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Project Information

Title of Project: Stimulation with Ebola VLPs containing and lacking RIG-I	
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Faculty Sponsor: Osvaldo Martinez	Faculty Department: Biology
Was this a capstone, senior thesis, or other degree culminating project? Yes	

Project Abstract

What was the purpose of this research? What were the planned outcomes? What did you do to achieve them? What were the actual outcomes?

Abstract: Ebola virus (EBOV), a filovirus family member, is a highly pathogenic virus that causes Ebola hemorrhagic fever (EHF) resulting in documented mortality rates in humans as high as 50%. Currently, the basic EBOV virus-like particle (VLP) vaccine contains the Ebola virus (EBOV) matrix VP40 and attachment glycoprotein (GP). VLPs are morphologically and biochemically similar to parental virus, yet because they lack a genome and cannot replicate, are safe enough to be used as vaccines. We hypothesize that the addition of a constitutionally active retinoic acid-inducible gene 1 (RIG-I) would enhance the ability of the vaccine to induce interferon-dependent immune functions yielding an improved vaccine. Expression of EBOV VP40 in 293T cells induces the spontaneous production of VLPs into the media supernatant and if expressed with EBOV GP, will produce VLPs studded with the attachment GP. Recombinant chimeric constitutively active (ca)RIG-I-VP40 matrix and a nonfunctional mutant L58A (mu)RIG-I-VP40 matrix genes were constructed to produce VLPs containing constitutively active and

nonfunctional RIG-I. Supernatant from 293Ts transfected with caRIG-I-VP40, muRIG-I-VP40 or VP40 along with GP expression plasmids were tested for the presence of VLPs. Western blotting of purified VLPs confirmed the presence of RIG-I in caRIG-I-VP40 and muRIG-I-VP40, but not VP40 containing VLPs. Monocyte-like and PMA-differentiated macrophage-like THP-1 Dual cells were treated with nothing, VP40+GP, caRIG-I-VP40+GP, muRIG-I-VP40+GP VLPs as well as LPS and a Vaccinia virus (VACV-70) positive controls and tested for induction of interferon (IFN) signaling. CaRIG-I containing, but not muRIG-I containing VLPs induced interferon signaling from both macrophages and monocytes. In this study we tested whether the addition of RIG-I to EBOV VLPs can activate human peripheral blood monocytes and found that RIG-I containing VLPs induce interferons from human blood monocytes.

Addition of retinoic acid-inducible gene 1 to enhance Ebola-like particle vaccine

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Abstract

Ebola virus (EBOV), a filovirus family member, is a highly pathogenic virus that causes Ebola hemorrhagic fever (EHF) resulting in documented mortality rates in humans as high as 50%. Currently, the basic EBOV virus-like particle (VLP) vaccine contains the Ebola virus (EBOV) matrix VP40 and attachment glycoprotein (GP). VLPs are morphologically and biochemically similar to parental virus, yet because they lack a genome and cannot replicate, are safe enough to be used as vaccines. We hypothesize that the addition of a constitutionally active retinoic acid-inducible gene 1 (RIG-I) would enhance the ability of the vaccine to induce interferon-dependent immune functions yielding an improved vaccine. Expression of EBOV VP40 in 293T cells induces the spontaneous production of VLPs into the media supernatant and if expressed with EBOV GP, will produce VLPs studded with the attachment GP. Recombinant chimeric constitutively active (ca)RIG-I-VP40 matrix and a nonfunctional mutant L58A (mu)RIG-I-VP40 matrix genes were constructed to produce VLPs containing constitutively active and nonfunctional RIG-I. Supernatant from 293Ts transfected with caRIG-I-VP40, muRIG-I-VP40 or VP40 along with GP expression plasmids were tested for the presence of VLPs. Western blotting of purified VLPs confirmed the presence of RIG-I in caRIG-I-VP40 and muRIG-I-VP40, but not VP40 containing VLPs. Monocyte-like and PMA-differentiated macrophage-like THP-1 Dual cells were treated with nothing, VP40+GP, caRIG-I-VP40+GP, muRIG-I-VP40+GP VLPs as well as LPS and a Vaccinia virus (VACV-70) positive controls and tested for induction of interferon (IFN) signaling. CaRIG-I containing, but not muRIG-I containing VLPs induced interferon signaling from both macrophages and monocytes. In this study we tested whether the addition of RIG-I to EBOV VLPs can activate human peripheral blood monocytes and found that RIG-I containing VLPs induce interferons from human blood monocytes.

Introduction

Ebola viruses (EBOVs) are ssRNA viruses that belong to the family *Filoviridae.* EBOVs can cause Ebola hemorrhagic fever (EHF) that may lead to death within the second week after the appearance of symptoms in infected humans (Manhanty and Bray, 2004). EHF is associated with deregulation of a cellular and humoral immune response. Hence, infection with EBOV can lead to the development of an inadequate immune response that results in unrestricted viral replication leading to EHF in humans and non-human primates. EHF results in high morbidity and mortality rates that varies 25-90% (Kadanali and Karagoz, 2015).

Virus-like particles (VLPs) are biochemically and morphologically similar to a parental virus yet are unable to replicate, because they lack a genome. Ebola virus-like particles (eVLPs) are produced by co-expressing EBOV VP40 protein and glycoprotein (GP) protein. The EBOV GP is the sole protein expressed on the surface of the virus and is essential in the attachment of EBOV to host cells. Whereas, VP40 is essential for the assembly and budding of new viral particles (Panchal et al., 2003. Expression of VP40 in 293T cells induces the formation of VLPs and if co-expressed with EBOV GP then the produced VLPs will be studded with the GP attachment protein. Since VLPs cannot replicate, they are safe to be used as a vaccine (Chroboczek et al, 2014).

Vaccines represent an important method to control the spread of infectious diseases. It is a biological preparation that induces active acquired immunity against a particular pathogen. VLPs can be used as a vaccine because they are safe, immunogenic, and express the same proteins as the parental pathogenic virus. VLPs contain a repetitive display of viral surface proteins that present B-cell and T-cell epitopes that trigger humoral and cellular immune responses, respectively. Antigen-presenting cells like Dendritic cells take up EBOV VLPs

presenting the antigen to T cells and B cells eventually leading to immune responses against EBOV antigens. EBOV VLPs have been used to vaccinate and protect animals. For example, a study showed that Kunjin virus replicon-based vaccines expressing EBOV GP protect against subsequent lethal EBOV infection in guinea pigs (Reynard et al, 2011).

VLPs can be manipulated to induce a stronger immune response. They can also be engineered to contain PRR ligands to act as an adjuvant. VLPs vaccines, in general, are not as immunogenic as an inactivated virus so we hypothesize that adding active retinoic acid-inducible gene-1(RIG-I), a PRR, to the VLPs will generate VLPs that are more immunogenic. For example, it has been shown that glucopyranosyl lipid adjuvant formulated in stable emulsion (GLA-SE) combined with EBOV VLPs confers lasting protection against EBOV in mice (Melek et al, 2019).

RIG-I, an intercellular pattern recognition receptor (PRR) detects pathogen-derived RNA and induces production of anti-viral interferon cytokines to induce antiviral immunity. The N-terminal Caspase Activation and Recruitment Domains (CARDs) of RIG-I induces signaling that leads to the production of interferon (Luo et al, 2014). The central RNA helicase core and a C-terminal domain function to sense viral RNA structure. The production of interferon helps activate both innate and adaptive immunity. Therefore, we attempted to enhance the immunogenicity of EBOV VLPS by adding RIG-I. Recombinant chimeric constitutively active (ca) RIG-I VP40 matrix and a less functional mutant L58A (mu) RIG-I-VP40 matrix genes were generated in order to produce VLPs containing active and less functional RIG-I.

In our study, six different combinations of expression plasmid vectors, including plasmids containing functional or less functional RIG-I were transfected into 293T cells to produce FLAG-VP40+GP, RIG-I-wt+GP, RIG-I-wt+GPF88A, Rig-I-Mut+GP. Four days after

the transfection, supernatant containing VLPs were harvested and VLPs isolated. Beta lactamase-VP40 expression vectors allowed the production of beta-lactamase-VP40 chimeric proteins in all VLP preparations. Spiking each VLP prep with a small amount of beta-lactamase-VP40 chimeric which incorporated into each VLP prep allowed us to quantitate the VLPs by testing for beta lactamase activity for each VLP preparation. All VLPs were quantified using a beta lactamase assay. The amount of VLPs is proportional to the amount of the enzyme in VLPs hence the amount of beta lactamase was measured.

We will be studying whether the addition of RIG-I to VLPs can induce more interferon production than VLPs that do not contain RIG-I by using an interferon reporter THP-1Dual cells as well as using an anti-viral state assay. Human blood monocytes were isolated and treated with following VLPs; FLAGVP40+GPwt, RIG-I-wt+GP, Rig-I-Mut+GP, RIG-I-wt+GP F88A as well as LPS and VACV-70 positive controls. The addition of RIG-I to the VLP is expected to activate the production of interferon from VP40-RIG-I+GP-VLP-treated human blood monocytes. If the supernatant has interferon, then monocyte supernatants stimulate THP1-Dual cells and then emit light. Hence, Luminescence reading was used to determine the amount of interferon signaling induced. The goal of this study is to measure and compare the amount of interferon produced form monocytes stimulated with VLPs containing and lacking RIG-I activity.

Methods and Materials

Virus-like particle production

Transfection of 293T cells

293T cells were plated in 100mm dishes (6.8. million cells per plate). For transfection of different expression vectors, solution A was prepared by adding 1M $CaCl_2$, DNA and sterile water and solution B was prepared by adding 300uL 2X Hepes Buffered Saline (HBS) *(Table 1 and 2).* Solution A was added slowly to solution B dropwise while bubbling air through the solution B by using another pipette. This process was continued for 2 minutes until solution A was depleted. After 30 minutes post-incubation a fine precipitate developed, and the precipitate was added dropwise to the cells.

VLP's were harvested 72 hours post-transfection. Since the VLPs are secreted into the media, media was harvested, plates were scraped, and PBS was used to wash cells to harvest any VLPs clinging to the cells. The media was centrifuged for 8 minutes at 1100 rpm to eliminate cells from the VLP prep. The supernatant obtained was added on top of 5 ml Sucrose in ultra-cent centrifuge tubes. VLPs in the supernatant were centrifuged at 100000g for 2 hours at 10^0C. The Supernatant was aspirated, the VLP pellet was washed with PBS and was re-centrifuged for 2 hours. After centrifuging, supernatant was aspirated out and 100uL of sodium Tris EDTA (100mM NaCl, 10 mM Tris, 1mM EDTA) was added to the pellet and stored on ice. The next day, the tube was gently resuspended, and pellets were transferred to new tubes. Enriched VLPS were stored in 4^0C for further use.

VLP Quantification

For VLP Quantification, PBS/Cytobuster (50% cytobuster in PBS) and 5uM fluorocilin (SIGMA) stocks were prepared. 50 ul of PBS/cytobuster and 5uM fluorocilin solution and 10 uL of VLPS were plated in 96 wells plate with clear bottoms and incubated at room temperature. The beta lactamase assay was used to measure the amount of beta lactamase present as all the VLPs were spiked with beta lactamase. The plate was read at a wavelength of 535 nm, 495 nm excitation, and medium shake for 5 seconds before the first read at each time interval. The fluorescence measurement was recorded for 20 minutes, 30 minutes and 3.5 hours post-incubating using SpectraMax i3x (Molecular Devices, San Jose, CA, USA) (Figure 1).

Isolation and Stimulation of Human Monocytes

Two anonymous blood samples were collected and diluted 1:2 with Hank's Buffered Saline solution (HBSS). Ficoll-Paque Plus was underlaid with diluted blood and was centrifuged for 30 minutes at 400 x g at 20^0C. The buffy layer was collected, resuspended in HBSS and centrifuges at 100 x g for 10 minutes. The pellet obtained was resuspended in 1X MojoSort Buffer. Human TruStain FcX™ was added and incubated for 10 Minutes at room temperature. Biotin-Antibody Cocktail containing antibodies against all lineages (for example T cells, B cells and NK cells) except against monocytes was then added and kept in ice for 15 minutes. The antibody coated cells were resuspended and Streptavidin Nanobeads (metal) were added and incubated on ice for another 15 minutes. Cells were washed with MojoSort buffer and centrifuged at 300 x g for 5 minutes. The supernatant was discarded, and the buffer was added. The solution was then kept on the MojoSort magnet for 5 Minutes and then supernatant was collect and stored in 96 well plate at 37 degrees Celsius with RPMI + 5% human serum.

Human monocytes were isolated and two separate individuals monocytes in 96 well plates were stimulated with VLPs; FLAGVP40+GPwt, RIG-I-wt+GP, Rig-I-Mut+GP, RIG-I-wt+GP F88A as well as LPS and VACV-70 positive controls by adding them in triplicate to the plate (Table 3). The plates were centrifuged for 1 hour at 2000 at 4 °C and then incubated at 37 C. The monocytes were treated for 48 hours and the supernatant was harvested. If the monocytes were stimulated by the treatments, then there should be interferon secreted in the supernatant. In order to test whether there was interferon in the supernatant, 20 microliters of the human monocyte supernatant were added to THP-1 Dual reporter cells which emit light when interferon stimulates them. The stimulation of THP-1 Dual cells by interferon in monocyte supernatants were analyzed by luminescence reading (Figure 2).

Table 1. Volumes and mass of DNA added to each plasmid preparation.

Plasmid	VP40		RIGI-Wt		RIGI M2-1		EBOV GP		GP F88A		Flag VP40		VSV-g	
	uL	ug	uL	ug	uL	ug	uL	ug	uL	ug	uL	ug	uL	ug
1	5.37	4	19.31	12			4.21	4						
2	5.37	4			21.12	12	4.21	4						
3	5.37	4	19.31	12			-		16.00	4				
4	5.37	4	19.31	12			-		16.00	4				
5	5.37	4					4.21	4			22.68	12		
6	13.43	10											23.81	10

Table 2. Volumes of solutions added

P.	$CaCl_2$	Sterile H_20	HBS
1	144	427	600
2	144	425	600
3	144	415	600
4	144	415	600
5	144	423	600
6	144	418	600

Table 3. Volumes of VLPs and controls added for monocyte stimulation with 1:1 eq Beta Lactamase

GPF88A #1	GPF88A #2	Mut RIG I	FLAG VP40	RIG-1 wt / VSV #1	RIG-1 wt / VSV #2	LPS control (1 ug/mL)	VacV control (100ug/mL)
20	19.1	11.5	5.94	14.6	16.61	1	7.5

Results

We made VLPs to test whether the RIG-I containing VLPs can induce more interferons from human cells than control VLPs. VLPs were successfully produced and quantified using a beta lactamase assay. Since all VLP preps were spiked with a small amount of VP40-beta lactamase which incorporates itself randomly into each VLP prep, the amount of beta lactamase activity is proportional to the amount of that VLP. The average relative beta lactamase units of 20 minutes, 30 minutes and 3 hours post-incubating of GPF88A#1 was 61234.17, GPF88A#2 was 61946.3, Mutant RIG-I was 89364.2, FLAG VP40 was 156293.5, RIG-I wild type/VSV #1 was 75285.2, and RIG-I wildtype/VSV #2 was 62441.2 (Figure 1). The results show that FLAG VP40 has higher average beta lactamase units (156293.5) than others. Hence, FLAG VP40 has higher beta lactamase activity. Using the beta lactamase units for each VLP prep all functional assays were performed using equal amounts of VLPs.

Once VLPS were successfully produced and quantified, two healthy human individual's monocytes were isolated and stimulated with VLPs; FLAGVP40+GPwt, RIG-I-wt+GP, Rig-I-Mut+GP, RIG-I-wt+GP F88A as well as LPS and VACV-70 positive controls by adding them in triplicate to a 96 well plate for 48 hours. If the monocytes are stimulated by the treatment, then the monocytes should produce interferon into the supernatant. The supernatant was then added to THP-1 Dual reporter cells to test for the presence of interferons. If the supernatant has interferon, then the reporter cells would be stimulated (interferon signaling) and then emit light. Therefore, the amount of light emitted is proportional to the amount of interferon signaling induced. The luminescence reading shows the amount of light emitted from individual wells of a plate.

The luminescence readings from monocyte 1 treated with GPF88A#1 was 454.66, GPF88A#2 was 390, mutant RIG-I was 474.33, FLAG VP40 was 481, VSV-g-VLPs was 419,

and RIG-I containing VLPs was 1673.3 units (Figure 2). LPS and VACV were used as positive controls and their luminescence reading were 538.6 and 598.5 units respectively (Figure 2). Whereas, THP-1 Dual reporter cells alone and a blank (nothing) were used as negative controls and their luminescence reading were 483.6 and 416.l units respectively. Figure 2 shows that the monocytes treated with RIG-I containing VLPs (RIG-Iwt+VP40+GP) has at least double luminescence units compared to GPF88A#1(VP40+RIG-I-wt+GP F88A), GPF88A#2(VP40+RIG-I-wt+GP F88A), Mut Rig-I(VP40+RIG-IM2-1+GP), FLAG VP40(VP40+FLAGVP0), VSV-g-VLPs(VP40+VSV-g) and negative controls(blank and THP Dual cells only). Not only that, RIG-I containing VLPs shows higher luminescence reading (1673.3) than LPS (538.6) and VACV-70 (598.5) positive controls too.

The luminescence readings from monocyte 2 treated with GPF88A#1 was 478, GPF88A#2 was 509, mutant RIG-I was 719.33, FLAG VP40 was 499, VSV-g-VLPs was 583, and RIG-I containing VLPs was 1179 units (Figure 2). LPS and VACV positive controls showed 586.6 and 415 luminescence units respectively. Whereas, the negative control showed 387 units. Hence, it indicates that RIG-I containing VLPs (RIG-Iwt+GP) shows more amount of interferon stimulation in comparison to GPF88A#1, GFF88A#2, Mut Rig-I, FLAG VP40, VSV-g-VLPs and negative controls.

Discussion and Conclusion

EBOV VLPs were successfully produced by transfecting 293T cells. The beta lactamase assay was used to quantify the amount of VLPs produced. Since FLAG VP40 has higher beta lactamase units (156293.5), that is at least double the amount in comparison to GPF88A#1(61234.17), GPF88A#2 **(**61946.3), Mutant RIG-I (89364.2), RIG-I wild type/VSV #1 (75285.2), and RIG-I wildtype/VSV #2 (62441.2), it has more beta lactamase activity hence more VLPs production. Studies have shown that the plasmids that express VP40 and GP proteins coordinate to produce VLPs for budding and release efficiently (Jonathan J M et al, 2015). Our results support the fact that VP40 helps in making VLPs, but we also interpreted that the addition of RIG-I to VP40 may decrease the efficiency of VLP production since all of our samples containing VP40-RIG-I chimeric protein showed less VLPs production except FLAG VP40 which lacks RIG-I. Furthermore, the FLAG protein is only 8 amino acids long.

Two healthy human individual's monocytes were isolated and treated with VLPs; FLAGVP40+GPwt, RIG-I-wt+GP, Rig-I-Mut+GP, RIG-I-wt+GP F88A as well as LPS and VACV-70 positive controls, and the interferon production was compared by using interferon assay. RIG-I containing VLPs(RIG-I-wt+GP) showed more luminescence readings (1673.3 units) in comparison to GPF88A#1 (454.66 units), GPF88A#2 (390 units), mutant RIG-I (474.33 units), FLAG VP40 (481units), VSV-g-VLPs (419 units) and negative control(483.6). This result is consistent with RIG-I containing VLPs that produce more interferons and are relatively more immunogenic. Moreover, RIG-I containing VLPs showed more luminescence units than positive controls, Lipopolysaccharides (538.6) and VACV-70(598.5). LPS and VACV-70 were used as positive controls as they stimulate other immune cells, but our data

shows that the error bars overlap between the negative control and positive controls which means that monocyte 1 and monocyte 2 did not respond to the treatments with controls. Although these stimulants work well with other immune cells, we have not tested them against untouched human monocytes. Hence, we may interpret that VACV and LPS do not stimulate untouched human monocytes. Not only that, luminescence readings of negative controls are similar to GPF88A#1, GPF88A#2, Mut Rig-I, FLAG VP40, and VSV-g-VLPs indicating no/less interferon production. However, GPF88A (RIG-I-wt+GP F88A) #1 and #2, also have functional RIG-I but they did not induce monocytes. It is because they lack functional EBOV GP, a structural protein, which is essential for infection and spread of EBOV, as it aids in attachment and coordination of the viral entry into host cells (Jonathan J M et al, 2015). GPF88A is rather a mutated GP at position 88 from amino acid F to A and is unable to enter the cell as it lacks functional EBOV GP.

In addition, RIG-I is a PRR localized in the cytoplasm that detects viral RNA so if VLP is unable to enter the cell then RIG-I cannot be activated to make interferons. Therefore, GPF88A is a good control to prove that it is important for VLP to enter the cell. Our study suggests that addition of RIG-I to EBOV VLP (VP40, RIGI-wt+EBOV GP) induces monocytes, leading to interferon signaling and probably an enhanced immune response. Since our results show that RIG-I-containing VLPs(VP40, RIGI-wt+EBOV GP)) induce monocytes to produce interferon, other assays like ELISA and Flow Cytometry can also be used to compare and further prove that the RIG-I containing VLPs produce statistically significant amount of interferon in comparison to negative control. Our study suggests that adding RIG-I to VLPs may allow them to induce interferon from targeted human cells.

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Figures

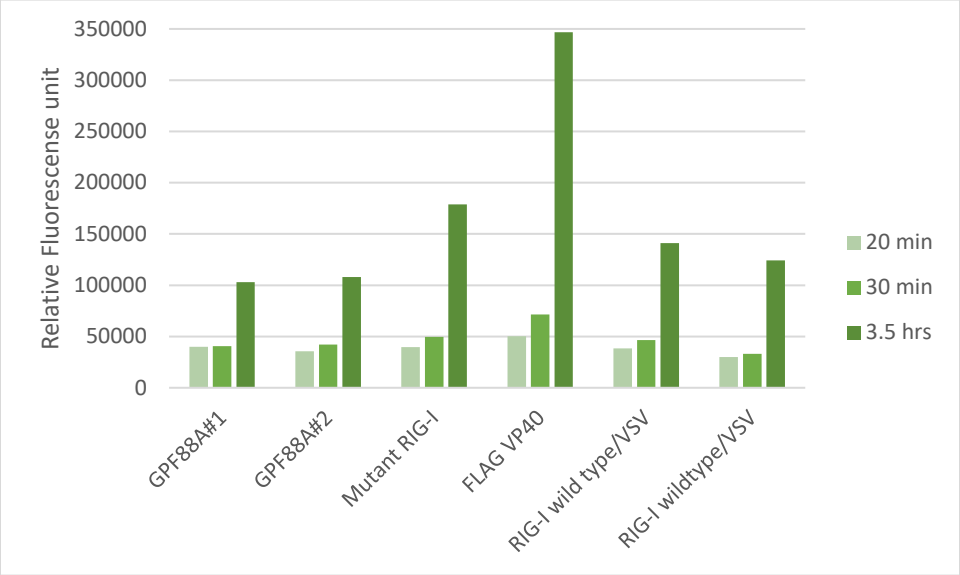

Figure 1: VLPs quantification using Beta-lactamase Assay and relative fluorescence.

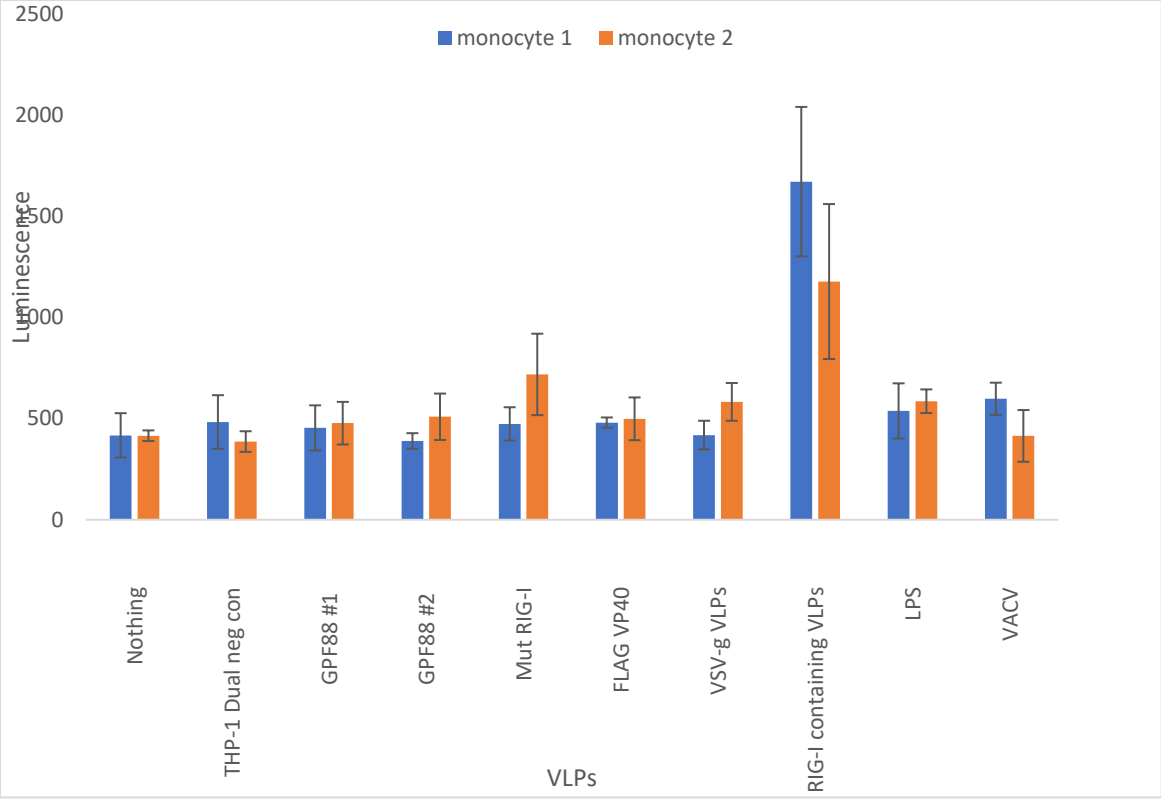

Figure 2: Luminescence reading representing the stimulation of THP-1 Dual IFN reporter cells using monocyte supernatants.

Figure Legends

Figure 1. VLPs Quantification using Beta-Lactamase Assay. VLP preps, FLAGVP40+GPwt, RIG-I-wt+GP, Rig-I-Mut+GP, RIG-I-wt+GP F88A, and VSV-g, and PBS/cytobuster-fluorocin solution were plated in black microplate with clear bottom. The plate was read at a wavelength of 535 nm, 495 nm excitation, and medium shake for 5 minutes and the fluorescence spectra was taken at 20 minutes, 30 minutes and 3.5 hours post-incubation at room temperature to visualize VLPs quantification using SpectraMax i3x (Molecular Devices, San Jose, CA, USA). The relative fluorescence represents the beta lactamase activity. Since all the VLP preparations included VP40-betalactamase to facilitate the reaction with fluorocilin, the beta lactamase activity in the VLP represents the amount of that VLP.

Figure 2. Stimulation of THP-1 Dual IFN reporter cells using monocyte supernatants. Two healthy human individual's monocytes were isolated and treated with VLPs; FLAGVP40+GPwt, RIG-I-wt+GP, Rig-I-Mut+GP, RIG-I-wt+GP F88A, THP Dual cells only (neg control), nothing, as well as LPS and VACV-70 positive controls. In order to test the production of interferons, the human monocyte supernatant was added to THP reporter Dual cells which emit light when interferon stimulates them. The graph shows the luminescence reading that represents the stimulation of THP-Dual cells.