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The Effects of Cell-Derived Extracellular Vesicles on the Innate Immune System

Madeline Bruhn

The Effects of Cell-Derived Extracellular Vesicles on the Innate Immune System

by

Madeline Bruhn

Under the Direction of Baozhong Wang, Ph.D.

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ABSTRACT

Influenza remains a major global health issue, demanding the need for more effective vaccine strategies. This thesis searches into a novel approach, employing cell-derived extracellular vesicles (EVs) to boost influenza vaccine effectiveness. It dives into the impact of EVs on the innate immune system and their role on a cellular basis. Using cell culture and in vitro immune assays, the composition and immunomodulatory traits of EVs from antigen-presenting cells are researched. The study systematically examines the interactions between these EVs and key components of the innate immune system, unveiling the mechanisms driving their immunostimulatory effects. This research yields crucial insights into EVs' potential as immunomodulators in influenza vaccines. Additionally, determining insights into the identification and validation of adjuvants that enhance EV-mediated immune responses marks a pivotal stride toward the advancement of novel influenza vaccines, increasing their efficacy and protection against diverse viral strains.

INDEX WORDS: Influenza vaccine, extracellular vesicles, adjuvants, innate immune system, immunomodulation, antigen-presenting cells

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by

Madeline Bruhn

Committee Chair: Dr. Baozhong Wang

Committee: Dr. Lanying Du

Electronic Version Approved:

Office of Academic Assistance – Graduate Programs

Institute for Biomedical Sciences

Georgia State University

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DEDICATION

This thesis is dedicated to my friends and family that have shown me much support throughout this process.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..... **V**

LIST OF FIGURES ..... **VIII**

1.INTRODUCTION **1**

2.METHODS **5**

2.1 Cell Culture **5**

2.2 EV Characterization and Conjugation **5**

2.3 Stimulating Cells **6**

2.3.1 JAWSii ***6***

2.3.2 RAW264.7 ..... ***6***

2.4 Collecting Samples for FACS and ELISA **7**

2.5 ELISA **7**

2.5.1 Capture antibody ***7***

2.5.2 Blocking ***8***

2.5.3 Standards and Samples ***8***

2.5.4 Detection Antibody ***8***

2.5.5 Avidin-Horseradish Peroxidase (Av-HRP) ***8***

2.5.6 Substrate ***9***

2.6 Flow Cytometry **9**

3. RESULTS **9**

3.1 JAWSii ELISA and FACS **9**

3.2 RAW264.7 ELISA and FACS 11

4. DISCUSSION 14

5. CONCLUSION 15

6. FUTURE DIRECTIONS 15

REFERENCES 17

VITAE 20

LIST OF FIGURES

Figure 1. The History of Influenza Viruses A and B. 2

Figure 2. Exosome Budding and Structure 4

Figure 3. EV Characterization and HA3 Conjugation 6

Figure 4. JAWSii FACS Data 10

Figure 5. JAWSii Cytokine ELISA Data 9

Figure 6. RAW264.7 ELISA Data 11

Figure 7. RAW264.7 FACS Data 12

Figure 8. RAW264.7 FACS gated F4/80+ CD86+ 13

1. INTRODUCTION

Influenza is a powerful virus that has history of pandemic virality. Throughout time, there have been outbreaks that have caused millions of deaths from the Spanish flu of 1918 to the Swine flu outbreak of 2009 (1). This virus is rapidly changing and therefore an international health concern. Thankfully, there are vaccines available to prevent transmission and infection, however, due to the rapid mutation rate of the virus, the vaccines are not always the most effective. Vaccine effectivity is a major issue for researchers worldwide in their attempts to develop solutions to viruses. Influenza is a spherical virus of four different types, A,B,C, and D, comprised of proteins and RNA. Influenza A and B are the types of Influenza that can affect humans with seasonal epidemics (3). Of these proteins, there are two glycoproteins called neuraminidase (N) and haemagglutinin (H). There are 18 different H types and 11 N types that can be comprised to form the specific Influenza virus subtype, i.e. H1N1 (3). Commercial Influenza vaccines are different every year to accommodate for antigenic shift and changing viruses. The FDA's Vaccines and Related Biological Products Advisory Committee (VRBPAC) makes the final decision on what specific strain and components to target with the seasonal vaccine after meeting with the World Health Organization examining global data and using prediction data analysis to predict which strains will be the most prevalent (4).

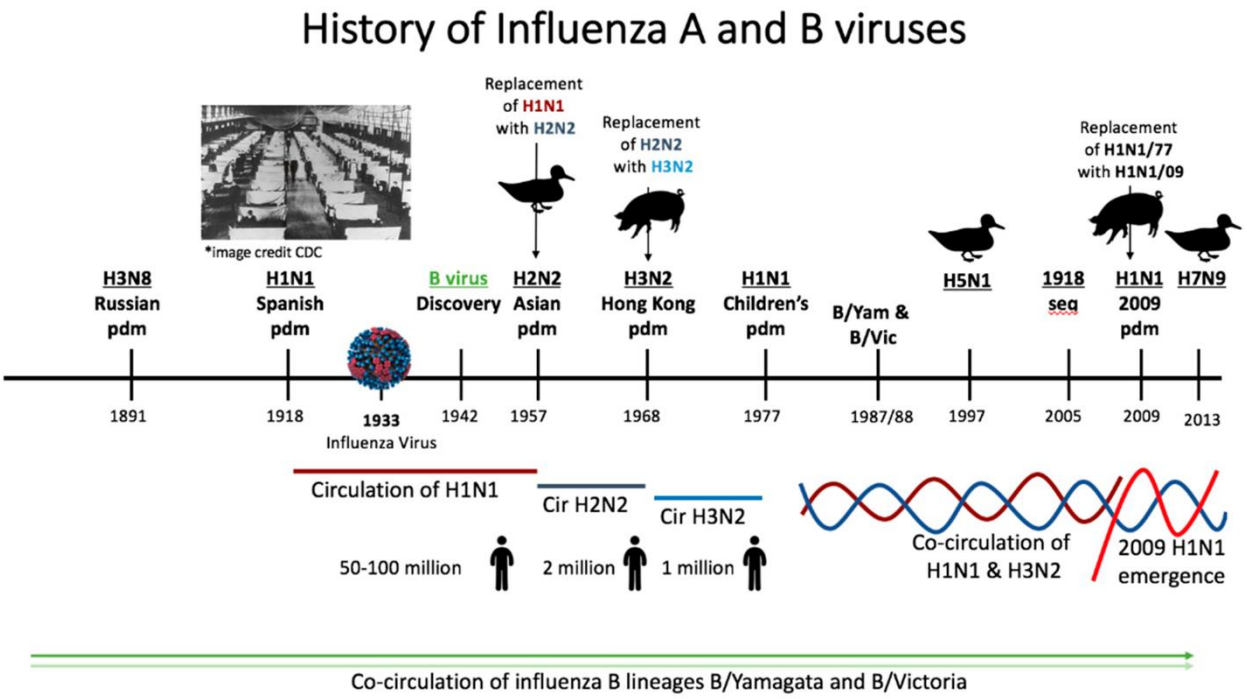

Figure 1. The History of Influenza Viruses A and B.
This timeline shows the major Influenza A and B pandemics (17). The Spanish H1N1 is the first known Influenza pandemic that has affected humans. The chart shows the different combinations of Hemagglutinin and Neuraminidase that have made history of being detrimental and have formed the early years of research that establishes how Influenza can recombine and mutate over the years.

One of the reasons the vaccines are not the most effective in comparison to other viral vaccines is due to these preseason predictions not accurately predicting the most prevalent strain. While this may not change anytime soon, there are other ways to increase vaccine effectivity. One of these being adding adjuvants to vaccines. An adjuvant is an additive to a vaccine that works to elicit a more intense immune response to the body (5). These can be composed of organic or inorganic matter based on the potential vaccine's proposed composition and function. Some of the most used adjuvants in FDA approved vaccines are mineral salts Aluminum Hydroxide and Aluminum Phosphate (6). However, these are not the only types of adjuvants; they can be made from microparticles, emulsions, and other particles

(7). Each style of adjuvant then has a different pathway or function to how they can increase a vaccine's efficiency. However, the mechanisms of action still need research into how exactly they function and elicit stronger immune responses. There are a few known explanations to their function which include stronger localized immune responses by "cellular recruitment" at vaccine locations, increasing cytokines and chemokines regulation, and increased APC function in the innate immune system (5).

The innate immune system is made up of many different types of cells that act as the first line of defense for the body by physical and chemical barriers. Comprised of physical barriers such as skin and mucous and cellular receptors and responses such as white blood cells and inflammatory cytokines (8). The physical barrier of the skin and mucosal layers prevents foreign substances from entering the body and causing harm. The blood-brain barrier is another example of this type of physical barrier defense by specializing the entrance between the two to specific immune cells and mediators instead of an open flow into the central nervous system (9). Antigen presenting cells (APCs) are the link between the innate immune system and the adaptive immune system that function by presenting antigens to T-lymphocytes (10). The first type of APC is dendritic cells (DCs). These cells function by presenting the processed antigens to the adaptive immune system by triggering B and T lymphocyte reactions (11). They uptake the viral antigen, process it, and then based of that, they prime a T cell to react to that antigen. Another type of APC is a macrophage that phagocytizes damaged tissue and foreign objects designated for erasure along with secreting cytokines after inflammatory stimulation (12). Macrophages are important for breaking down these infected particles and inducing these inflammatory cytokines to stimulate the adaptive immune response. Finally, B-lymphocytes are

a type of APC that function in communication with CD4+ and CD8+ cells to elicit an immune response (13). These three types of APCs work in series in the innate immune system to immediately respond to pathogens and prevent their function. They communicate with the adaptive immune system if any additional immune response is needed after the initial layer of defense.

Extracellular vesicles (EVs) function in cell-to-cell communication and can make a good target for delivery of items into and out of cells. They can communicate through interactions between cell surface receptors along with intracellular delivery (14). Inflammatory processes in the innate immune system can be linked to EVs by their secretion from innate immune cells (15). Their ability to deliver antigens directly to a target cell makes them valuable in potential vaccine and drug development. This type of specialized delivery would make their use as an adjuvant relevant for future work. Manipulation of EVs for use as adjuvants is emerging as a popular research topic as researchers are beginning to understand the mechanisms behind the pathways and how to manipulate them into directly completing their intended task.

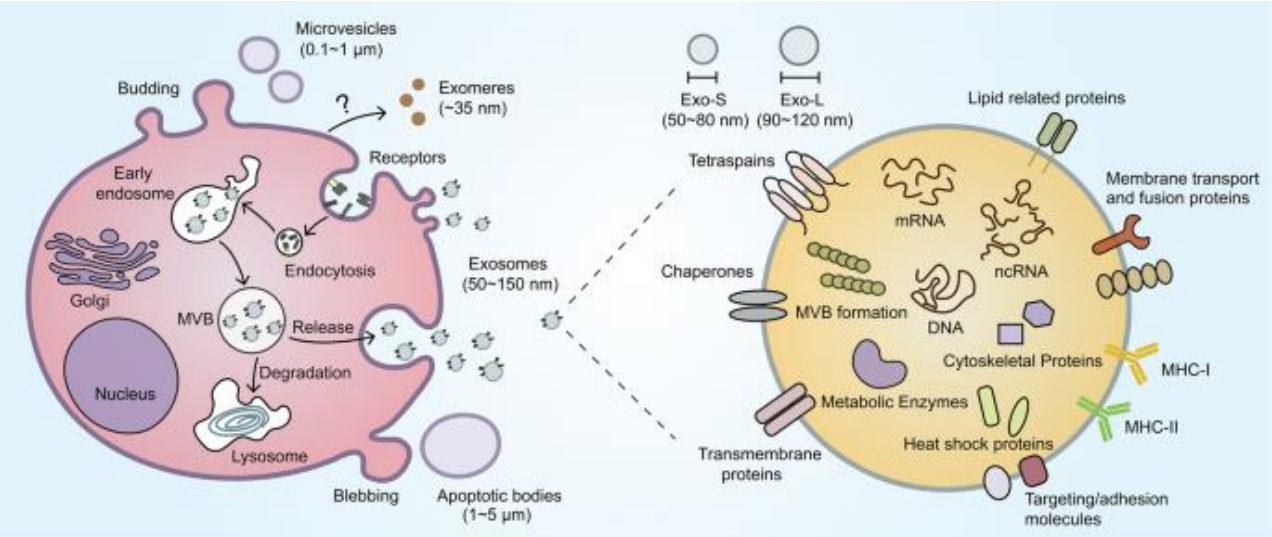

Figure 2. Exosome Budding and Structure
Exosomes, a type of Extracellular Vesicles, are shown here participating in cellular communication through endocytosis, modification, and release (left). Structure of endosome is also displayed on the right hand side (18).

2.METHODS

2.1 Cell Culture

Cell lines JAWSii and RAW264.7 were chosen for cell cultures. JAWSii is a murine immortalized dendritic cell line derived from bone marrow. These cryopreserved cells were recovered following ThermoFisher Scientific's guidelines. Once recovered, the cells are resuspended in culture plates with a complete medium comprised of 4-5 ml DMEM, 5ng/ml murine GM-CSF, 20% Fetal Bovine Serum, 10 U/ml penicillin, and 100 µg/ml streptomycin at one million cells per plate. JAWSii cells were then seeded onto non-tissue culture treated in 24-well plates at 500,000 cells/well density in 1 ml complete medium and medium was changed every 2-3 days until 70-80% confluence was reached. After this, they were passaged by discarding culture medium, rinsing with 5ml PBS, incubating with 1ml trypsin-EDTA for 3-5 minutes to detach cells, neutralizing the trypsin-EDTA with 4 ml complete medium and transferring to T75 flask. This process is repeated until cells are mature. RAW264.7 cells were cultured in the same manner but with their optimal conditions: no murine GM-CSF and the complete medium was alpha-MEM rather than DMEM. RAW264.7 is a macrophage cell line immortalized from an Abelson leukemia virus tumor in BALB/c mice.

2.2 EV Characterization and Conjugation

EVs purified from 293T cells were gifted from mentor, Dr. Wandi Zhu. 293T cells were cultured in a complete medium and then ultracentrifuged for 500gX10min, 5000gX30min, and then 27800rpmX2h. The culture supernatant and tested using dynamic light scattering (DLS) to confirm EV's presence. Images are taken using confocal microscopy to further confirm the

presence. Influenza-derived Aichi HA3 were conjugated on the surface of these EVs by incubation and then western blot using EV cell surface markers TSG101 and Alix.

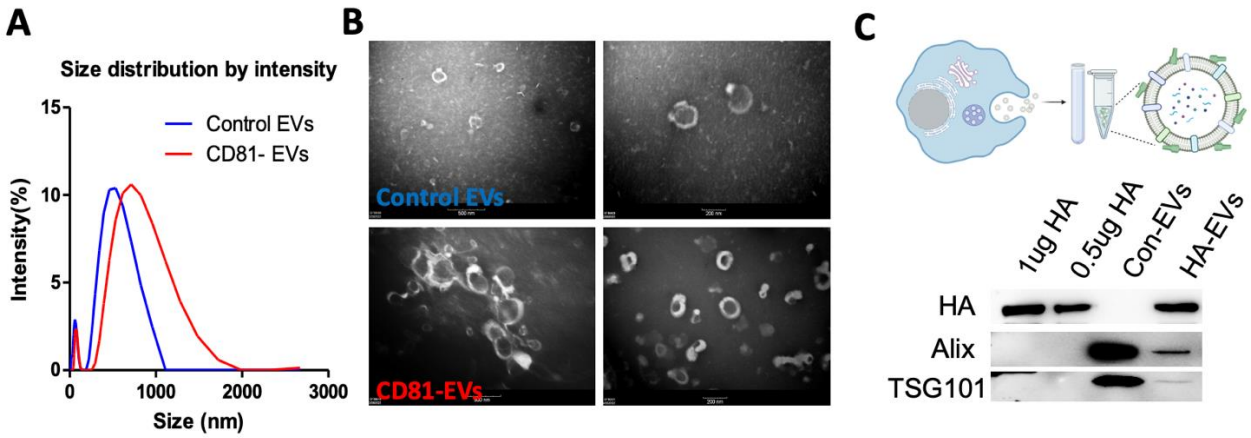

Figure 3. EV Characterization and HA3 Conjugation
Figure 3A. displays DLS data confirming the size and intensity of the purified EVs. Figure 3B. shows images confirming the size of the EVs in comparison to control. Figure 3C. western blot data confirms HA conjugation on EV surface.

2.3 Stimulating Cells

2.3.1 JAWSii

The cultured JAWSii cells were then stimulated with 2ul Soluble HA per well, 1 ul MPLA per well, 10 ul EV-Has per well, and 10 ul EVs per well with three of each type and three left unstimulated as a control. They were then incubated overnight for future use.

2.3.2 RAW264.7

The cultured RAW264.7 cells were then stimulated with 2ul Soluble HA per well, 1 ul MPLA per well, 10 ul EV-Has per well, and 10 ul EVs per well with three of each type and three left unstimulated as a control. They were then incubated overnight for future use. .

2.4 Collecting Samples for FACS and ELISA

Supernatant is transferred from wells into a tube. The wells are then washed with 200 ul PBS. This is discarded and then 200 ul trypsin is added. After digestion, 300ul fresh medium is added to stop the reaction and everything is transferred to a new tube. The supernatant tube and the digested tubes are then centrifuged at 2000 rpm x 5 minutes. The supernatant was collected from the undigested tubes and then frozen for ELISA cytokine assay and the supernatant from the digested tubes was discarded. The pellet from the digested cells is then resuspended with 50 ul PBS with 2% FBS in same tube. For JAWSii samples CD11-APC, CD40-PE, CD80-PLT, CD86-APC, CD86, CD8, and CD16-132 for the first attempt at a 1:250 dilution and for the second attempt and the RAW264.7 samples CD16/32, CD86APC, F4/80, CD11APC, MHCii, and CD11b were used at a 1:250 dilution. Samples were then incubated at room temperature for 20 minutes kept out of light. After this, 1mL of FACS staining buffer was added and centrifuged at 2000 rpm for five minutes and then supernatant discarded. The pellet is then resuspended in 150 uL FACS buffer. 150 ul 4% PFA was used to fix cells and incubated overnight at 4°C.

2.5 ELISA

An adapted general ELISA procedure from BD Biosciences was performed as follows:

2.5.1 Capture antibody

Purified anti-cytokine (tnf-a cap, IL 12cap, IL 6 cap, IL iB cap) capture antibody was diluted to 4 ng/ml and plate sealed to prevent evaporation during incubation overnight at 4°C

2.5.2 Blocking

Plate was brought to room temperature (RT), capture antibody solution removed, washed twice with PBS/Tween® (PBST), and blocked non-specific binding by adding 150 µl of Blocking Buffer (BSA) per well. Plate was sealed and incubated at RT for one hour and then washed three times with PBST.

2.5.3 Standards and Samples

Standards and samples (diluted in Blocking Buffer/Tween® h) were added at 100 µl per well. Standards started at were IL-6 at 20ng/ml, IL-1B at 25.8ng/ml, IL-12 at 14ng/ml, and TNF-a at 18ng/ml and the dilution starts at a 2x dilution. Plate sealed and incubated for 2 hours at 37°C or overnight at 4°C and then washed three times with PBST.

2.5.4 Detection Antibody

Biotinylated anti-cytokine detection antibody was diluted to 0.5-2 µg/ml in Blocking Buffer/Tween® at a 1:250 dilution. 50 µl of diluted antibody was added to each well. Plate was sealed and incubated for 1.5 hours at 37°C and washed three times with PBST. The detection antibodies were for TNF-a, IL-6, IL-12 and IL-1B.

2.5.5 Avidin-Horseradish Peroxidase (Av-HRP)

Streptavidin-HRP (cat. no. 554066) was diluted at a 1:1000 ratio in Blocking Buffer/Tween® and 50 µl per well of the mixture was added. Plate was sealed and incubated at RT for one hour and then washed five times with PBST.

2.5.6 Substrate

100 µl of TMB was immediately dispensed into each well and incubated at RT (5-80 min) for color development. Reaction was then stopped by adding 100uL stop buffer and optical density was read with a microplate reader set to 405 nm.

2.6 Flow Cytometry

Samples were read on BD FACSDiva™ Software in appropriate parameters and then analyzed on GraphPad.

3. RESULTS

3.1 JAWSii ELISA and FACS

The first experiment we completed was the ELISA and FACS for the JAWSii cells after they reached confluence, and the samples were prepared for testing. First, a cytokine ELISA was

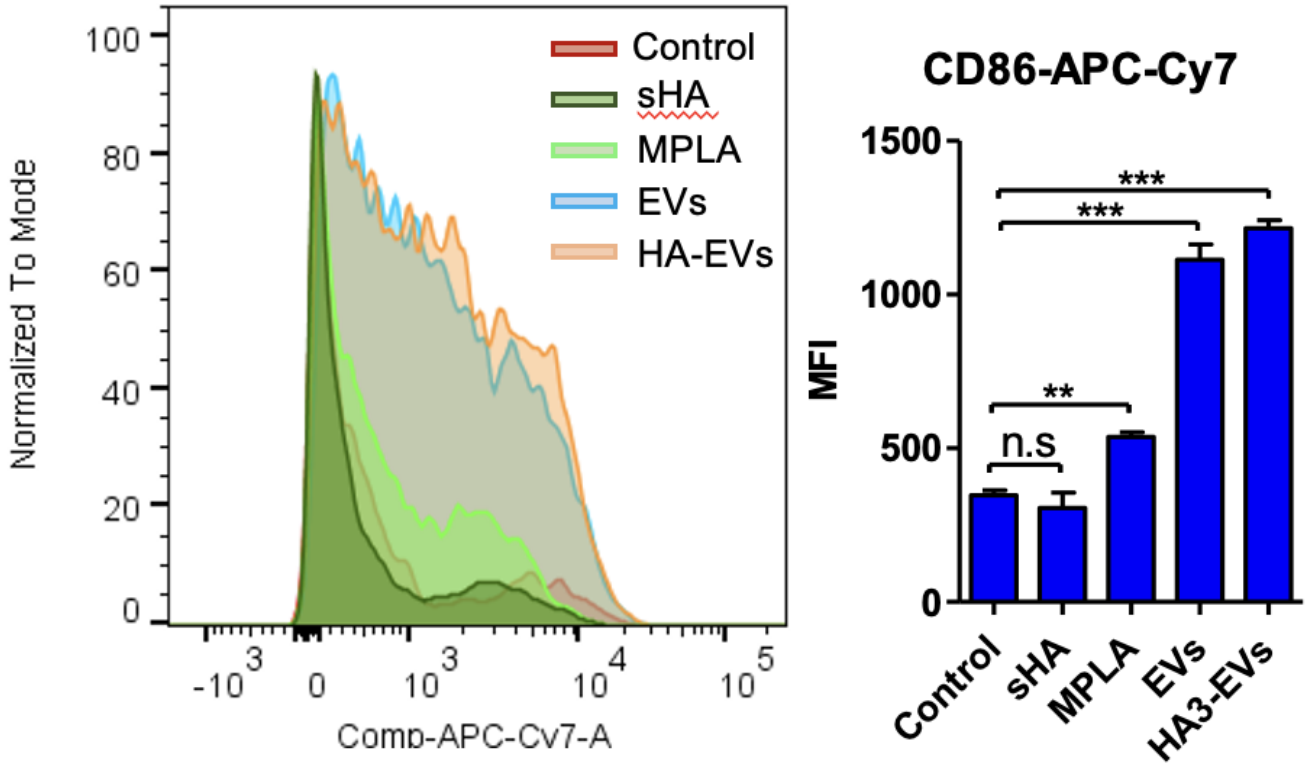

Figure 4. JAWSii Cytokine ELISA Data
EVs and HA3-EVs equally stimulated increased IL-6, TNF-a and IL-1β expressions in JAWSII cells.

performed with the cytokines being TNF-a. IL-1B, IL-6, and IL-12. Next, a FACS study was performed with several cell markers.

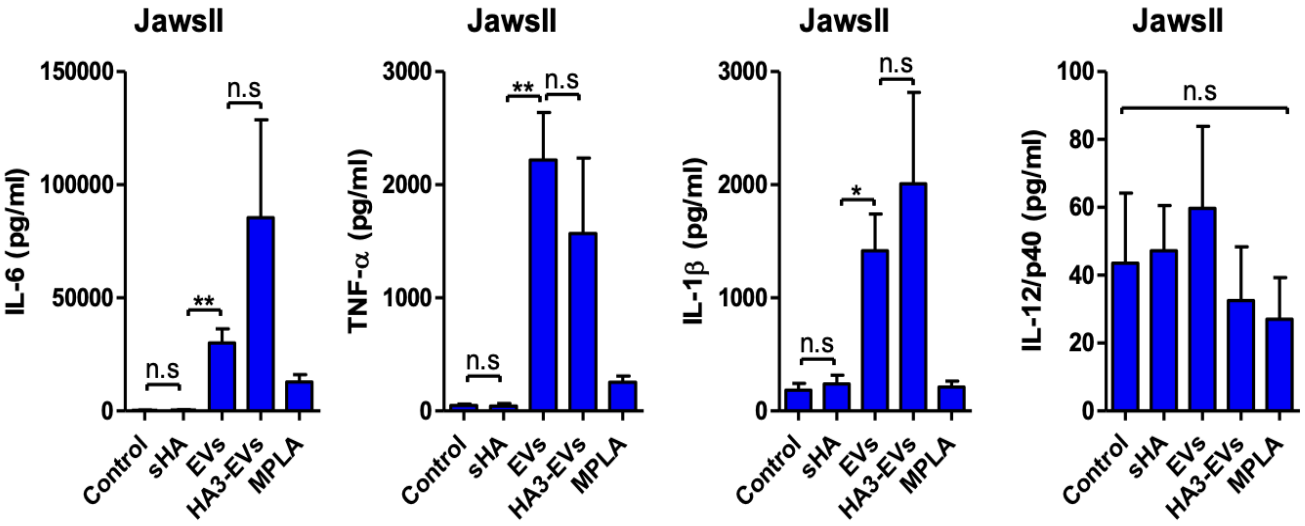

Figure 5. JAWSii FACS Data

EVs, HA3-EVs and MPLA stimulate significant increased CD86 expression, but not CD40 and CD80 expressions in JAWSII cells.

The cytokine ELISA assay results showed that EVs and HA3-EVs equally stimulated increased IL-6, TNF-a and IL-1β expressions in JAWSII cells. The negative control, soluble HA (sHA), and positive control, MPLA were not able to significantly stimulate these cytokines. The FACS study data also show EVs and the HA3-EVs to have increased CD86 expression compared to MPLA, sHA, and the control.

3.2 RAW264.7 ELISA and FACS

Next, the same experiments were conducted in RAW264.7 cells with the same cell markers and cytokines. Figure 5 shows the ELISA data for the RAW264.7 cells. In these cells, EVs and HA3-EVs were able to stimulate IL-6 and TNF-a, however, they were not able to stimulate IL-1B or IL-12.

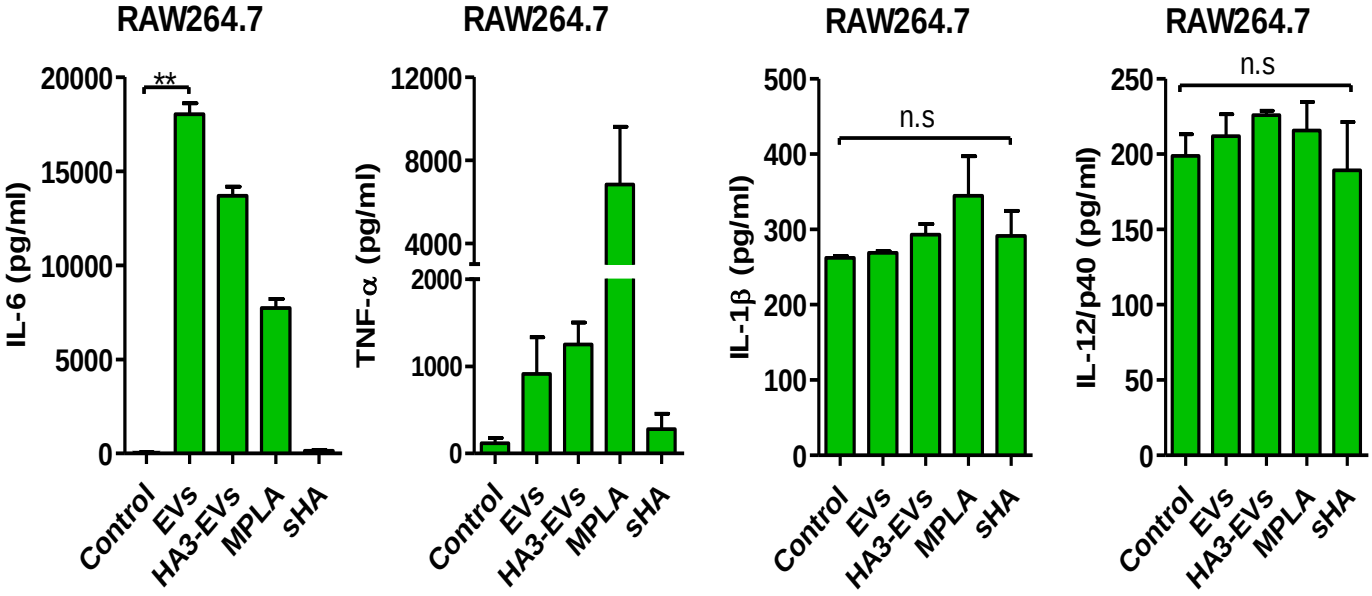

Figure 6. RAW264.7 ELISA Data
These four graphs display cytokine ELISA data for IL-6, TNF-a, IL-1B, and IL-12, respectively.

After completing the cytokine ELISA study, FACS was performed as well to determine the cell populations. Figure 5 shows the RAW264.7 cells FACS data gated at CD11b+CD11c- conditions. EVs, HA3-EVs and MPLA stimulated IL-6 expressions in RAW264.7 cells. There was a tendency of increased TNF-a after EVs, HA3-EVs and MPLA treatment. The expression of TNF-a needs to be further determined. EVs, HA3-EVs and MPLA could not stimulate IL-β and IL-12 expressions in RAW264.7 cells.

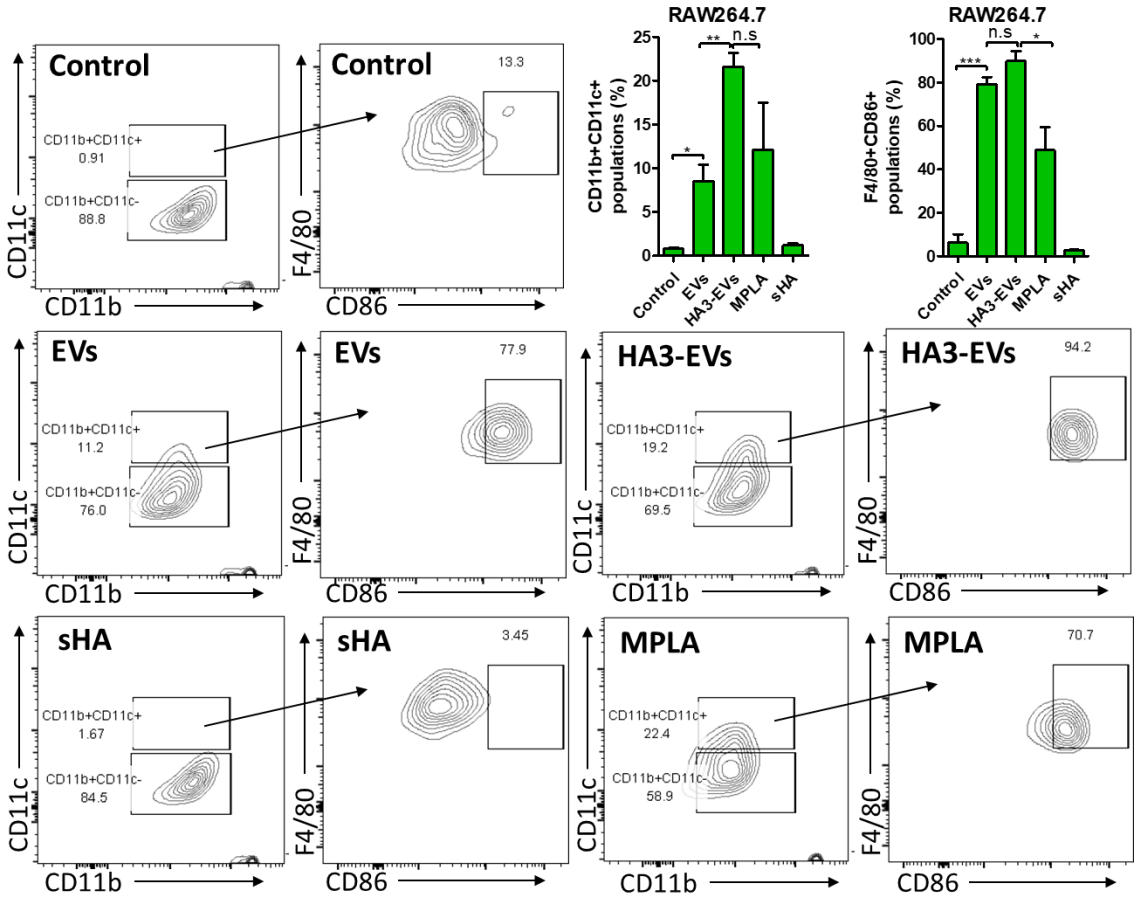

Figure 7. RAW264.7 FACS Data
EVs, HA3-EVs and MPLA treatment increased F4/80+CD86+ populations in CD11b+CD11c- cells.

This data shows the populations at CD11b+CD11c+ for HA3-EVs, EVs, and MPLA in addition to F4/80+CD86+ populations. The next figure, Figure 7, shows these F4/80+CD86+ populations more clearly. EVs, HA3-EVs and MPLA treatment increased F4/80+CD86+ populations in CD11b+CD11c+ cells.

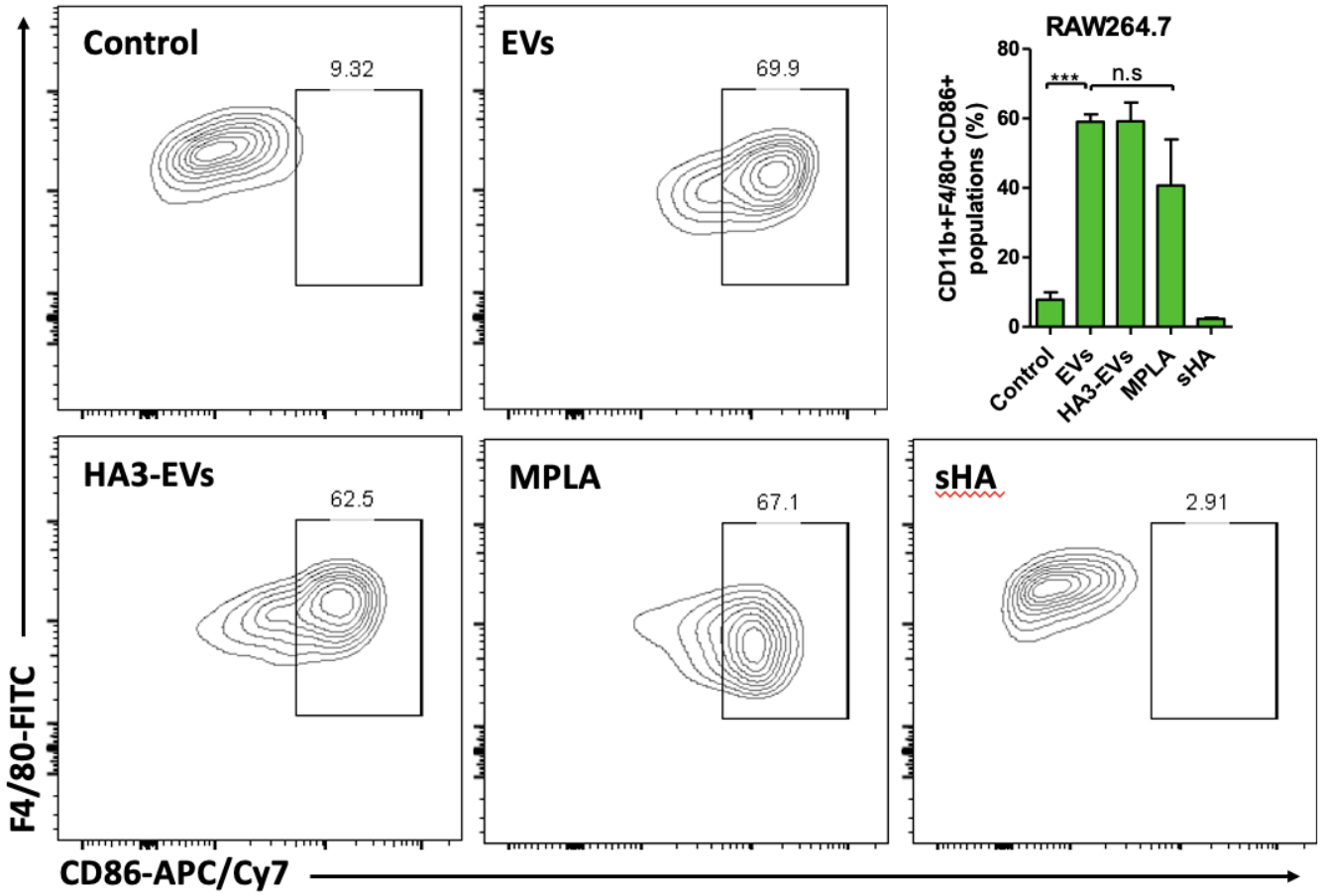

Figure 8. RAW264.7 FACS gated F4/80+ CD86+
EVs, HA3-EVs and MPLA treatment increased F4/80+CD86+ populations in CD11b+CD11c+ cells.

In this figure, there are clear populations of EVs, MPLA, and HA3-EVs in the F4/80 and CD86 double positive regions while the control and sHA do not have cell populations in these regions. EVs, HA3-EVs and MPLA treatment increased F4/80+/CD86+ populations in CD11b+CD11c- cells. EVs, HA3-EVs and MPLA stimulated macrophage activation. More activation markers are needed for further evaluation to determine the type of macrophage and to further confirm the T cell response.

4. DISCUSSION

Aims of this study were to explore EVs as adjuvants and how they are able to stimulate immune cells. Additionally, to study the stimulation of influenza HA conjugated EVs on innate immune cells. Based off these aims, the study was conducted with certain cell markers and cytokines that play important roles in the immune system. In the JAWSii ELISA, the data shows that the EVs and HA3-EVs were able to stimulate IL-6, IL-1B, IL-12, and TNF-a with the HA3-EVs able to stimulate more intensely than regular EVs for IL-6 and IL-1B. The JAWSii cell line is an immortalized bone marrow derived dendritic cell line from p53-deficient mice chosen for this study due to dendritic cell's role in the innate immune system. The ELISA results conclude that there was a heightened inflammatory response and an activation of immune cells.

The RAW264.7 cells were also able to stimulate some of the cytokines, however, not all that JAWSii were able to. This data shows that EVs, HA3-EVs, and MPLA were able to stimulate IL-6 with a significant increase. There was a tendency of increased TNF-a after EVs, HA3-EVs and MPLA treatment. The expression of TNF-a needs to be further determined. EVs, HA3-EVs and MPLA could not stimulate IL-β and IL-12 expressions in RAW264.7 cells. This cell line was chosen to study effects on macrophages as it is a macrophage-like cell line.

Both the JAWSii and RAW264.7 cells had upregulation of CD86 and F4/80 in the cellular populations. These are macrophage cell markers, so a macrophage population is confirmed in these gated conditions for EVs, HA3-EVs, and MPLA for RAW264.7 and JAWSii cells. CD86 also functions as a dendritic cell maturation marker so the increased expression of CD86 in EVs and influenza conjugated HA3-EVs confirms the dendritic cells present. Along with CD80, CD86 functions as a costimulatory signal in immune cell response as activators of T cells. They either

activate or inhibit T cell response by binding to CD28 or CTLA-4, another costimulatory signal pair (16). The innate immune system is involved here as APCs function as a link between the innate and adaptive immune responses. These innate immune cells are presenting adjuvanted activity with the increased upregulation of CD86 in the EVs and HA3-EVs which then stimulates a T cell response, an adaptive immune system component. These T cells are then able to differentiate and divide. This activation thus demonstrates adjuvanted behavior by activating an immune response.

5. CONCLUSION

Aims of this study were to explore how EVs function in the innate immune system in early stages, especially to learn about the influenza conjugated EVs, HA3-EVs. Due to the upregulation of CD86 and F4/80 along with increased cytokine stimulation in these cell lines that are dendritic cell and macrophage derived, adjuvanted behavior is observed. These influenza HA3 conjugated EVs stimulated activation and maturation of DCs and activation of macrophages in vitro. Overall, Flow Cytometry and ELISA demonstrate the effects that EVs and Influenza HA3 conjugated EVs have on innate immune cells by stimulating pro-inflammatory cytokines and markers for activation and maturation such as CD86 and F4/80. The increased expression of cytokines and cell surface markers after EVs stimulation indicated that EVs alone had adjuvant effects to enhance immune responses.

6. FUTURE DIRECTIONS

Exploring more on the innate immune system in early stages is important in understanding cellular processes in immunity. To better understand this, there are a couple future directions to explore. First, the adjuvant experiments can be done in BMDC cells. This

would give more data outside of experiments in immortalized cell lines. BMDC cells were cultured for this series of experiments, but a successful isolated culture was not able to be obtained. However, the same FACS and ELISA study would be done and repeated with a primary cell culture. In addition, more cytokines and cell markers would be beneficial to be included in these studies to further confirm the type of stimulation is clearly presenting adjuvanted behavior. Some of these cell markers include CD40 to drive T cell activation, CD206 to upregulate anti-inflammatory macrophage activation. Additional cytokine ELISA components include LPS elicit a pro-inflammatory M1 macrophage response and IL-4 or IL-13 to stimulate M2 macrophage activation in bone marrow derived macrophages. Next, being able to determine the immunogenicity of the HA3-EVs in vivo would be helpful to explore their effect on the innate immune system response. Finally, the HA3 conjugated EVs could be used as a potential mucosal vaccine through intranasal delivery in future due to the non-toxic property of EVs in a future study.

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VITAE

Madeline Bruhn

Atlanta, GA | madi.bruhn98@gmail.com | (229) 289-1284 | LinkedIn

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Education

Georgia State University
Atlanta, GA
Master of Interdisciplinary Studies in Biomedical Science and Enterprise
December 2023 (Expected)
Graduate Research Assistantship
Translational Biomedical Student Association

University of Georgia
Athens, GA
Bachelor of Science in Biology, Minor in Spanish
May 2021
Undergraduate research position in antibiotic resistance lab
Recipient of Stethoscope Magazine Leara Scholarship

University of Valencia
Valencia, Spain
Study abroad coursework in Spanish literature and culture
July 2019

Experience

Georgia State University
Atlanta, GA
Graduate Research Assistant
August 2022- Present

- Completing thesis in microbiology and genomics with self-created protocol that meets all regulatory requirements.
- Analyze data in collaboration with mentor along with analyzing secondary research.
- Maintained quality assurance and quality standards in all data collection and interpretation.

Brash Coffee Roasters
Atlanta, GA
Barista
December 2022- Present

- Prepare espresso beverages and serving to customers.
- Educate customers on techniques and varieties in coffee roasting and brewing.

Café on Lumpkin
Athens, GA
Server
June-July 2022

- Provided efficient wait service to create customer satisfaction.
- Delegated tasks to ensure efficient order of operations.

Collective Harvest
Athens, GA
Retail Associate
June-July 2022

- Assessed customer needs and reaching solutions.
- Organized inventory to provide optimal shopping experience.

Dermatology Associates of Tallahassee
Tallahassee, FL
Clinical Assisting Student
May 2021-June 2022

- Obtained client medical history, medication information, symptoms and allergies.
- Taught patients about medications, procedures and care plan instructions.
- Completed clinical procedures and gathered patient data for interpretation by physician.
- Assisted in surgeries and clinical procedures.
- Compliant with HIPAA and all other necessary clinical practices and healthcare quality standards.

ICNA Relief USA- Shifa Clinic Athens
Athens, GA
Lead Volunteer
January 2019-May 2021

- Teamed with community organizations and government agencies to increase outreach and service utilization.
- Managed multi-line phone system with over 50 incoming calls each day, directing individuals to desired personnel and providing general information about clinical and food bank operations.
- Assisted and shadowed physicians in their care of patients

Skills

Language: English and Spanish
Laboratory: Polymerase Chain Reaction, Western Blot Assay, ELISA, qPCR, DNA sequencing, RNA Translation, Transfection, & Transformation, Cell Culture, Flow Cytometry, FACS, Data Analysis, Biobanking, Attention to Detail, Accessioning, cGMP

Computer: Sufficiency in Microsoft Office Suite, Excel, PowerPoint, Python Coding, Stata, Canva, Statistical Software, Computer Literacy, Data Mining, Data Analysis

Licenses & Certifications

Enterprise Design Thinking Practitioner
January 2023

- Issued by IBM
- Acquired knowledge of applying Enterprise Design Thinking and its value

Python Ready! Badge -- Quantitative Data Analysis Coding Training
February 2023

- Awarded for completing requirements in group Software & Coding Training
- Recipient must score 100% on quiz assessment.

SPSS Ready! Badge -- Quantitative Data Analysis Software Training
March 2023

- Digital credential awarded for completing SPSS workshops training offered by Georgia State University Library's Research Data Services (RDS) department and demonstrating learning of content via quiz assessment.

Grad Data Jam! Badge -- Analyzing, Visualizing, & Communicating for Maximum Impact
May 2023

- **Awarded to a Georgia State University Graduate student enrolled in the credit-bearing course and attended live or pre-recorded sessions, self-selected from the following topic areas:**
  (1) quantitative and qualitative data analysis and visualization software/coding/tools
  (2) web design with html/CSS coding
  (3) presentation design
  (4) secondary research techniques