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**Proposal Cover Sheet**

**Term: Fall X Spring \_\_\_\_\_ Year \_\_\_\_\_\_**

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Major Biology

Have you identified a research mentor for a senior thesis (if applicable)?

\_\_\_\_\_ Yes \_\_\_\_\_ No.

If yes, please identify.

Name: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Title of Proposal:**

Dengue Induced apoptosis in the presence of TNF-α

**Checklist:**

All required portions of the first submission are included X Yes \_\_\_\_\_ No

I had an external reviewer read the proposal X Yes \_\_\_\_\_ No

If Yes, who Wendy Crowell When October 13, 2011

I authorize the use of this proposal as an example in future courses X Yes \_\_\_\_\_ No

***Abstract***

The relationship between the host immunological response to infection of dengue virus has been difficult to ascertain. Cytokines such as TNF-α play a role in cellular apoptosis which has been assumed to contribute to the overall pathogenesis of the virus, however the mechanism is unknown. To delineate the complex interaction between the virus and the host, TNF-α will be introduced to host endothelial cells and apoptosis will be observed. Cells that have undergone apoptosis will be analyzed via TUNEL assays and real-time PCR.

***Key Terms:*** Dengue virus, apoptosis, and TNF-α.

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***Problem Statement***

Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) result from a viral infection of the dengue virus (DENV). Now considered one of the most prominent arthropod-borne infectious viruses, transmitted by *Aedes* mosquitoes, the dengue epidemic has spread to all parts of the world such as Africa, South Asia, South America, and most recently there has been an outbreak in Key West, Florida (Yen 2008). Dengue virus is a ssRNA virus that pertains to the *Flaviviridae* family, genus *Flavivirus.* The viral genome contains a single open reading frame (ORF) which encodes for three structural proteins: core (C), membrane (M), and envelope (E) proteins, with seven nonstructural proteins (Nasirudeen 2009). These proteins contribute to receptor mediated endocytosis (uptake of the virus into the cell) and the replication and production of new viruses once inside the host cell.

Dengue has four different viral strains (DENV-1, DENV-2, DENV-3, and DENV-4) which enable the virus to undergo antibody- dependent enhancement (ADE). There are four antigenically different serotypes for each strain which allow the body to produce lifelong immunity to that particular strain via antibody response (Brown 2009). However, once infected with a second strain, the antibody produced previously enhances viral infection in the host cell, giving rise to life-threatening DHF/DSS. Once dissimilation of the virus takes place in the host, it is believed that DENV contributes to plasma leakage and bleeding tendencies which may play a role in the immunopathogenesis of dengue virus (Rothman 1999). A mechanism proposed to elucidate this immune response is activated by pro-apoptotic gene expression in DENV induced apoptosis of endothelial cells. However, it is currently not known if the virus, the host immune system, or the interaction of both the host and the virus is responsible for this response (Chen 2007)

Dengue virus pathogenesis is not very well understood due to the inability of *in vivo* studies. Most knowledge about the body immunological response to the virus is acquired from i*n vitro* testing of infected cells from patients that have undergone viral infection. It has been hypothesized that programmed cell death of infected host endothelial cells contributes to vascular damage due to increased vascular permeability which contributes to sever hemorrhage of DHF/DSS (Yen 2008). More specifically, DENV- induced apoptosis has shown to play a role in the viruses overall pathogenesis, but the exact mechanism is unknown. Apoptosis triggered by anti-body enhanced DENV was also found to be caspase-dependent (Brown 2009). In recent studies, p53- dependent apoptosis has shown upregulation in pro-apoptotic genes such as caspase-1 which plays a role in production of cytokines such as tumor necrosis factor- alpha (TNF-α) (Nasirudeen and Liu 2008). Cytokines are believed to play a role in intracellular communication, notifying neighboring cells of cell death and therefore contributing to the global response of the immune system.

***Research Objective***

Since limited information is available on DENV-induced apoptosis and whether or not it contributes to the increase of pathogenesis of viral infection, this study will be conducted to better understand the role of pro-apoptotic gene expression of tumor necrosis factor- alpha (TNF-α) when presented to host endothelial cells. *In vitro* testing of the apoptotic endothelial cells real- time PCR analysis and TdT- mediated dUTP nick- end labeling (TUNEL) assays will identify apoptotic cells and determine whether TNF-α induces apoptosis in host cells infected with DENV versus uninfected cells. TNF-α exposure to endothelial cells is hypothesized to promote apoptosis of infected endothelial cells.

***Methods***

*Cell Culture*

Human umbilical vein vascular endothelial cell line (HUV-EC) will be obtained from the American Type Culture Collection (ATCC CRL-1730), Manassas, VA. HUV-ECs will be grown in an ATCC-formulated medium (F-12K Medium), (ATCC 30-2004). To make the complete growth medium, 0.1 mg/ml heparin, 0.03-0.05 mg/ml endothelial cell growth supplement (ECGS), and 100 U/mL penicillin will be added; supplemented to a final concentration of 10% fetal bovine serum, according to the propagation instruction provided by ATCC. The cells will be incubated at 37oC in humidified air containing 5% CO2 for storage.  
*Preparation of virus stock*

Dengue type 2 viral strain 16681 will be propagated in C6/36 mosquito cell line and cultured in Medium 199 prior to infection, provided by MyBioSource. Once received, addition of new media will be added and the HUV-ECs will be harvested for a 5 day period, cell debris will be removed by centrifugation, and the virus supernatant will be aliquoted and stored at -70oC until used. Virus will be titrated using the methods provide by Espina (2003). Cells will be seeded at a concentration of 1x106 in24-well plates and then incubated at 37oC for a period of 5 -7 days. Plaques will be visualized by staining with 1% crystal violet at performed by Espina (2003).

*Infection of endothelial cells*

Monolayers of adhered endothelial cells will be trypsinized and resuspended in growth medium. Virus culture fluid will be added to three 24-well plates containing the HUV-EC at a final concentration of 4x104 PFU/mL and incubated at 37oC in an atmosphere of 5% CO2 during various time intervals of one, two, and three days. Uninfected endothelial cells will serve as a control. Endothelial cells with only the virus present will also serve as a control.

*Introduction of TNF-α*

Human TNF-α recombinant protein provided by eBioscience (14-8329-63) will be introduced by micropipette technique at quantities of 5 µg during each of the time intervals described previously. After exposed to TNF-α, the cells will be tested for the presence of apoptotic cells in time intervals of 30 minutes, 1 hour, 2 hours, and 5 hours. Endothelial cells presented with the TNF-α, but without the virus, will serve as a control.

*Terminal deoxynuceotidyl transferase- mediated dUTP nick-end labeling (TUNEL) assay*

TUNEL assays will be performed on the endothelial cells to detect the presence of DNA strand breaks by the binding of deoxynucleotidyl trasferase to the 3’ OH ends of the DNA. The TUNEL assay provided by Gavrieli’s commercial kit will be followed according to the manufacturer’s protocol. The cells will be visualized under the fluorescent microscope for determination of apoptosis. Apoptotic cells will be counted.

*Detection of TNF-α by reverse transcription real time PCR*

To detect the presence of the TNF-α in the apoptotic endothelial cells, which will determine whether the cause of apoptosis is because of the cytokine, PCR will be conducted. RNA will be extracted from the cells and will be reverse transcribed to cDNA. The TNF-α will be amplified with techniques performed by Chen (2007) in which Real-Time PCR detection is used. The sequences of TNF-α primers are 5’-ATCCGCCTGGAGTTCTGGAA-3’ will be used to identify TNF-α.

*Detection of DENV through nucleic acid sequenced- based amplification (NASBA)*

Nucleic acid from DENV will be isolated and amplified as performed by the manufacturer’s instructions (Organon Teknika). Nucleic acid detection on the apoptotic cells will be performed by incubation of the amplification products and a dengue virus- specified probe then analyzed through gel electrophoresis as performed by Usawattanakul (2002).

*Statistical Analysis*

The difference between the experimental group and the control will be analyzed using analysis of variance. Statistical significance will be considered by *P*-values < 0.05. The Statistical Package for the Social Sciences will be employed.

***Timeline***

January 1, 2011

* Endothelial cells received and seeded, new medium will be prepared, and supplementary nutrients will be added. Incubation until infection with virus.
* Dengue-2 virus received, new medium will be added, and cells will be harvested for 5 day period
* Recombinant TNF-α received and preserved.

January 6, 2011

* Cell debris will be removed by centrifugation and the virus supernatant will be aliquoted and stored at -70oC until added to endothelial cell culture.

January 8, 2011

* Virus will be titrated. Cells will be incubated for 5-7 days.

January 15, 2011

* Infection of endothelial cells with viral culture fluid.
* Observation of cells every 1, 2, and 3 days.
* Each day observed, TNF-α will be added. Once added, testing of apoptosis and of the presence of TNF-α and DENV will be conducted in the given time frames of 30 minutes, 1 hour, 2 hours, and 5 hours.

January 20, 2011

* Data will be processed and analyzed.

***Significance of Expected Results***

Dengue research conducted for the understanding of the pathogenesis of the virus is crucial because little is known about the mechanism of how DENV infects its hosts. Deciphering the relationship between the virus and the immunological response of the host is key in understanding how DENV pathogenesis occurs in response to the presence of cytokines such as TNF-α. Programmed cell death or apoptosis of neighboring cells may be caused by an indirect relationship between virus- infected cells and TNF-α, therefore contributing to the spread of the virus. On the other hand, apoptosis of virus- infected cells may be due to a direct relationship between the virus and the host immune system. Expanding on the information already provided about the relationship between the host and the virus will potentially affect the rate of which a vaccine is produced to combat the virus.

***Equipment***

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| Laboratory | Facility will be provided by Florida Gulf Coast University. |
| Basic laboratory materials | Pipettes (1mL, 5mL, 10mL, and 25mL)  Micropipette Tips  Micropipetter  15mL Conical Vials  T25 Flasks  Disposable Waste Container  Plastic Gloves  Lysol Disinfectant  70% Ethanol Spray Bottle  All of these materials are needed for proper, sanitary cell sub- culturing technique. |
| Electric pipette aid | Pipette is need for easy extraction of virus and nutrients from one flask to the next. |
| Fluorescent microscope | Viewing one’s culture is essential for knowledge and evaluation of the experiment performed. |
| Centrifuge | Centrifuge is need for the collection of the virus or cells without the media containing the waste. |
| Incubator | Cells are grown and maintained in an incubator at an optimal temperature of 37°C and a pH of 7.4.  The temperature inside the incubator is usually  controlled by water and the pH is controlled by a CO2 buffering system which controls the amount gas available. |
| Biohood | All laboratory experiments requiring the use of media preparation need to be conducted in the bio hood. The hood contains a air flow system and a ultraviolet light that prevents contamination. |
| PCR | PCR machine is necessary for DNA analysis. |

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