of 4G2 ranging from 200-800 ng, and time post infection from 24-72 hrs. Importantly, we got up to 50% of DENV2 -infected U937 with 200 ng of 4G2 and MOI of 10 at 24 hours post infection (Figure 1). However, the percentage of infection decreased when the time post infection was increased as shown in Figure 2-3. In order to get more than 50% infection, we attempted to optimize the condition by varying level of 4G2 ranging from 50-200 ng, and time post infection from 24-72 hours at MOI of 10. Surprisingly, our experiment found the lowest suboptimal condition of purified 4G2 at 50 ng, that prone U937 monocyte cell line to be infected up to 70% of infection after 24 hours post infection as shown in Figure 4 and 5. Nevertheless, the high percentage of infection resulted in increasing of cellular apoptosis or death, leading to the decreasing for percentage of DENV2 infection when the time was increasing up to 72 hours.

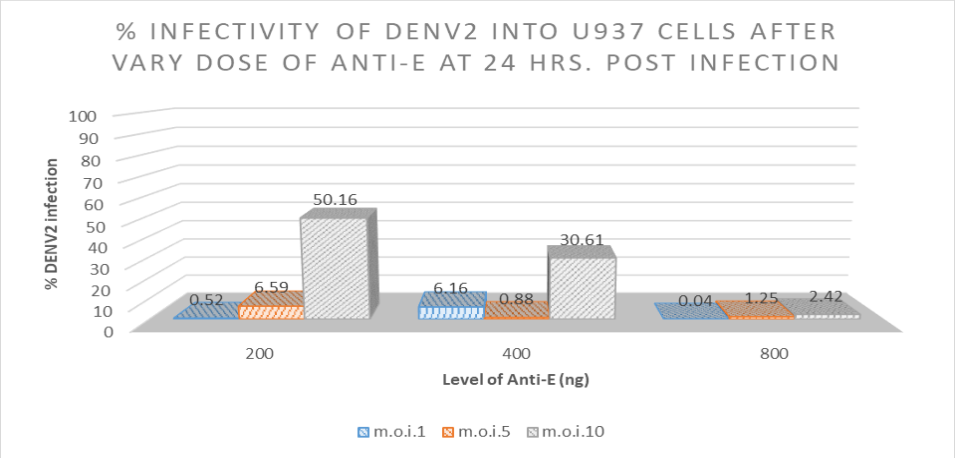


Figure 1. The infectivity of DENV2-infected U937 monocyte cell line in antibody-dependent enhancement model by varying viral dose and level of anti-E or purified 4G2 at 24 hours post infection.

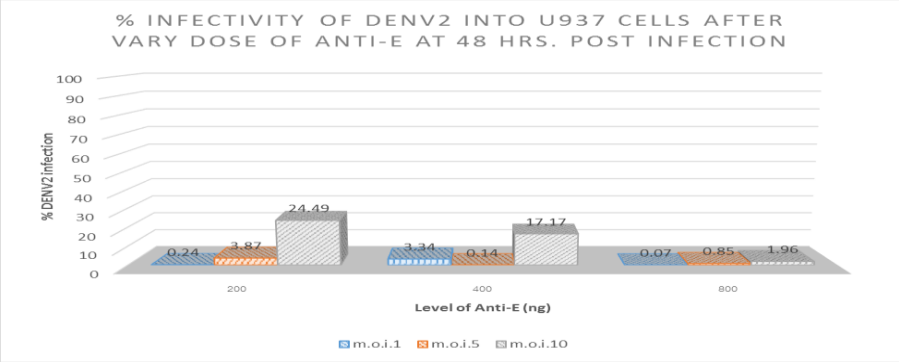


Figure 2. The infectivity of DENV2-infected U937 monocyte cell line in antibody-dependent enhancement model by varying viral dose and level of anti-E or purified 4G2 at 48 hours post infection.

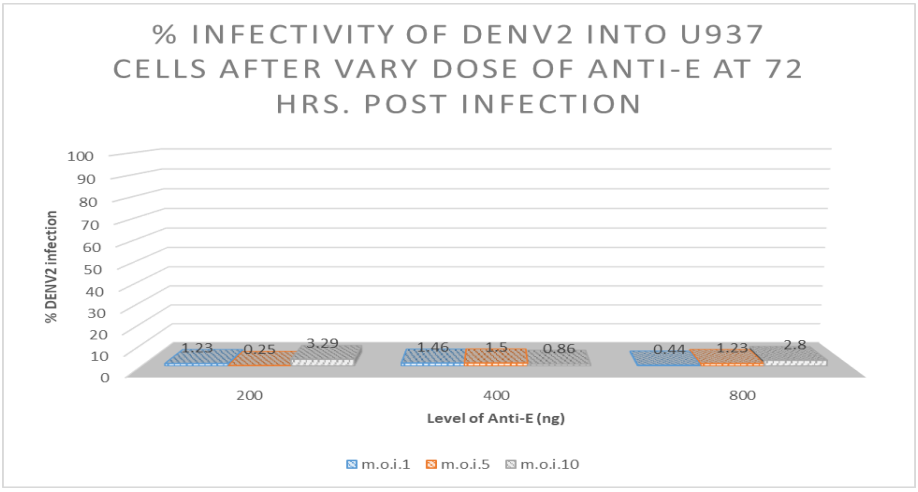


Figure 3. The infectivity of DENV2-infected U937 monocyte cell line in antibody-dependent enhancement model by varying viral dose and level of anti-E or purified 4G2 at 72 hours post infection.

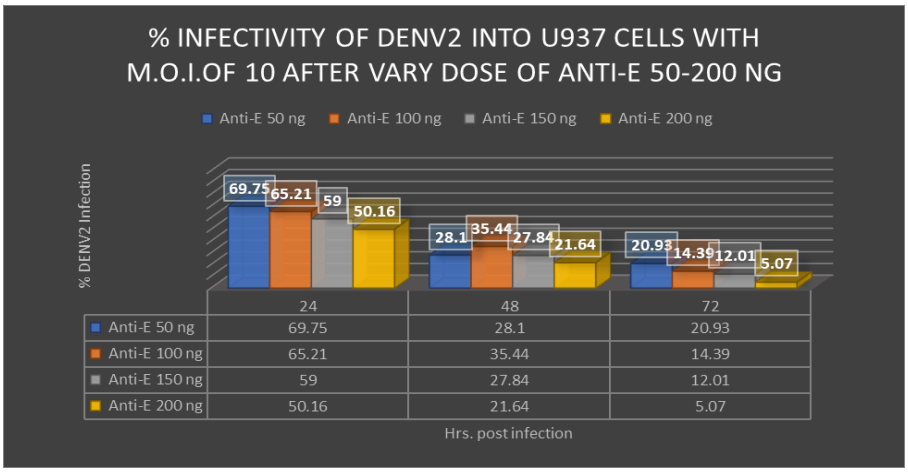


Figure 4. The infectivity of DENV2-infected U937 monocyte cell line in antibody-dependent enhancement model by using MOI of 10 and varying level of anti-E or purified 4G2 from 50-200 ng and time post infection 24- 72 hours.

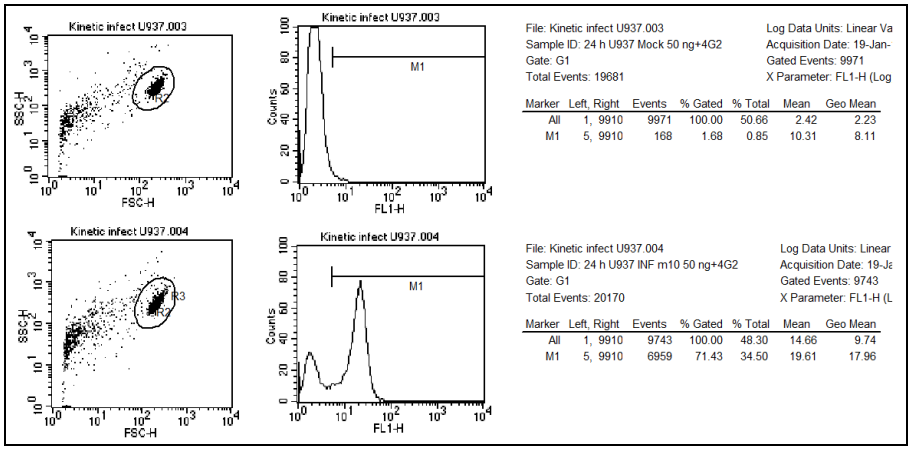


Figure 5. The infectivity of DENV2-infected U937 monocyte cell line in antibody-dependent enhancement model by using MOI of 10 and 50 ng of anti-E at 24 hours post infection was performed by using intracellular staining and then, analyzed by using Flow cytometer.

2.1.2 Characterization of CD137 signaling in DENV –mediated apoptosis in monocytes

According to the crucial role of monocytes, several reports demonstrated the apoptotic induction upon DENV infection. Monocytic cells is well known to study CD137 ligand signaling, however, there were no reports revealing the CD137 signaling in DENV –mediated apoptosis in monocytes. To determine whether CD137 signaling triggers apoptosis in DENV-infected monocytes, human monocytic cell lines with myeloid lineage, U937 will be employed to infect with DENV serotype 2 by using antibody-dependent enhancement model with suboptimal condition including 50 ng. of anti-E, MOI of 10, and performing the kinetic time points in every 12 hours post infection starting from 24- 72 hours to study the minor change of DENV2-infected U937 for induction of apoptosis. The quantitative of apoptotic cells, CD137 mRNA, surface CD137 receptor and ligand expression level, will be measured upon DENV infection as shown in proposed model in Figure 6. As expected, we detected the trend of upregulating for CD137 receptor and ligand in mRNA level but not reached statistically difference when compared between mock and infected cells in each time point, as shown in Figure 7-8. More importantly, we detected the trend of upregulating for surface CD137 receptor in protein level but not reached statistically significant difference when compared between mock (non-infected cells) and infected cells in each time point as shown in Figure 9. On the other hand, we surprised that not only found the trend of increasing for surface CD137 ligand expression in protein level but also statistically

significant difference at 60 hours post infection in DENV2-infected cells compared with mock, p value < 0.01, as shown in Figure 10. Interestingly, we discovered the increasing of apoptotic cells in DENV2-infected cells compared with mock cells only at 48- and 60-hours post infection with statistically significant difference, p value equal 0.006 and 0.001, respectively as shown in Figure 11 and 12. DENV2-infected U937cells conferred up to 60-70% infection at 24 hours post infection. The percentage of infected cells decreased along with the increasing of time post infection as shown in Figure 13. Each time point has been shown the error bar, which indicated the mostly same amount percentage of DENV2 infection for each experiment.

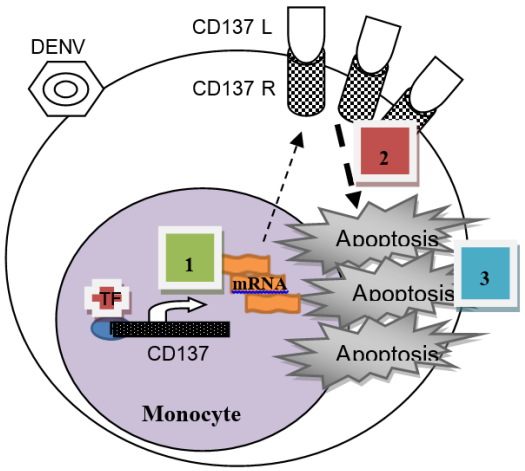


Figure 6. The proposed model represented the CD137-mediated apoptosis in monocytes

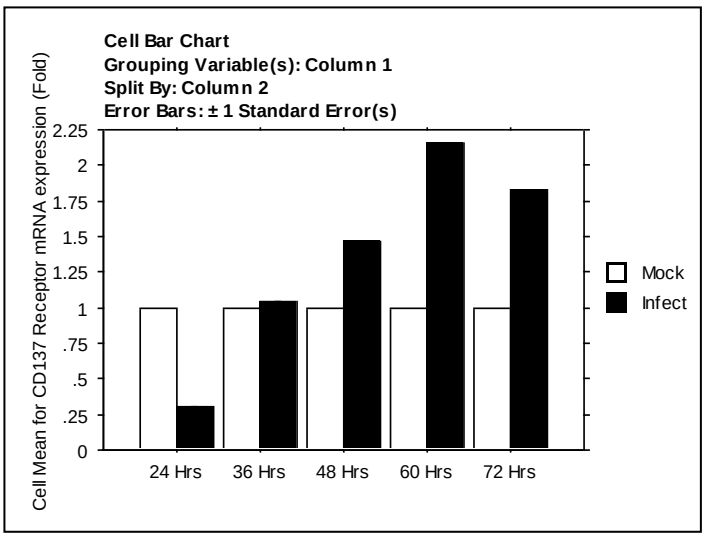


Figure 7. The fold change CD137 receptor mRNA expression level of DENV2-infected U937 cells in antibody-dependent enhancement model by using MOI of 10 with 50 ng of 4G2 and time post infection 24- 72 hours. All three independent experiments were included and analyzed the statistical significance by using Stat view program. Asterisks indicate a significant difference among two groups (*p < 0.05 and **p<0.01).

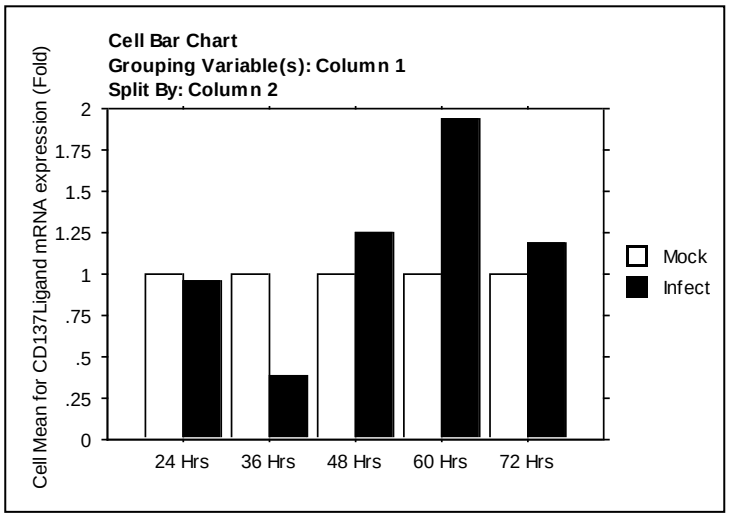


Figure 8. The fold change CD137 ligand mRNA expression level of DENV2-infected U937 cells in antibody-dependent enhancement model by using MOI of 10 with 50 ng of 4G2 and time post infection 24- 72 hours. All three independent experiments were included and analyzed the statistical significance by using Stat view program. Asterisks indicate a significant difference among two groups (*p < 0.05 and **p<0.01).

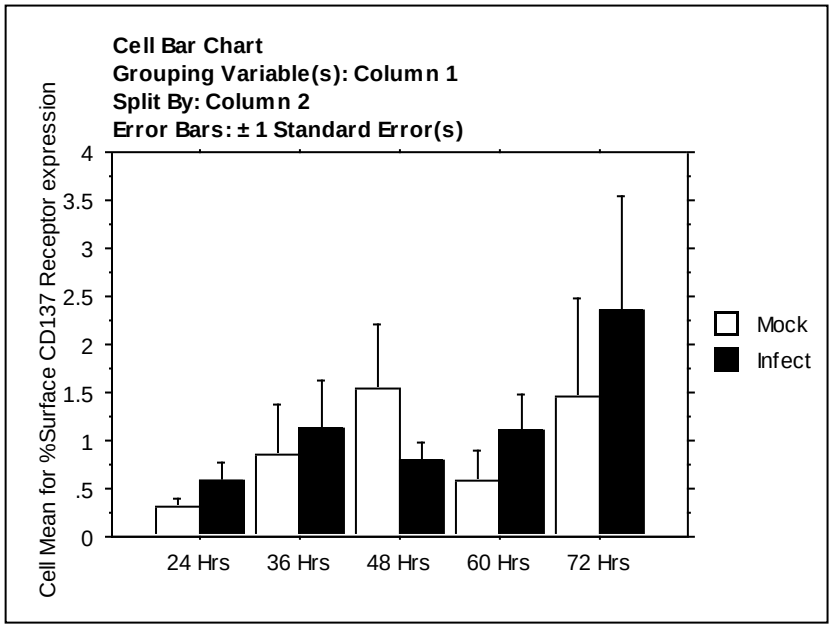


Figure 9. The surface CD137 receptor expression level of DENV2-infected U937 cells in antibody-dependent enhancement model by using MOI of 10 with 50 ng of 4G2 and time post infection 24- 72 hours. All three independent experiments were included and analyzed the statistical significance by using Stat view program. Asterisks indicate a significant difference among two groups (*p < 0.05 and **p<0.01).

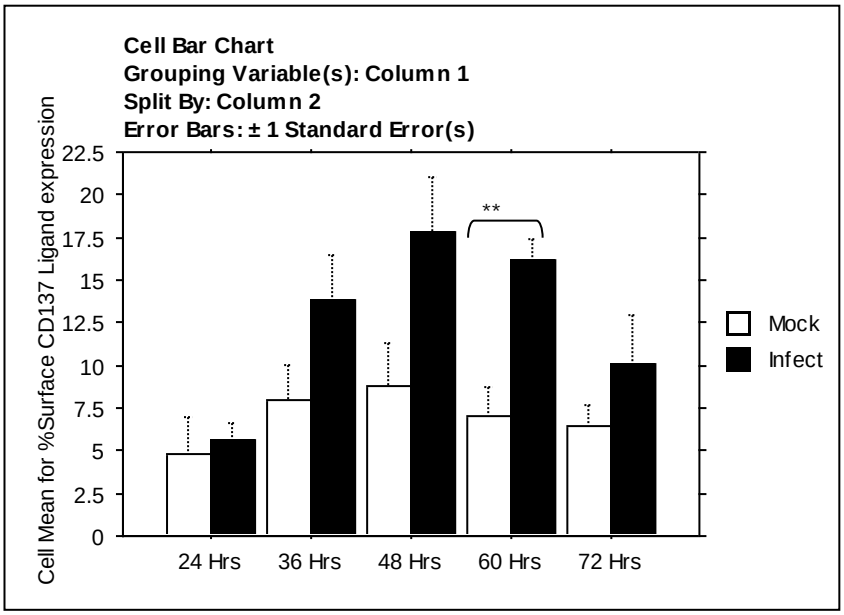


Figure 10. The surface CD137 ligand expression level of DENV2-infected U937 cells in antibody-dependent enhancement model by using MOI of 10 with 50 ng of 4G2 and time post infection 24- 72 hours. All three independent experiments were included and analyzed the statistical significance by using Stat view program. Asterisks indicate a significant difference among two groups (*$p < 0.05$ and **$p<0.01$).

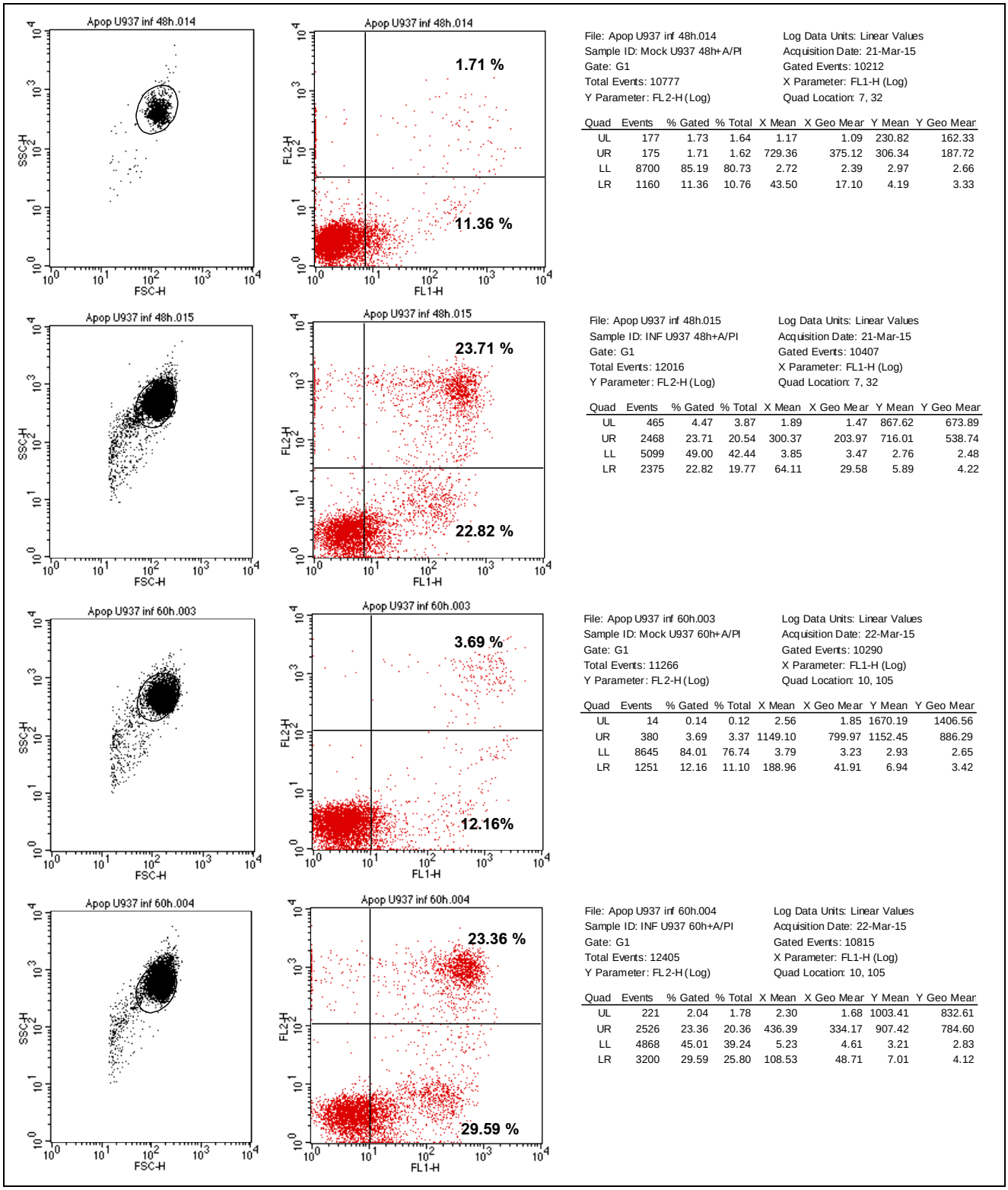


Figure 11. Dot plots represented the percentage of apoptotic cells in DENV2-infected U937 in comparison with mock at 48- and 60-hours post infection.

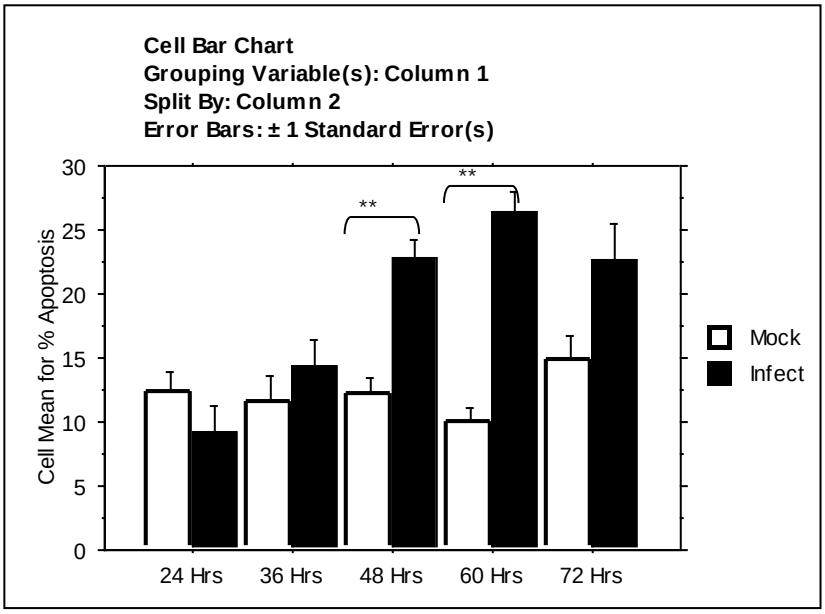


Figure 12. The percentage of apoptotic cells in DENV2-infected U937 in comparison with mock along with time course 24-72 hours post infection. All three independent experiments were included and analyzed the statistical significance by using Stat view program. Asterisks indicate a significant difference among two groups (*p < 0.05 and **p<0.01).

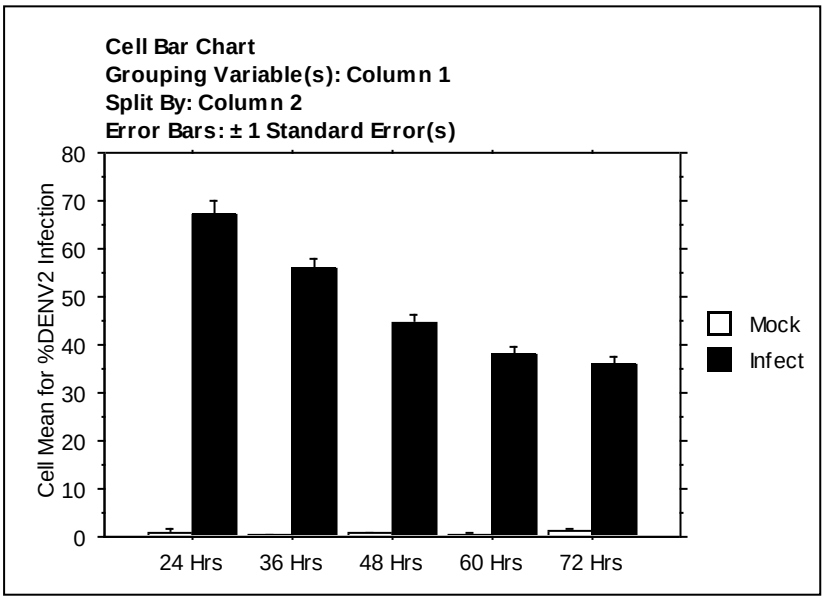
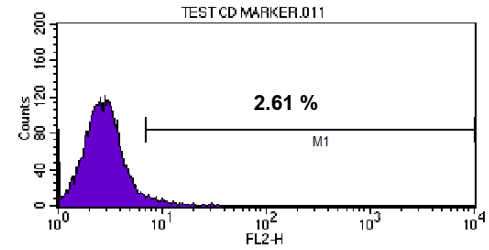


Figure 13. The infectivity of DENV2-infected U937 monocyte cell line along the time course of infection starting from 24-72 hours post infection.

2.2 To determine whether CD137 receptor/CD137 ligand interaction between hepatocytes and monocytes potentiates DENV –mediated apoptosis

2.2.1 Determination of specific marker for monocyte cells line, U937

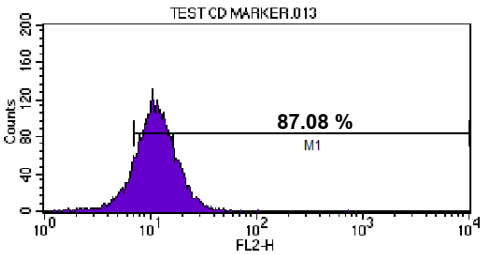
Several CD markers, which widely expressed along with monocytic cells were drawn into this study, such as CD14, CD80, CD45, CD11, and CD40. Basically, CD14 served as monocytic marker but it can be expressed in Hepatocytic cells (HepG2) approximately more than 50%. Hence, CD14 did not represent the differentiation marker between U937 and HepG2. Other CD markers, CD11, CD40, and CD80, were not expressed on U937. The best CD monocytic CD marker was CD45, which can be expressed on surface of U937 more than 80% and not shown on the surface of HepG2, compared with their isotype control antibodies, as shown in Figure 14.



File: TEST CD MARKER.011
Sample ID: U937 isotype igg1 PE gate
Tube:
Acquisition Date: 30-Sep-17
Gated Events: 11016
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G1
Total Events: 26988

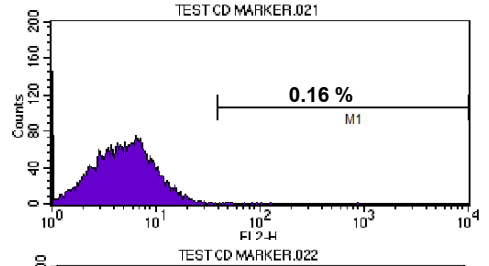
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	11016	100.00	40.82	3.02	2.73	57.94	2.69	2
M1	7, 9910	288	2.61	1.07	10.27	9.66	44.22	8.74	7



File: TEST CD MARKER.013
Sample ID: U937 CD45RA PE gate
Tube:
Acquisition Date: 30-Sep-17
Gated Events: 10915
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G1
Total Events: 25353

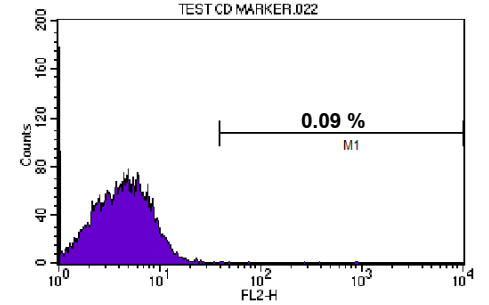
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10915	100.00	43.05	12.06	11.00	69.61	11.04	10
M1	7, 9910	9505	87.08	37.49	13.02	12.21	65.95	11.76	10



File: TEST CD MARKER.021
Sample ID: HepG2 isotypr PE
Tube:
Acquisition Date: 30-Sep-17
Gated Events: 10265
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G1
Total Events: 29391

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10265	100.00	34.93	5.86	4.74	157.28	4.87	1
M1	39, 9910	16	0.16	0.05	112.19	71.41	171.46	51.86	39



File: TEST CD MARKER.022
Sample ID: HepG2 CD45RA PE
Tube:
Acquisition Date: 30-Sep-17
Gated Events: 10299
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G1
Total Events: 28006

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10299	100.00	36.77	5.11	4.11	253.30	4.26	1
M1	39, 9910	9	0.09	0.03	286.24	137.14	118.90	72.99	40

Figure 14 The percentage of surface CD45 marker expression between hepatocytic cell (HepG2) and monocytic cells (U937) in comparison with their isotype control antibodies.

2.2.2 Examination of CD137 receptor/ligand interaction between hepatocytes and monocytes in DENV –mediated apoptosis

Coculture method between HepG2 and U937 was employed in this study with 4 different coculture conditions as following; HepG2 Mock and U937 Mock, HepG2 Mock with DENV-infected U937, U937 Mock with DENV-infected HepG2, and DENV-infected HepG2 and U937. These cells were harvested at 48- and 60-hours post infection and examined for cellular apoptosis by gating CD45-positive cells, which is specific marker to differentiate U937 from HepG2, in order to ascertain the correct number of apoptotic cells only from monocytes. Interestingly, we detected the trend of increasing for apoptotic cells in U937 when cocultured with DENV2-infected HepG2 cells in comparison with noninfected- HepG2 and -U937 cells at 48 hours post infection but reached the statistically significant difference at 60 hours post infection with p value less than 0.0001. As expected, DENV-infected HepG2 and -U937 cells had shown the high number of apoptotic cells containing U937 cells with statistically significant difference, p value less than 0.01, at 60 hours post infection as shown in Figure 15.

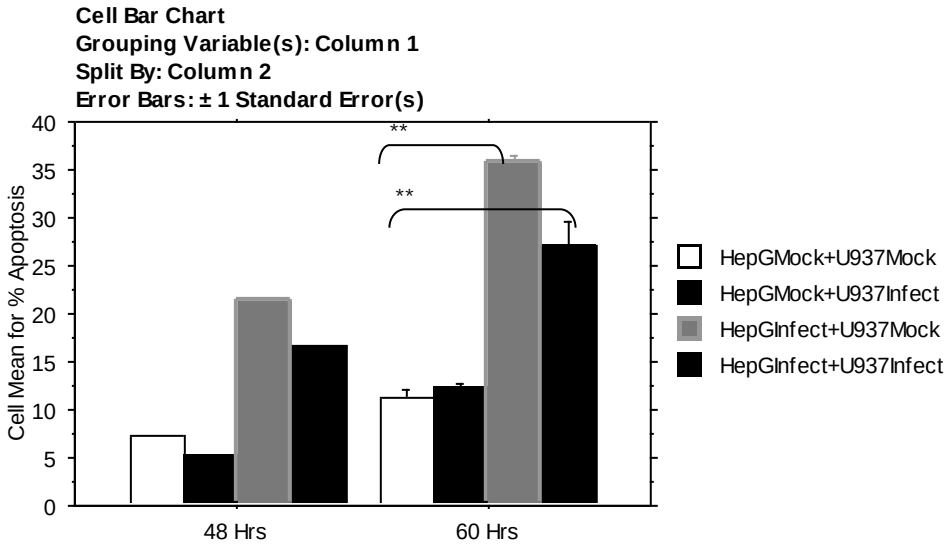


Figure 15. The percentage of apoptotic cells in U937 cell line with different coculture conditions along with time course 48- and 60-hours post infection. All three independent experiments were included and analyzed the statistical significance by using Stat view program. Asterisks indicate a significant difference among two groups (*$p < 0.05$ and **$p<0.01$).

2.3 To determine how to block CD137 signaling passing through the interaction between monocytes and its targets for reduction of apoptosis induction

2.3.1 The effect of specific inhibitors targeting CD137 receptor/ligand interaction upon DENV-infected monocyte for reduction of apoptosis induction

The activity of CD137 ligand can be expressed when interacts with its receptor by initiation of apoptosis. CD137 ligand blocking peptide and antagonist anti-CD137 ligand antibody were utilized upon DENV-infected U937 cells line with different time point, starting at 36, 48, and 60 hours post infection. We found that there was no effect when incubated longer than 12 hours (at 48- and 60-hours post infection) (data not shown). Therefore, we further test its activity only at 36 hours post infection with 0.5 and 5 μg/ml of blocking peptide and antibody, respectively. Surprisingly, only blocking peptide demonstrated the reduction in the percentage of apoptotic cells in U937 cells when compared with untreated DENV-infected U937 cells and blocking antibody-treated cells with statistically significant difference, p value less than 0.05, at 36 hours post infection as shown in Figure 16 and 17. In order to discriminate the infectivity with different treated cells, we further tested the percentage of DENV-infected U937. Up to 80% infection at 36 hours post infection were demonstrated in Figure 18. Each treated cells and untreated cells have been shown the error bar, which indicated the mostly same amount percentage of DENV2 infection for each experiment. In order to obtain the increasing time for blocking activity, we tried to increase dose up to 2 ug of blocking CD137L peptide and then harvested cells at 48 hours post infection and measured apoptosis. Obviously, we detected that the increasing dose of blocking CD137L peptide resulted in the decreasing of apoptotic cells with statistically significant difference, p value less than 0.0001, in comparison with untreated group and blocking antibody-treated cells. Moreover, the percentage of apoptotic cells was quite similar to noninfected cells (mock), as shown in Figure 19.

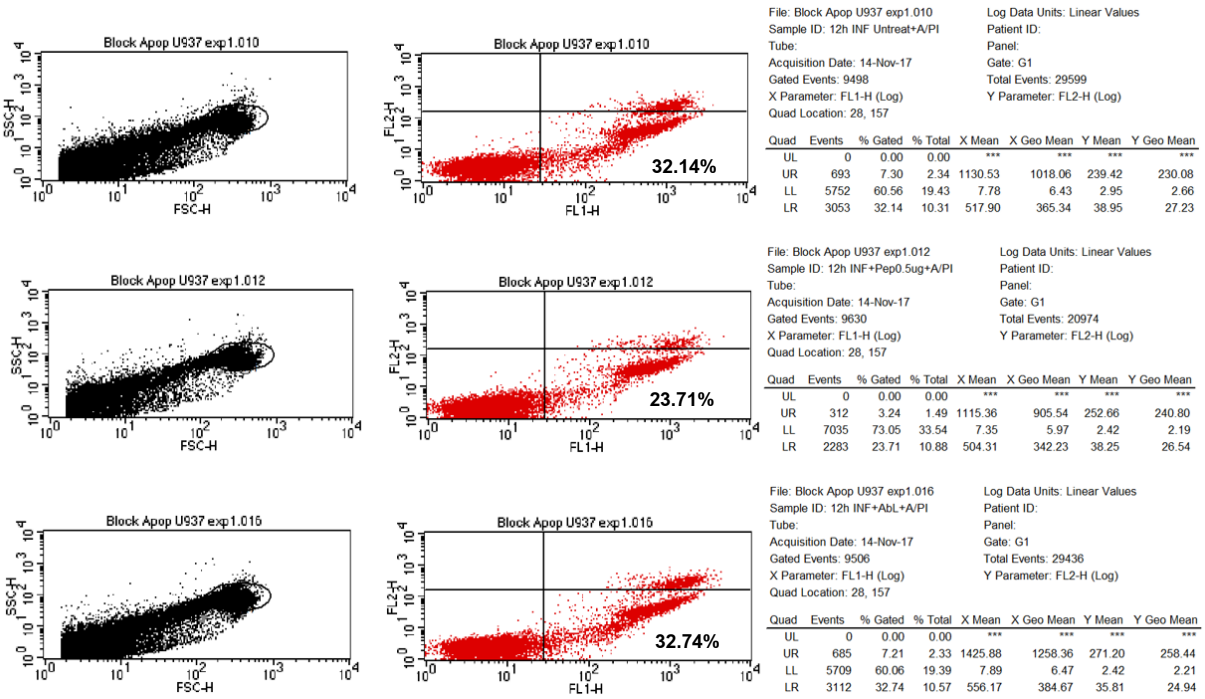


Figure 16. Dot plots represented the percentage of apoptotic cells in DENV2-infected U937 when incubated with either blocking -peptide or -antibody in comparison with untreated group at 36-hours post infection.

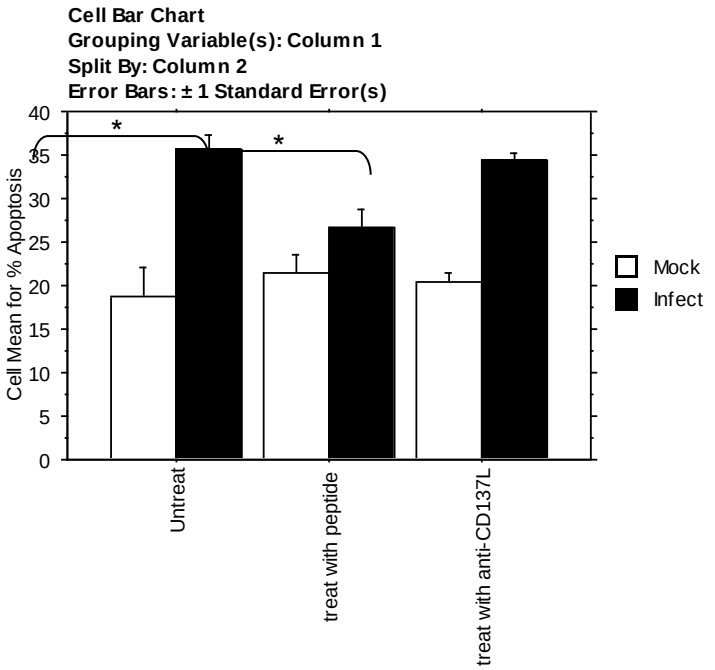


Figure 17. The percentage of apoptotic cells in U937 cell line when incubated with either blocking -peptide or -antibody in comparison with untreated group at 36-hours post infection. All three independent experiments were included and analyzed the statistical significance by using Stat view program. Asterisks indicate a significant difference among two groups (*$p < 0.05$).

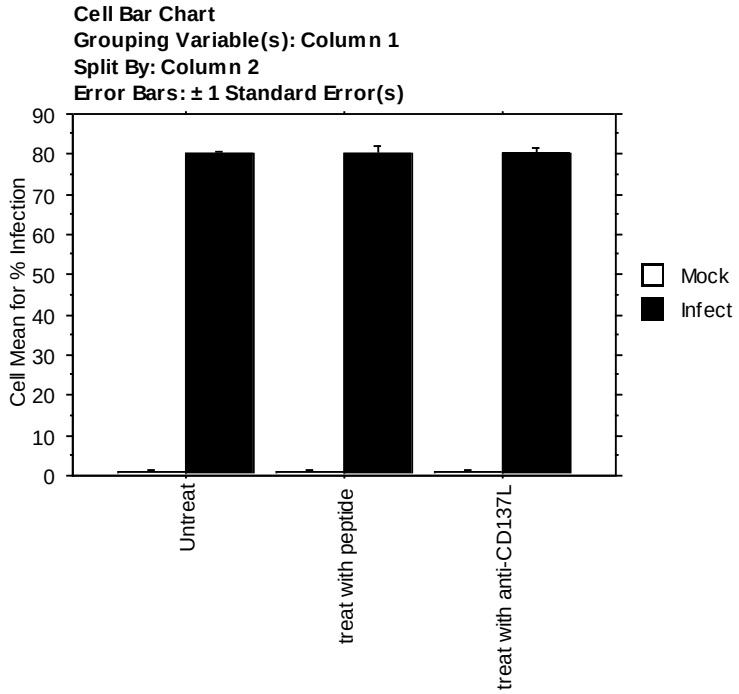


Figure 18. The infectivity of DENV2-infected U937 monocyte cell line when incubated with either blocking -peptide or -antibody in comparison with untreated group at 36-hours post infection.

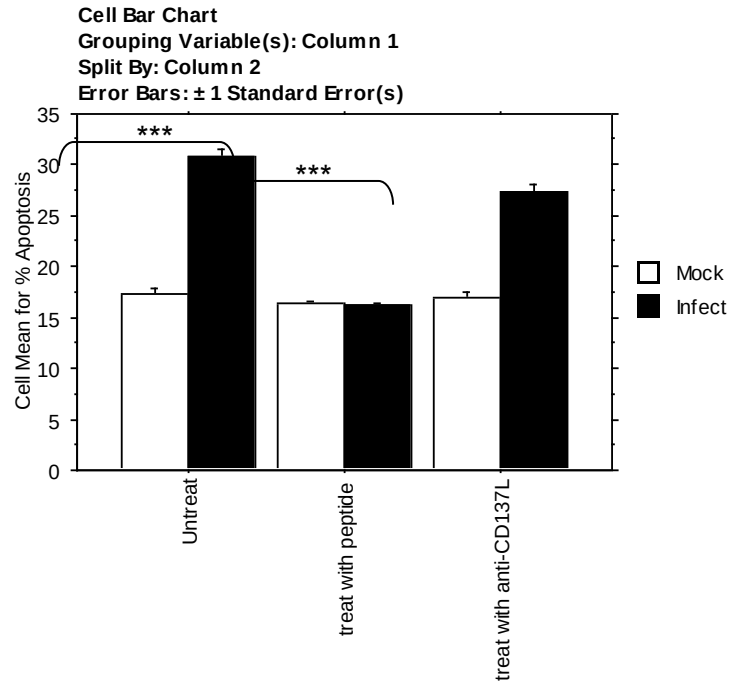


Figure 19. The percentage of apoptotic cells in U937 cell line, when increased amount of blocking CD137L peptide up to 2 ug/ml, compared with blocking anti-CD137L treated- and untreated- group at 48 hours post infection. All three independent experiments were included and analyzed the statistical significance by using Stat view program. Asterisks indicate a significant difference among two groups (***$p < 0.0001$).

2.3.2 The effect of specific inhibitors targeting CD137 receptor/ligand interaction between DENV-infected monocyte and hepatocyte for reduction of apoptosis induction

Previous report revealed the high number of apoptotic HepG2 cells at 60 hours post infection as well as mRNA and surface protein expression of CD137 receptor and CD137Ligand. Moreover, our previous result in coculture experiment demonstrated the trend of augmentation for apoptosis in U937 when cocultured with HepG2, especially 60 hours post infection, see Figure 15, similar to DENV-infected U937 cells, see Figure 12. Therefore, we prior treated cocultured cells with 2 ug/ml of blocking CD137L peptide and harvested at 60 hours post infection. Surprisingly, we finally discovered the high decreasing number of apoptotic U937 cells, when cocultured with DENV-infected HepG2, in comparison with untreated group and reaching the statistically difference, as shown in Figure 20 and 21. More importantly, the percentage of apoptotic cells was quite similar to noninfected HepG2 and U937 cells (mock).

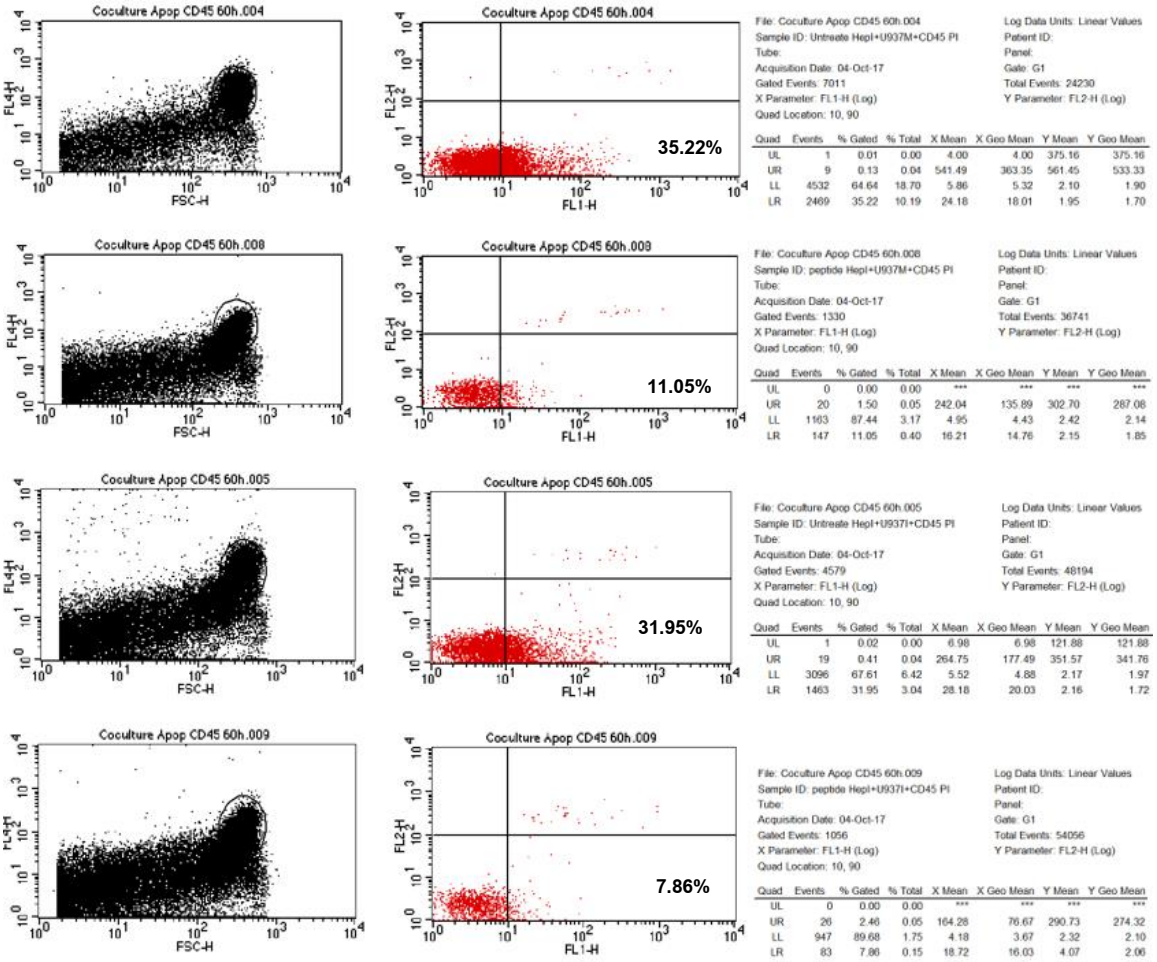


Figure 20. Dot plots represented the percentage of apoptotic cells in DENV2-infected HepG2 and U937 in comparison between blocking CD137L peptide and untreated group at 60-hours post infection.

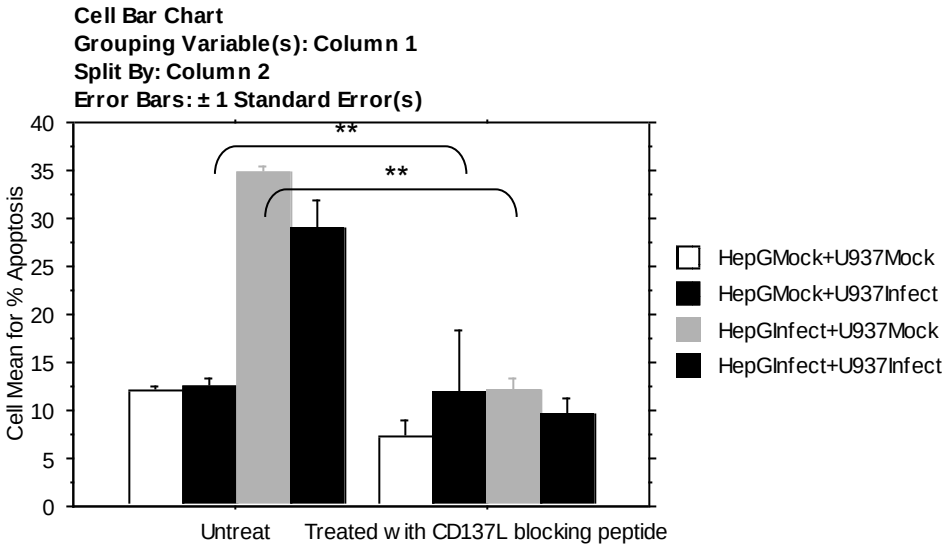


Figure 21. The percentage of apoptotic cells in U937 cell line with different coculture conditions, which treated with 2 ug/ml of blocking CD137L peptide, were harvested at 60-hours post infection. All three independent experiments were included and analyzed the statistical significance by using Stat view program. Asterisks indicate a significant difference among two groups (**p<0.01).

3. Conclusion and Discussion

3.1 DENV-infected monocyte potentiated apoptosis by up-regulation the expression of surface CD137

Monocyte cells line, U937 can be infected with Dengue virus serotype 2 (DENV2) by using antibody-dependent enhancement model. Previous reports demonstrated that the suboptimum level of antibody to help DENV2 infection to U937 cells was 200 ng of anti-E, 4G2 or 3H5 with high Multiplicity of Infection (MOI) of 10. These condition can increase the percentage of infection from 0.1+ 0.09 up to 23+ 8 (23) at 72 hours post infection. We firstly titrated the lowest suboptimal condition of purified 4G2 at 50 ng, that prone U937 monocyte cell line to be infected up to 70% of infection after 24 hours post infection. This condition yielded the high infectivity and reproduced with the same manner to study the role of DENV infection in U937 monocytic cells line. In order to characterize the CD137 signaling in DENV –mediated apoptosis in monocytes, we firstly demonstrated the statistically significant increasing of apoptotic cells in DENV2-infected U937 compared with non-infected cells at 48- and 60-hours post infection. At 60 hours post infection, our finding surprisingly detected the increasing amount of surface CD137 ligand expression with statistically significant difference between infection and mock. Even though, the level of CD137 receptor and

ligand mRNA, also surface CD137 receptor protein, could not reached statistically significant but reveling the trend of Up-regulating in DENV2-infected U937 more than mock. Overall results indicate the importance role of CD137 signaling in mediated the apoptosis in monocyte cell line, U937, especially CD137 ligand, which can kill any cells-bearing CD137 receptor, such as DENV2-infected HepG2 at 48-60 hours post infection. Both these phenomenon in difference type of cells lineage might be the reason for cell-to cell contact upon DENV-mediated apoptosis.

3.2 Blocking CD137L peptide significantly reduced the number of apoptotic U937 cells upon DENV-infection

The interaction between CD137 receptor and its ligand plays an importance role in apoptosis. Several reports revealed the effect of specific inhibitor, which targets CD137 ligand to block CD137 signaling in monocyte. This CD137 ligand blocking antibody can increase allograft survival in corneal-transplanted mice *in* vivo (24). We primarily reported that only Blocking CD137L peptide statistically significant decreased the amount of apoptotic U937 cells upon DENV infection at 36-hour post infection in comparison with antagonist CD137L antibody and untreated groups. In order to discriminate the infectivity with different treated cells, we obtained more than 80% infection with different treated cells at 36 hours post infection, which indicated the mostly same amount percentage of DENV2 infection for each experiment. According to the stability of blocking peptide, it was no longer than 12 hours. Hence, we tried to increase amount of peptide up to 2 ug/ml and incubated longer than 12 hours. At 48 hours post infection, we surprised that the activity of blocking CD137L peptide was still functionable and statistically significantly reduced the number of apoptotic U937 cells upon DENV infection compared with antagonist CD137L antibody and untreated groups. More importantly, the percentage of apoptotic cells was quite similar to noninfected cells. Overall results implied that the interaction between CD137 receptor and its ligand potentiated apoptosis in DENV-infected monocytic cells line, U937. This blocking CD137L peptide can be applied to target molecules, particularly CD137 ligand, which decreases the apoptosis of monocytes, thereby alleviating DENV-mediated disease severity during DENV infection. Moreover, our blocking peptide, CD137L blocking peptide was designed as the mapping on C-terminal part of CD137 ligand ectodomain, which interacts for its receptor. Therefore, our blocking peptide might interfere the interaction both soluble and transmembrane CD137 ligand with CD137 receptor.

3.3 The interaction between hepatocyte and monocyte cells lines during DENV infection potentiated apoptosis of monocyte cells line

Bidirectional signaling determines the stage of cell upon receiving the CD137 signaling, including proliferation, differentiation, and especially apoptosis(9). Previous reports revealed the apoptotic induction during DENV infection in many cell types, such as monocytes and hepatocytes, which correlated to the disease severity. Therefore, it might be possible that CD137 signaling will trigger apoptotic event in both cell types by interaction of CD137 receptor/CD137 ligand between hepatocytes and monocytes upon DENV infection. However, there is no precise data for the expression of CD137 receptor and its ligand, depending on the cell type and the stage of cells. In order to discriminate U937 from HepG2, we found that CD14 can express little amount on U937 but it highly expressed on surface of HepG2 up to 50%. Another CD marker, CD45 expressed more than 80% on the surface of U937 but no expression on surface of HepG2 cells. CD45 served as a specific CD marker for U937 to differentiate from HepG2 cells but there was no precise specific marker for HepG2 cells. Therefore, we can quantitate only the number of apoptotic U937 cells in this cocultured experiment. According to time course during DENV infection, we found that both U937 and HepG2 cells demonstrated the high degree of apoptotic events at 48- and 60-hours post infection. We revealed the statistically significant difference in amount of apoptotic U937 cells when cocultured with HepG2 cells, especially DENV-infected HepG2 cells and noninfected U937 cells, at 60 hours post infection in comparison with noninfected HepG2 and U937 cells. As expected, DENV-infected HepG2 and -U937 cells had shown the high number of apoptotic cells containing U937 cells with statistically significant difference in the same manner. It might be possible that DENV-infected hepatocyte cells line can induce the expression of CD137 Receptor on monocyte, leading to apoptosis of monocyte cells line. The future study will examine the expression of inducible CD137 Receptor on the surface of monocyte cells line during DENV infection with cocultured conditions.

3.4 Blocking CD137L peptide significantly minimized the amount of apoptotic U937 cells when cocultured with DENV-infected HepG2 cells

According to our previous results, we selected the time course of infection at 60 hours post infection because our cocultured experiment demonstrated the significant increasing amount of apoptotic U937 cells when compared to others. Moreover, our previous reports revealed the expression of surface CD137 both ligand

and receptor on these cells as well as the induction of apoptosis event. We firstly demonstrated the effect of blocking CD137L peptide at 2 ug/ml for reduction of apoptotic U937 cells when cocultured with DENV-infected HepG2 cells at 60 hours post infection. Overall results implied the important role of CD137 ligand expression on both monocytic cells and hepatocytic cells for triggering apoptotic events either monocyte or hepatocytes or both for bidirectional signaling. Future study, we appreciate to ascertain the specific surface marker in HepG2 cells line and test further by using either blocking peptide or antagonist antibody to diminish apoptotic HepG2 cell, leading to alleviate the tissue injury or disease severity upon DENV infection.

In summary, DENV-infected monocytic cells line, U937, resulted in the augmentation of cellular apoptosis by upregulation of both mRNA and surface protein expression for CD137 receptor and its ligand. Cocultured experiment between HepG2 and U937 during DENV infection demonstrated the importance role of interaction between CD137 receptor and its ligand to trigger the apoptosis in U937 cells line. Blocking CD137 ligand peptide diminished the apoptotic events in DENV-infected U937 cells line and also reduced the number of apoptotic U937 cells when cocultured with DENV-infected HepG2 cells. These phenomena indicated the crucial role of CD137 receptor and ligand interaction to control cellular apoptosis and severity of disease.

4. References

1. Gubler DJ. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev. 1998;11(3):480-96.

2. Gibbons RV, Vaughn DW. Dengue: an escalating problem. Bmj. 2002;324(7353):1563-6.

3. Anderson R. Manipulation of cell surface macromolecules by flaviviruses. Advances in virus research. 2003;59:229-74.

4. Clyde K, Kyle JL, Harris E. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. Journal of virology. 2006;80(23):11418-31.

5. Huerre MR, Lan NT, Marianneau P, Hue NB, Khun H, Hung NT, et al. Liver histopathology and biological correlates in five cases of fatal dengue fever in Vietnamese children. Virchows Arch. 2001;438(2):107-15.

6. Limonta D, Capo V, Torres G, Perez AB, Guzman MG. Apoptosis in tissues from fatal dengue shock syndrome. J Clin Virol. 2007;40(1):50-4.

7. Alderson MR. Regulation of immune responses by the ligands for CD27, CD30, and 4-1BB. Circ Shock. 1994;44(2):73-6.

8. Schwarz H, Valbracht J, Tuckwell J, von Kempis J, Lotz M. ILA, the human 4-1 BB homologue, is inducible in lymphoid and other cell lineages. Blood. 1995;85(4):1043-52.

9. Shao Z, Schwarz H. CD137 ligand, a member of the tumor necrosis factor family, regulates immune responses via reverse signal transduction. J Leukoc Biol. 2011;89(1):21-9.

10. Nagila A, Netsawang J, Suttitheptumrong A, Morchang A, Khunchai S, Srisawat C, et al. Inhibition of p38MAPK and CD137 signaling reduce dengue virus-induced TNF-alpha secretion and apoptosis. Virol J. 2013;10:105.

11. Nagila A, Netsawang J, Srisawat C, Noisakran S, Morchang A, Yasamut U, et al. Role of CD137 signaling in dengue virus-mediated apoptosis. Biochem Biophys Res Commun. 2011;410(3):428-33.

12. Jessie K, Fong MY, Devi S, Lam SK, Wong KT. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. The Journal of infectious diseases. 2004;189(8):1411-8.

13. Kou Z, Quinn M, Chen H, Rodrigo WW, Rose RC, Schlesinger JJ, et al. Monocytes, but not T or B cells, are the principal target cells for dengue virus (DV)

infection among human peripheral blood mononuclear cells. Journal of medical virology. 2008;80(1):134-46.

14. Karin M, Lin A. NF-kappaB at the crossroads of life and death. Nat Immunol. 2002;3(3):221-7.

15. Torrentes-Carvalho A, Azeredo EL, Reis SR, Miranda AS, Gandini M, Barbosa LS, et al. Dengue-2 infection and the induction of apoptosis in human primary monocytes. Memorias do Instituto Oswaldo Cruz. 2009;104(8):1091-9.

16. Couvelard A, Marianneau P, Bedel C, Drouet MT, Vachon F, Henin D, et al. Report of a fatal case of dengue infection with hepatitis: demonstration of dengue antigens in hepatocytes and liver apoptosis. Human pathology. 1999;30(9):1106-10.

17. Limjindaporn T, Netsawang J, Noisakran S, Thiemmeca S, Wongwiwat W, Sudsaward S, et al. Sensitization to Fas-mediated apoptosis by dengue virus capsid protein. Biochemical and biophysical research communications. 2007;362(2):334-9.

18. Netsawang J, Noisakran S, Puttikhunt C, Kasinrerk W, Wongwiwat W, Malasit P, et al. Nuclear localization of dengue virus capsid protein is required for DAXX interaction and apoptosis. Virus research. 2010;147(2):275-83.

19. Alderson MR, Smith CA, Tough TW, Davis-Smith T, Armitage RJ, Falk B, et al. Molecular and biological characterization of human 4-1BB and its ligand. Eur J Immunol. 1994;24(9):2219-27.

20. Schwarz H, Arden K, Lotz M. CD137, a member of the tumor necrosis factor receptor family, is located on chromosome 1p36, in a cluster of related genes, and colocalizes with several malignancies. Biochemical and biophysical research communications. 1997;235(3):699-703.

21. Michel J, Langstein J, Hofstadter F, Schwarz H. A soluble form of CD137 (ILA/4-1BB), a member of the TNF receptor family, is released by activated lymphocytes and is detectable in sera of patients with rheumatoid arthritis. Eur J Immunol. 1998;28(1):290-5.

22. Puttikhunt C, Ong-Ajchaowlerd P, Prommool T, Sangiambut S, Netsawang J, Limjindaporn T, et al. Production and characterization of anti-dengue capsid antibodies suggesting the N terminus region covering the first 20 amino acids of dengue virus capsid protein is predominantly immunogenic in mice. Arch Virol. 2009;154(8):1211-21.

23. Diamond MS, Edgil D, Roberts TG, Lu B, Harris E. Infection of human cells by dengue virus is modulated by different cell types and viral strains. Journal of virology. 2000;74(17):7814-23.

24. Asai T, Choi BK, Kwon PM, Kim WY, Kim JD, Vinay DS, et al. Blockade of the 4-1BB (CD137)/4-1BBL and/or CD28/CD80/CD86 costimulatory pathways promotes corneal allograft survival in mice. Immunology. 2007;121(3):349-58.

5. Output (Acknowledge the Thailand Research Fund)

5.1 International Journal Publication

Some part of this project was plan to publish by Biochemical and Biophysical Research Communications (BBRC) in the topic **" Blocking CD137 receptor/ligand interaction by CD137 ligand peptide alleviates the Dengue virus-mediated apoptosis in monocyte cells line"**

5.2 Research Utilization and Application

Overall results from this study indicate the basic knowledge in pure science and further test to apply in clinical trial in order to diminish tissue injury and disease severity during late state of Dengue viral infection.

5.3 Others e.g. national journal publication, proceeding, international conference, book chapter, patent

Some part of result was presented the progression of research by using poster presentation at The Regent Cha Am Beach Resort, Phetchaburi province on Jan 6-8, 2016.