12-13-2023

Induction of Inflammasome Activation in Macrophage Cells by Polyethyleneimine-Modified Graphene Oxide Nanoparticles

Ibinabo Lilly-Tariah
Georgia State University

Induction of Inflammasome Activation in Macrophage Cells by Polyethyleneimine-Modified Graphene Oxide Nanoparticles

by

Ibinabo Lilly-Tariah

Under the Direction of Dr Baozhong Wang, Ph.D.

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Interdisciplinary Studies

In the Institute for Biomedical Sciences

Georgia State University

2023

ABSTRACT

Vaccine development has been a cornerstone of modern medicine and the use of adjuvants in vaccine development has been a phenomenon present since the early 1900's. Nanoparticles offer unique advantages as adjuvants due to their adjustable properties and their ability to serve as antigen carriers. In this study, we investigate the influence of polyethyleneimine-modified graphene oxide nanoparticles (GO-PEI) on the activation of the inflammasome, a crucial component of the innate immune system, in macrophage cells. Narrowing our focus on testing the successful activation of the inflammasome and its mechanism of activation, we found that GP nanoparticles could induce the activation of the inflammasome in vitro and its mechanism of action involved cathepsin B release and generation of reactive oxygen species as downstream secondary activating signals. This finding is significant and illustrates GP nanoparticles' usefulness in the development of more effective vaccines due to their unique properties.

INDEX WORDS: Inflammasome, Graphene oxide, Macrophage.

Induction of Inflammasome Activation in Macrophage Cells by Polyethyleneimine-Modified Graphene Oxide Nanoparticles

by

Ibinabo Lilly-Tariah

Committee Chair: Baozhong Wang

Committee: Lanying Du

Electronic Version Approved:

Office of Academic Assistance – Graduate Programs

Institute for Biomedical Sciences

Georgia State University

October 2023

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to my committee chair and principal investigator, Dr Baozhong Wang for his guidance and tutelage. I want to also extend my gratitude to Dr Lanying Du for being a part of my thesis committee. I would also like to extend my heartfelt gratitude to my mentor Dr Chunhong Dong as this project would not have been possible without her guidance and patience. Again, I would like to express my thanks to all the other members of the Wang laboratory for their assistance, support and encouragement throughout this entire process. And finally, I would like to express my heartfelt gratitude and thanks to my family and friends for their support, encouragement and faith in me as I would not be here without them.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

BMDM – Bone Marrow-Derived Macrophages

BSA – Bovine Serum Albumin

cRPMI – Complete Roswell Park Memorial Institute

DCFDA - Dichlorodihydrofluorescein Diacetate

DMEM - Dulbecco's Modified Eagle's Medium

DPBS - Dulbecco's Phosphate Buffered Saline

EDC - 1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide

FBS – Fetal Bovine Serum

GO – Graphene Oxide

GP/GO-PEI – Polyethyleneimine-modified Graphene oxide

HA – Hemagglutinin Protein

IMDM - Iscove's Modified Dulbecco's Medium

M-CSF – Macrophage Colony-Stimulating Factor

NEAA – Non-essential Amino Acids

P/S – Penicillin/Streptomycin

PBS – Phosphate-Buffered Saline

PBST - Phosphate-Buffered Saline with Tween 20

PEI - Polyethyleneimine

PMA - Phorbol 12-Myristate-13-Acetate

RBC – Red Blood Cell

ROS – Reactive Oxygen Species

THP-1 – Human Monocytic Cell Line Isolated from Peripheral Blood of an Acute Monocytic Leukemia Patient

TMB - 3,3’,5,5’-Tetramethylbenzidine

1. INTRODUCTION

In the world of biomedical science and medicine, one of the major facets that is subject to heavy research is the topic of vaccination. Vaccination has played a major role in the advancement of medicine, the treatment of diseases and the betterment of public health. There are various forms of vaccines including but not limited to whole virus vaccines, sub-unit vaccines, split vaccines and live attenuated vaccines etc. With all the various types, their mechanism of action may differ, but they all share a primary aim which is to activate the adaptive immune system to generate an immunological response that primes the host against future infections. This usually results in a more robust and rapid immunological response in the case of future infections.

Vaccine adjuvants have been a phenomenon present since the early 1900's. Aluminum salts were amongst the first adjuvants used in the development of vaccines specifically, diphtheria and tetanus vaccines, and are still widely used to this day as a common adjuvant. Adjuvants play a pivotal role in vaccine development as they can enhance protective immunity and when compared to other vaccine types like live attenuated vaccines, they provide an improved safety profile and can help address issues concerning vaccine supply by allowing vaccine dose sparing and vaccine consistency [1]. In recent times, vaccine adjuvant designs have been made as such as they specifically target the pathogen of interest thereby activating the appropriate pathways [1].

The use of nanoparticles as adjuvants in vaccine development has come a long way since its inception in the late 1900's. These particles as used in vaccine formulations offer a variety of benefits like allowing for enhanced immunogenicity and stability of antigens, targeted

delivery, and slow release [2]. The types of nanoparticles used in vaccine formulations range from inorganic particles to virus-like particles each offering their very own unique properties that help in the targeting and enhancing of immunological response.

Our research focuses on studying the adjuvant mechanism of our nanoparticle of interest, polyethyleneimine-modified graphene oxide nanoparticles. Otherwise known as GO-PEI or GP nanoparticle. Prior research on the use of polyethyleneimine functionalized graphene oxide nanoparticles as a vaccine nanoplatform for hemagglutinin protein 3 (HA3) showed that intranasal vaccination with influenza GP-HA nanoparticles induced robust humoral and cellular immune responses in vivo and thus conferred broad protection against challenge by homologous and heterologous influenza viruses in mice [3]. This finding showed the potential of nanoparticle-based vaccines being used to overcome obstacles associated with intranasal vaccine delivery but also shows the possibility of GP nanoparticles being used as a suitable adjuvant for vaccine developments and treatments.

To further study the adjuvant mechanism of our inorganic nanoparticle adjuvant, we focus on studying the particle's ability to activate the inflammasome. Inflammasomes are components of the innate immune system that play a role in sensing and detecting pathogenic microbes and molecules as well as molecules derived from host proteins [4]. Upon detection, they induce inflammation that triggers other inflammatory pathways that then result in the activation of the adaptive immune system. Hence inflammasomes serve as a bridge between innate and adaptive immunity which makes it an attractive target for vaccine developers.

1.1 Inflammasomes

Our immune system offers numerous interconnected systems in the maintenance of a host's functions. The systems and components that make up the immune system of an organism are diverse and far-reaching but interdependent at the same time. An organism's primary system of defense, the innate immune system, offers different levels of detection and protection against foreign materials. One of the components that make up and play a major role in innate immunity is inflammasomes.

Inflammasomes, a mechanism of cellular innate immune response, are a system of complex protein structures that help with the sensing and inflammatory response of a host cell to foreign or toxic matter. They are multimeric protein complexes that assemble in response to a cytosolic innate immune receptor detecting pathogen-associated molecular patterns (PAMPS) or damage-associated molecular patterns (DAMPS) [5]. They are believed to also shape or modulate the activity of the adaptive immune system. Successful activation of an inflammasome could result in an inflammatory response in a host by the release of certain cytokines that further activate and regulate certain lymphocytes, particularly T cells, as well as regulate the activation of other inflammasomes in neighboring cells. Numerous types of inflammasomes are often activated by different sensors responding to different PAMPS or toxins. The mechanisms that underlie the majority of the inflammasomes are still unknown and poorly understood, hence prompting the need for more extensive study. The most well-studied and understood inflammasome pathway is the NLRP3 inflammasome (**Fig. 1**). One of the pathways through which this inflammasome can function is the canonical pathway [6]. The inflammatory response elicited by the inflammasome in most cases occurs through a cascade of

events such as the production, cleavage and release of caspase -1 protein, IL-1β and IL-18 cytokines, gasdermin D, and in some cases, the induction of cell death via pyroptosis.

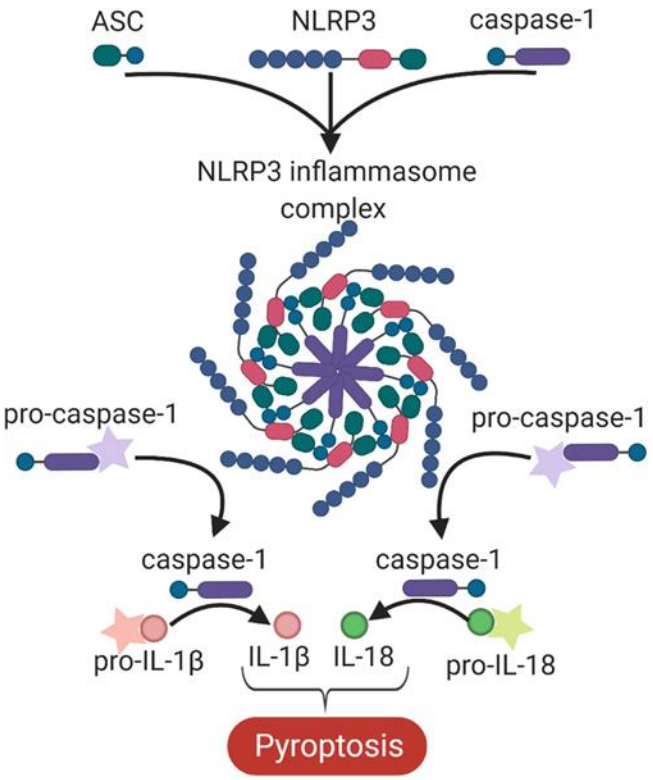

Figure 1: Structure of an NLRP3 Inflammasome
Source: O'Brien, W.T., Pham, L., Symons, G.F. et al. The NLRP3 inflammasome in traumatic brain injury: potential as a biomarker and therapeutic target. J Neuroinflammation 17, 104 (2020).

1.1.1 Indicators of a Successful Inflammasome Activation

From what is understood of the inflammasome pathway, particularly through the study of the NLRP3 inflammasome, we know that there are two main stages to the activation of the inflammasome in the canonical pathway. The first stage, otherwise known as the priming stage, involves the recognition of PAMPS by certain pattern recognition receptors (PRRs). As the name suggests, this step primes the cell for successful activation of the inflammasome and typically results in the transcription of NLPR and pro-IL-1β through the activation of the NF-κB pathway [7]. The second stage, otherwise known as the activation stage, involves one or more secondary

signals or activators that then cause the assembly of the inflammasome and its subsequent activation through the cleavage of pro-Caspase-1 protein to Caspase-1 (amongst others) which then cleaves pro-IL-1β cytokine to IL-1β, pro-IL-18 cytokine to IL-18 and gasdermin D (GSDMD) to N-term GSDMD [7]. These factors, the production of active Caspase-1, active IL-1β cytokine, IL-18 cytokine and N-term GSDMD, usually serve as the basis of confirmation of successful inflammasome activation.

Some of the primary signals that serve to prime the inflammasome pathway are microbial ligands such as LPS recognized by TLRs or cytokines such as TNF-α [8]. Some of the secondary signals involve several PAMPS and DAMPS such as particulates, bacteria, viruses, and pore-forming toxins [7]. Under noninfectious conditions, extracellular ATP and K+ efflux lead to the activation of the NLRP3 inflammasome through a different receptor [8]. Endogenous and exogenous particulates, such as CPPD crystals, cholesterol crystals, amyloid β, silica crystals, asbestos, and alum, promote lysosomal damage and release cathepsin B into the cytosol, leading to the NLRP3 inflammasome activation [8]. Calcium influx could also be another signal that activates the inflammasome through the generation of mitochondrial ROS [8].

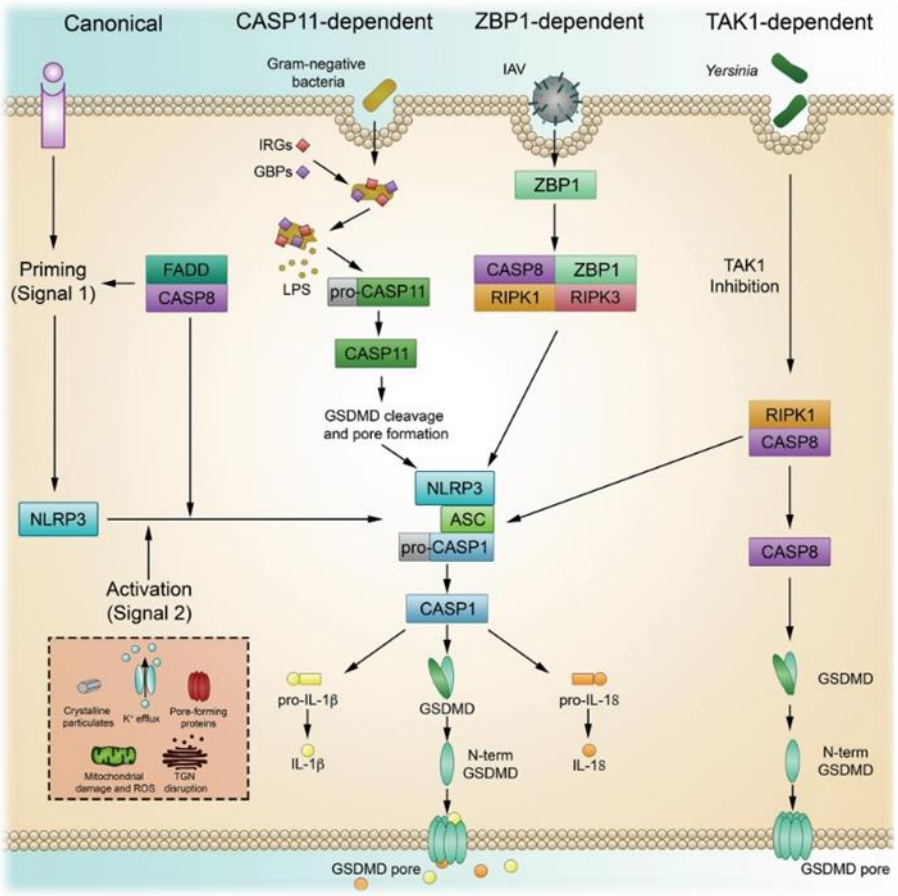

Figure 2: NLRP3 Inflammasome Activation Pathway.
Priming and activation signals involved in the canonical pathway of NLRP3 inflammasome.
Source: Christgen, S., Place, D. E., & Kanneganti, T. (2020). Toward targeting inflammasomes: Insights into their regulation and activation. Cell Research, 30(4), 315-327.

1.2 Polyethyleneimine-Modified Graphene Oxide Nanoparticles

Graphene oxide is a two-dimensional monomolecular structure. Its hydrophilic nature and large surface area allow for biochemical and bioconjugation reactions that facilitate the functionalization of proteins, antibodies and DNA fragments [9]. This particle offers a number of advantages, thus why it has been studied and used in prior research and is useful to the study of inflammasomes.

Polyethyleneimine (PEI) is a hydrophilic cationic polymer and when conjugated with another particle can serve as a suitable adjuvant for mucosal vaccination. PEI itself has been

found to have a potent mucosal adjuvant effect [10]. The functionalization of graphene oxide nanoparticles with polyethyleneimine confers the particle a positive surface charge and flexibility for conjugation with various vaccine components. Additionally, GP nanoparticles display characteristics such as high antigen loading capacity, immunostimulant properties and self-adjuvant properties. These qualities are useful in the designing of an efficient and immunostimulatory vaccine.

With our study, to better understand the adjuvant mechanism elicited by polyethyleneimine-modified graphene oxide nanoparticles, we investigate the nanoparticle's ability to induce the activation of inflammasomes in macrophage cells, which are cells of the innate immune system. We hypothesized that the polyethyleneimine-modified graphene oxide nanoparticles were capable of inducing inflammasome activation in macrophage cells. We were able to confirm our hypothesis by the end of our study as we found that the GP nanoparticles were able to induce active Caspase-1 protein production and IL-1β cytokine secretion which are indicators of inflammasome activation in murine bone marrow-derived macrophage cells (BMDM) and THP-1 macrophage cells. We also sought to understand the mechanism of GP-induced inflammasome activation in those cells and therefore through the blocking of known secondary activation signals, were able to pin down some of the mechanisms involved in its activation.

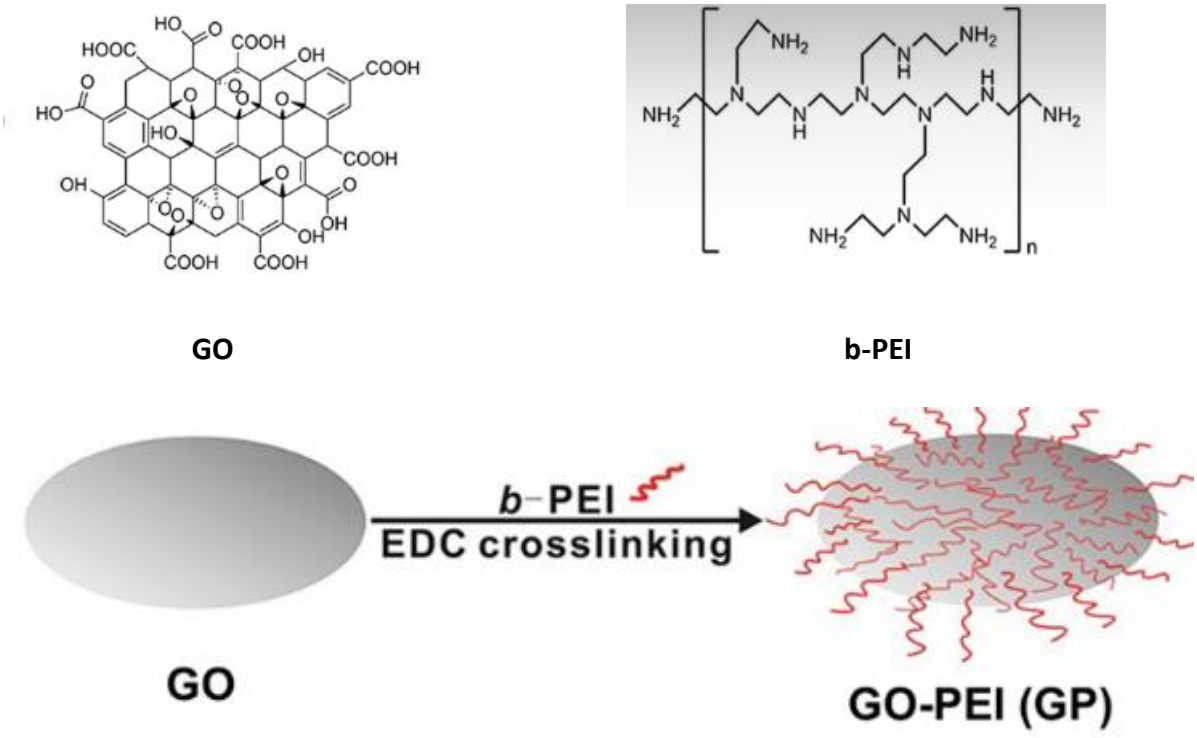

Figure 3: Illustration of GO-PEI Nanoparticle

Source: Dong, C., Wang, Y., Gonzalez, G. X., Ma, Y., Song, Y., Wang, S., Kang, S., Compans, R. W., & Wang, B. (2021). Intranasal vaccination with influenza HA/GO-PEI nanoparticles provides immune protection against homo- and heterologous strains. Proceedings of the National Academy of Sciences, 118(19), e2024998118.

2. METHODS

2.1 Experimental Design

BMDM and THP-1 macrophage cells were cultured to test the induction of inflammasome by GP nanoparticles. Inflammasome activation was tested in cells treated with adjuvants (LPS, MPLA, R848), GP, GP primed with LPS, GP-HA3. Positive control “LPS + ATP” and negative control “no treatment”. Western blot of caspase-1 cleavage and cytokine ELISA of IL-1β cytokine were evaluated to confirm successful inflammasome activation. We incubated macrophage cells with cathepsin B and potassium channel inhibitors (CA-074 Me and Amiodarone respectively) 30 minutes prior to particle incubation to study the mechanism of inflammasome activation.

2.2 Protocol: Polyethyleneimine-Modified Graphene Oxide Nanoparticles

Graphene oxide powder was dissolved in milli-Q water at a concentration of around 1 mg/ml. The graphene oxide solution went through sonication at multiple time points using a tip/probe type ultra sonication device in an ice-water bath. Particles were sonicated with a setting of 100% Amplitude and 5-second on/5-second off pulse. The size of the graphene oxide nanoparticle was measured at multiple time points post-sonication using a Malvern Zetasizer instrument. An excess amount of polyethyleneimine (PEI) was added to the graphene oxide nanoparticles when they were about 200 – 300 nm in size. The mass ratio of GO to PEI used is 1:6 (GO: PEI, 1: 6). The GO-PEI nanoparticles were then sonicated further for 30 minutes. To activate the nanoparticle, EDC is added to the GO-PEI suspension in two to three batches at a ratio of 1:6. With each addition of EDC, the suspension is sealed and stirred continuously with a magnetic stirrer at room temperature for 1-2 hours and then 16 hours (overnight). The GO-PEI suspension is then washed by centrifugation several times at a speed of 21130 rcf at 4°C. Size, zeta potential, and concentration of nanoparticles are determined before storage and use.

2.3 Protocol: Murine Bone Marrow-Derived Macrophage Cell Culture and Stimulation

Cells were extracted from the bone marrow of 6-8-week-old BALB/c mice (femur and tibia bones). To extract the cells, the femur and tibia bones were flushed with 2% FBS in PBS into a 15 mL tube. Cells were pelleted by centrifuging at 1700 rpm for 5 minutes. The pellet was resuspended in RBC lysis buffer for 4 minutes before adding IMDM medium. Cells were again pelleted by centrifuging at 1700 rpm for 5 minutes before plating in BMDM Culture Medium (84.05% IMDM, 14.5% heat-inactivated FBS, 1.45% NEAA, 5mL P/S mix) plus M-CSF (2000x dilution, 500 IU/mL). Cell culture is left to incubate at 37°C for 5 days with the medium

being added on days 3 and 5. To seed the cells, on day 6, the medium in the cell culture plate is replaced with 10mL of ice-cold DPBS and left to incubate for 5 minutes. The cells on the plates were scraped gently with a cell scraper and collected into a single 50 mL tube. Cells were pelleted by centrifugation at 270 xg at 4°C for 5 minutes. The cells were resuspended in 20mL BMDM medium and counted before seeding in a 24-well flat bottom culture plate at a density of 5 x 10^5 cells/well and volume of 1 mL/well. M-CSF was added to each well and cells were left to incubate overnight at 37°C. On the day of particle addition, the culture medium is removed, and cells are washed with DPBS (1mL per well). BMDM stimulation medium (89% DMEM, 10% heat-inactivated FBS, 1% P/S mix) is added to each well and left to incubate for 2 hours at 37°C [5].

2.3.1. Particle Addition.

Particles and adjuvants GP-HA3, GP, LPS, MPLA, R848, and positive control (LPS + ATP) are added to predetermined wells at specific concentrations. Particles are left to incubate at 37°C for 16 – 24 hours. GP-HA3 (GP: HA3 10: 5), positive control LPS + ATP (LPS: ATP 1: 100). For positive control well, 0.5M ATP is added 30 minutes prior to sample collection.

2.3.2. Collection.

Supernatant/medium is taken from each well and transferred to 0.5mL Eppendorf tubes and stored at -80°C prior to sample analysis. To collect cell lysate, lysis buffer (NP40 + protein inhibitor, 100x dilution) is added to each well and placed on ice before cell scraping. Cells are scraped and collected into 1.5mL Eppendorf tubes on ice. 4X Laemmli buffer is added to each tube and subsequently boiled at 99°C for 15 minutes. Samples are stored at -80°C prior to sample analysis.

2.3.3. Inhibition Experiment.

For inhibition experiments, we made use of cathepsin B inhibitor (CA-074 Me) and potassium channel inhibitor (Amiodarone). BMDM cells were pre-treated with CA-074 Me or amiodarone 30 minutes prior to particle incubation/treatment (20µM CA-074 Me and 10µM amiodarone).

2.4 Protocol: THP-1 Macrophage Cell Culture and Stimulation

THP-1 cell line retrieved from storage under nitrogen condition. 50 mL of cRPMI medium was prepared (45% RPMI medium, 50% FBS, 5% Penicillin/Streptomycin mix). THP-1 aliquot was added to a 15 mL Eppendorf tube containing 9 mL of cRPMI and centrifuged at 125xg for 5 minutes. The supernatant was discarded and cell pellets were plated with cRPMI medium and 2-mercaptoethanol in a 1000x dilution (0.05mM). Cell density was calculated, and cells were left to incubate in a 37°C incubator. Cell culture was maintained at a density of 4-8 x 10^5 cells/mL with a maximum limit of 1 x 10^6 cells/mL. Medium was renewed every 2-3 days. Cells were seeded in a 96-well flat bottom culture plate at densities of 2-4x10^5 cells/well. To stimulate the differentiation to macrophage cells prior to particle addition, 100–150nM of PMA was added to seeded cells and incubated for 24 - 72 hours. Cells were then left to rest by replacing the stimulation medium with complete cRPMI medium for 24 hours before particle addition.

2.4.1. Particle Addition.

Particles and adjuvants GP, LPS, MPLA, R848, and positive control (LPS + ATP) are added to predetermined wells at specific concentrations. Particles are left to incubate at 37°C for 16 –

24 hours. Positive control LPS + ATP (LPS: ATP 1: 100). For positive control well, O.5M ATP is added 30 minutes prior to sample collection.

2.4.2. Collection.

Supernatant/medium is taken from each well and transferred to 0.5 mL Eppendorf tubes and stored at -80°C prior to sample analysis. To collect cell lysate, lysis buffer (NP40 + protein inhibitor, 100x dilution) is added to each well and placed on ice before cell scraping. Cells are scraped and collected into 1.5 mL Eppendorf tubes on ice. 4X Laemmli dye is added to each tube and subsequently boiled at 99°C for 15 minutes. Samples are stored at -80°C prior to sample analysis.

2.4.3. Inhibition Experiment.

For inhibition experiments, we made use of cathepsin B inhibitor (CA-074 Me) and potassium channel inhibitor (Amiodarone). BMDM cells were pre-treated with CA-074 Me or amiodarone 30 minutes prior to particle incubation/treatment (20µM CA-074 Me and 10µM amiodarone).

2.5 Reactive Oxygen Species (ROS) Protocol

Quantitative and qualitative testing of ROS in THP-1 macrophage cells incubated with GP nanoparticles, GP nanoparticles primed with LPS, and positive control (LPS + ATP). Analysis was done using a fluorescence microplate reader and a fluorescence microscope respectively. We made use of the Abcam Cellular ROS Assay kit (ab113851 DCFDA/H2DCFDA – Cellular ROS Assay kit). Quantitative analysis: THP-1 macrophage cells were seeded in a dark 96-well flat bottom culture plate at a density of $5x10^5$ cells/well and left to incubate overnight at 37°C with PMA stimulation. Media removed and cells were washed twice with warm dPBS prior to staining with

diluted DCFDA solution in 1X buffer (20µM, 100µL/well). Cells were incubated with stain for 55 minutes and then washed once with 1X buffer (100µL/well). 100µL of 1X supplemental buffer (10% FBS in 1X buffer) is added to each well before incubation with particles at varying concentrations. Plate read with a fluorescence microplate reader at 2-hour and 4-hour time points (Ex/Em = 490/520nm). Qualitative analysis: THP-1 macrophage cells were seeded in a 24-well flat bottom culture plate at a density of $5x10^5$ cells/well and left to incubate overnight at 37°C with PMA stimulation. Media removed and cells were washed twice with warm dPBS before staining with diluted DCFDA solution in 1X buffer (20µM, 300µL/well). Cells were incubated with stain for 55 minutes (excluding background) and then washed 1-2 times with 1X buffer (300µL/well). 250µL of 1X supplemental buffer (10% FBS in 1X buffer) is added to each well before incubation with particles. The plate was imaged with a fluorescence microscope at 2-hour and 4-hour time points.

2.6 Western Blot Analysis

Cell lysates are boiled at 99°C for 15 minutes and further centrifuged at 14500 xg for 30 seconds to pellet debris prior to loading on SDS page gel (12% resolving). ~ 10 µL or 15 µL of each sample is loaded onto a 1.0 mm or 1.5 mm SDS page gel respectively. 3 – 4 µl of BioRad precision plus protein dual color standard is used in each sample run. Samples in the electrode chamber are run at 80 V for 50 minutes and then at 120 V until fully separated. Samples are blotted/transferred to a nitrocellulose membrane at 20 V for 1 hour using a semi-dry transfer cell. The nitrocellulose membrane is rinsed with TBST, blocked with 5% blocking buffer for 1 hour, and then incubated with primary and secondary antibodies as follows. BMDM (Primary antibody: mouse anti-caspase-1 antibody 1000x. Secondary antibody: Goat anti-mouse HRP

conjugated antibody 5000x). THP-1 macrophage (Primary antibody: Human anti-caspase-1 antibody 1000x. Secondary antibody: Goat anti-mouse HRP conjugated antibody 2000x). To visualize our control protein, β-actin, membranes are stripped with a mild stripping buffer prior to blocking and incubation with primary and secondary antibodies. BMDM (Primary antibody: Rabbit anti-β-actin antibody 2500x. Secondary antibody: Goat anti-rabbit IgG HRP conjugated antibody 5000x). THP-1 macrophage (Primary antibody: Rabbit anti-β-actin antibody 2500x. Secondary antibody: Goat anti-rabbit IgG HRP conjugated antibody 5000x). Chemiluminescence and colorimetric imaging were used to visualize the western blot.

2.7 Cytokine ELISA Analysis

96-well flat-bottomed Elisa plate was coated with IL-1β capture antibody (50µL/well, 250x dilution in 1x PBS. BMDM: Biolegend purified anti-mouse IL-1β capture antibody IgG, cat# 503502. THP-1: Biolegend purified anti-human IL-1β capture antibody IgG2b, cat# 511601) and left to incubate overnight at 4°C. The plate is washed 3x with PBST (300µL/well) and blocked with blocking buffer (200µL/well, 2% BSA in PBST) for 1 hour in a 37°C incubator. IL-1β standard, blocking buffer and samples are then added to the wells and incubated for 2 hours at 37°C or overnight at 4°C (50µL/well, 8ng/mL BMDM: Biolegend mouse IL-1β standard. THP-1: Biolegend human IL-1β standard). Plate is washed 3x and then incubated with detection antibody for 1 hour at RT (BMDM: Biolegend biotin anti-mouse IL-1β antibody. THP-1: Biolegend biotin anti-human IL-1β antibody, 250x dilution). The plate is washed 3x and then incubated with streptavidin HRP for 45 minutes at RT. (50µL/well, 1000x). The plate is washed 7x and incubated with TMB for 5 minutes at RT (50µL/well) before the addition of stop solution

(50µL/well, 2M H_2SO_4). The plate is read with an ELISA plate reader program set at a wavelength of 450nm.

3. RESULTS

3.1 Characterization of GP Nanoparticles

GP nanoparticles used in the experiment were characterized using size measurements, zeta potential and polydispersity index (PDI). Differential centrifugation produced nanoparticles of different sizes ranging from 100nm to 300nm (**Table 1**). Two GP nanoparticle stock solutions were used in our experiments: GP small (GP_S 111nm, 2µg/µL) and GP large (GP_L 189.3nm. 3.8µg/µL) made from a previous batch **(Table 1)**

	Batch I	**Batch II**
Average size (nm)	136.8	243.9
PDI	0.174	0.172
Zeta potential	54.8	37.9
Concentration (µg/µL)	0.21	3.8
	GP small	**GP large**
Average size (nm)	111	189.3
PDI	0.032	0.130
Zeta potential	42.7	52.0
Concentration (µg/µL)	2.0	3.8

Table 1: Characterization of GO-PEI Nanoparticles

3.2 Aim 1: Determination of Inflammasome Activation by GP Nanoparticle

3.2.1 GP Nanoparticles Induce IL-1β Cytokine Secretion

Among many events downstream of inflammasome activation, the cleavage of pro-caspase-1 into mature caspase-1 that facilitates the maturation of pro-interleukin (IL)-1β into IL-1β is one of the key events. In this study, we found that our GP nanoparticles were capable of inducing IL-1β cytokine secretion (**Fig. 4B**) and when primed with an adjuvant resulted in a more robust secretion of the cytokine IL-1β which served as one of our indicators for successful inflammasome activation. We see robust IL-1β cytokine secretion in both BMDM (**Fig. 4B**) and THP-1 macrophage cell line (**Fig. 5**). Several adjuvants were tested for priming of the inflammasome system. We made use of adjuvants R848, MPLA and LPS at the beginning stages of the experiments and continued with the use of LPS as our priming agent for the remainder of the experiments. In addition, we were able to confirm the cleavage of pro-caspase-1 to active caspase-1 (20kDa) in our BMDM cell line (**Fig. 4A**).

For the remainder of our experiments, LPS served as the priming signal to increase the pro-IL-1β availability in the cells for successful inflammasome activation [11]. Protocols on the use of engineered nanomaterials used LPS as a priming signal in addition to the nanoparticle of interest that served as the activating signal [11][12]. With the addition of the priming signal, a stronger IL-1β cytokine secretion can be noted as seen in **Fig. 5**.

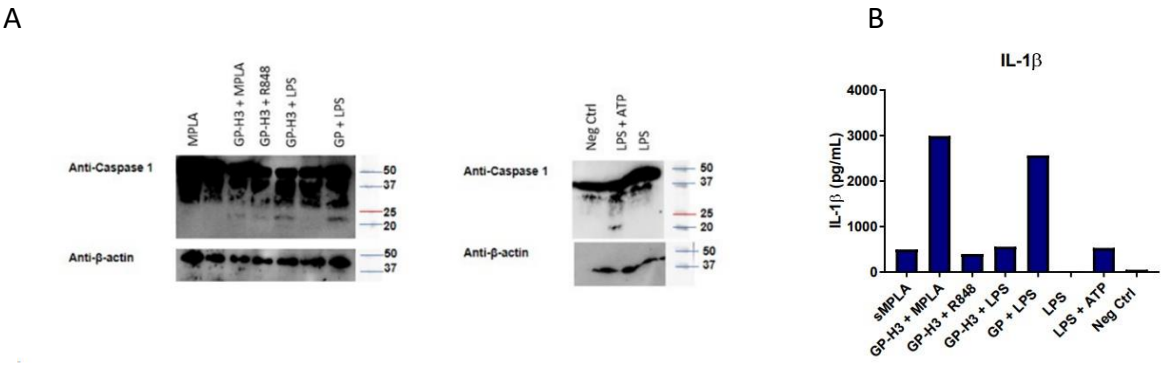

Figure 4: GO-PEI Nanoparticles Induce Inflammasome Activation in BMDM Cells.
A.) Western blot of Caspase-1 protein with Caspase-1 (p20) antibody B.) Cytokine ELISA of IL-1β secretion. *GPs size – 111nm, GP: HA3: Adjuvant – 10:5:1 – 30µg:15µg:3µg

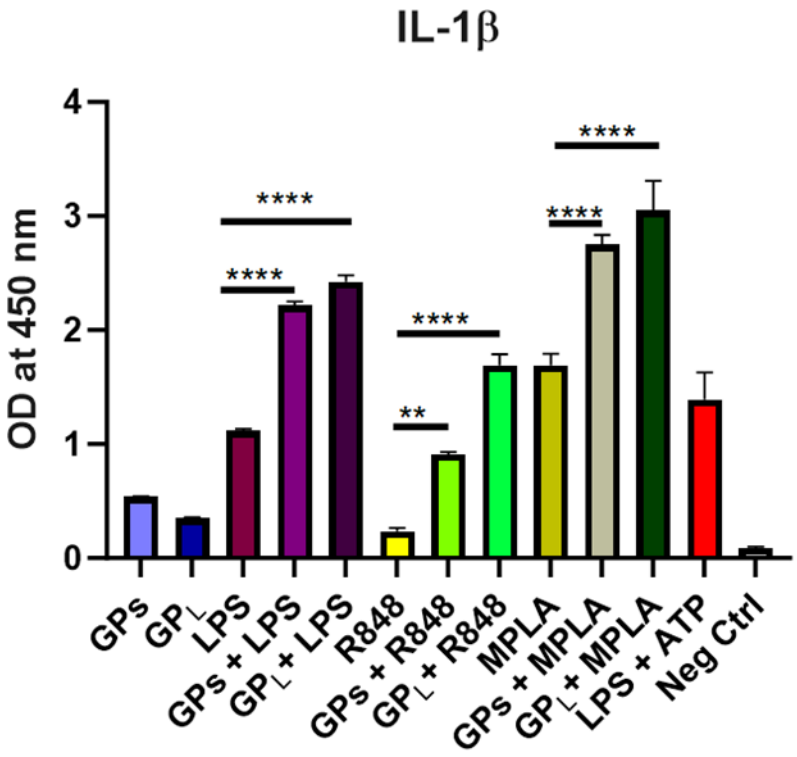

Figure 5: GO-PEI Nanoparticles Induce Inflammasome Activation in THP-1 Macrophage Cells.
Cytokine ELISA of IL-1β secretion. *GPs (111nm, 50µg/mL), GP_L (189.3nm, 50µg/mL), adjuvants: LPS, MPLA and R848 (5ug/mL).

3.2.2 Particulate Incubation Time Influences IL-1β Cytokine Secretion

To determine if particulate incubation time had an effect on the successful induction of inflammasome activation, we incubated THP-1 macrophage cells with GP nanoparticles for

varying periods of time. We detected for IL-1β cytokine secretion as our indicator for inflammasome activation. Macrophage cells were seeded and incubated with GP nanoparticles at a concentration of 50µg/mL. We tested three particulate groups: GP nanoparticles (50µg/mL), GP nanoparticles primed with LPS (5µg/mL) and positive control (LPS + ATP). Macrophage cells were incubated with the particles for 6, 16 and 24-hour incubation periods. Prior to cell culture supernatant collection. Macrophage cells incubated with GP nanoparticles for 16 hours appeared to have the highest secretion of IL-1β cytokine. IL-1β secretion plateaued and/or declined past the 16-hour incubation point (**Fig. 6**).

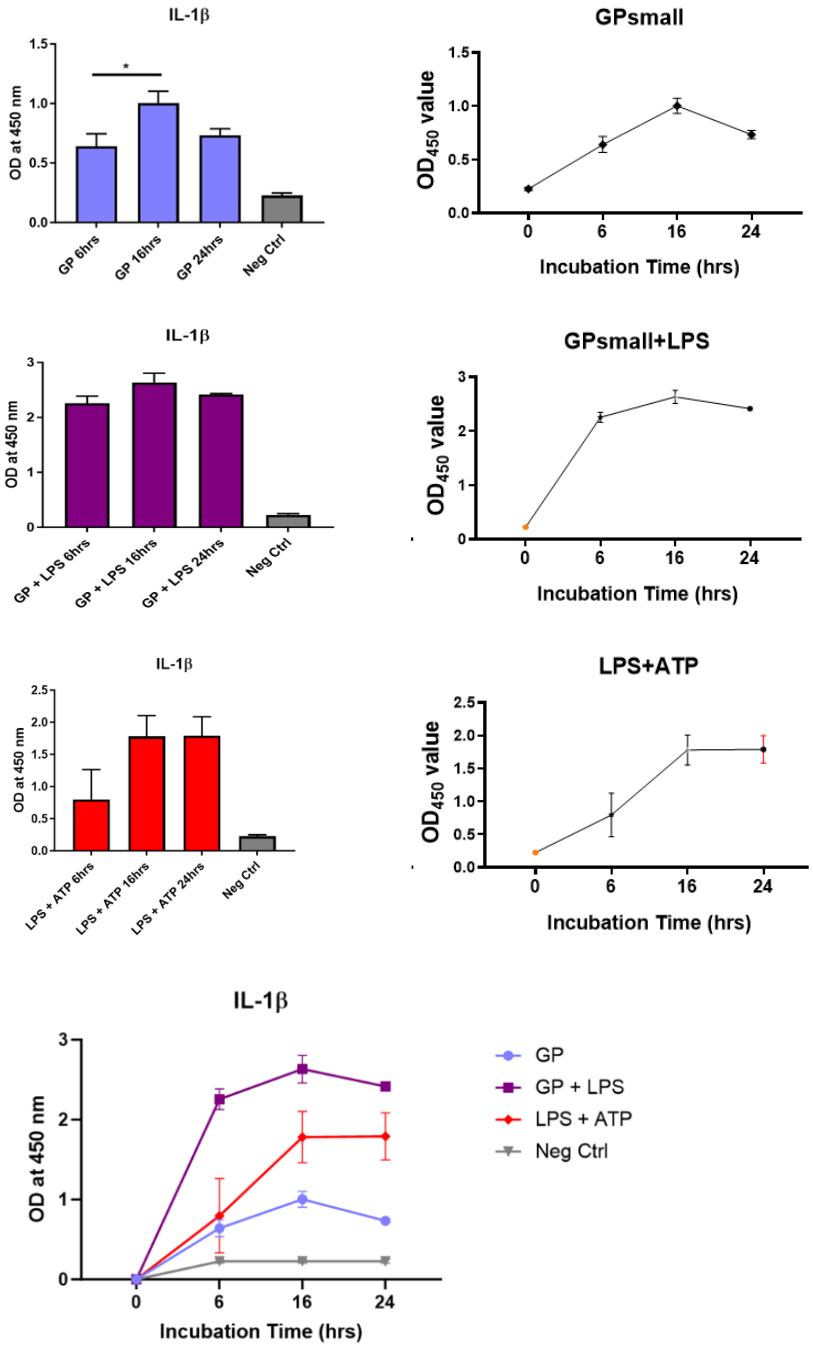

Figure 6: Optimal Particulate Incubation Time for Inflammasome Activation.
Incubation with GO-PEI nanoparticles, GO-PEI nanoparticles primed with LPS and positive control *GPs (111nm, 50µg/mL), LPS (5µg/mL)

3.2.3 Dose-Dependent Relationship Between GP Nanoparticle and IL-1β Cytokine Secretion

We sought to investigate if the induction of inflammasome activation by GP nanoparticles bore a dose-dependent relationship with the concentration of GP nanoparticles. We incubated THP-1 macrophage cells with GP nanoparticles at concentrations of 5, 10, 20, 50 and 100 μg/mL for a period of 24- hours. The levels of IL-1β secretion appeared to mirror a dose-dependent relationship with the concentration of GP nanoparticles as seen in **Fig. 7**. Optimal concentration of GP nanoparticles discovered to induce the most IL-1β cytokine secretion was 50μg/mL. IL-1β secretion declined at a concentration of 100μg/mL. This decline in the secretion of IL-1β could be the result of toxic effects from the use of an excessively high concentration of GP nanoparticles on the cell.

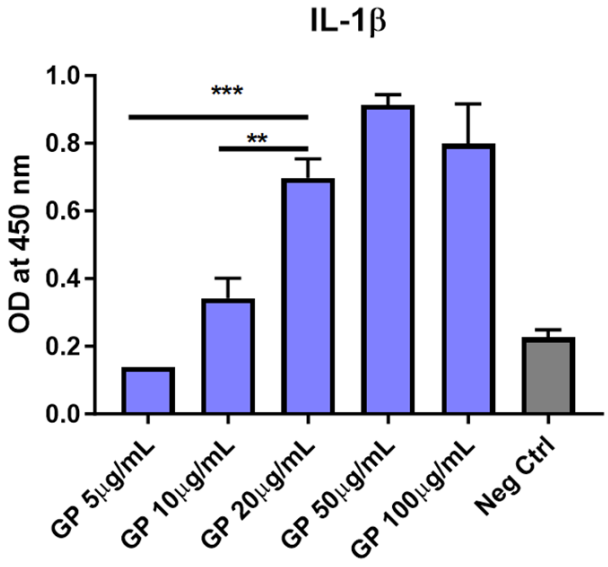

Figure 7: Dose-Dependent Relationship Between GO-PEI Nanoparticle Concentration and IL-1β Cytokine Secretion

*GPs (111nm)

3.3 Aim 2: Determination of Mechanism of Inflammasome Activation by GP Nanoparticle

3.3.1 Cathepsin B Inhibition Affects IL-1ϐ Secretion in BMDM and THP-1 Macrophage Cells

In determining the mechanism of GP-induced inflammasome activation, we blocked two known downstream activators (signals) that have been found to affect successful inflammasome activation. Cathepsin B, a lysosomal cysteine protease involved in the secondary activation of the inflammasome was blocked using a cathepsin B inhibitor, CA-074 Me. To prevent potassium efflux, another secondary signal that plays a role in inflammasome activation, potassium channels were blocked using a potassium channel inhibitor called Amiodarone.

We saw a significant decline in the amount of IL-1β secretion by cells incubated with cathepsin B inhibitor in both BMDM and THP-1 macrophage cell lines. Cells incubated with Amiodarone had no decline in the secretion of IL-1β cytokine (**Fig. 8**).

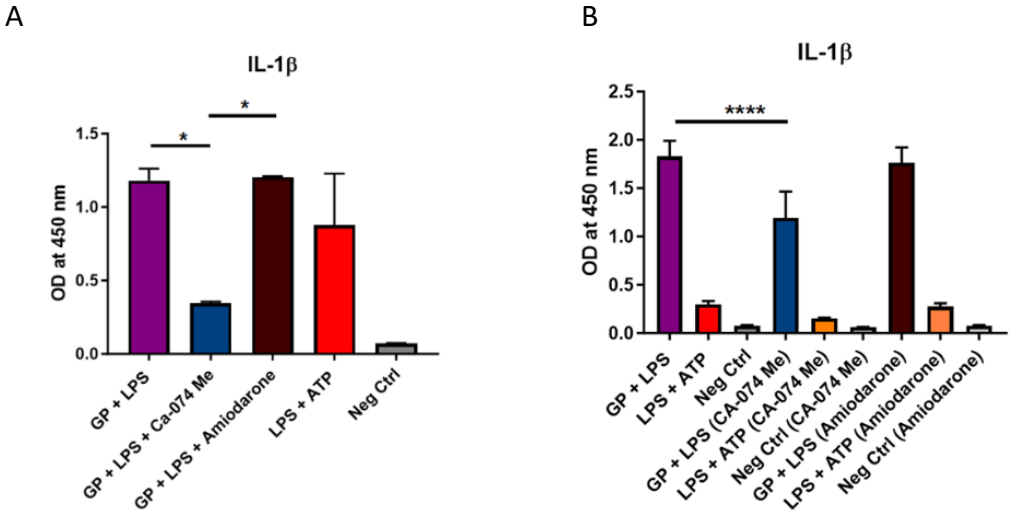

Figure 8: Mechanism of GO-PEI-Induced Inflammasome Activation.
A.) Inhibition in BMDM cell B.) Inhibition in THP-1 macrophage cell. *BMDM (GPs - 111nm, 50µg/mL; LPS - 5µg/mL; CA-074 Me - 20µM; Amiodarone - 10µM). THP-1 macrophage (GP_L – 189.3nm, 40µg/mL; LPS – 4µg/mL; CA-074 Me – 20µM; Amiodarone – 20µM)

3.3.2 GP Nanoparticles Induce Generation of Reactive Oxygen Species

To determine if the generation of ROS was a mechanism by which GP nanoparticles induced inflammasome activation in macrophage cells, we detected the presence of ROS after incubation with GP nanoparticles, GP nanoparticles primed with LPS, and positive control TBHP. We also tested for the generation of ROS at varying concentrations of GP to establish a dose relationship between the generation of ROS and GP concentration. Previous studies on the induction of the NLRP3 inflammasome by engineered nanomaterials showed that one of the mechanisms by which several nanoparticles induced the NLRP3 inflammasome was through the generation of ROS [13].

Our results show that GP nanoparticles were capable of generating ROS. The amount of ROS generated by GP nanoparticles increased with time within 4 hours and appeared to be dose-dependent (**Fig. 9**). However, after a certain concentration (20µg/mL), there appeared to be a decline in the generation of ROS.

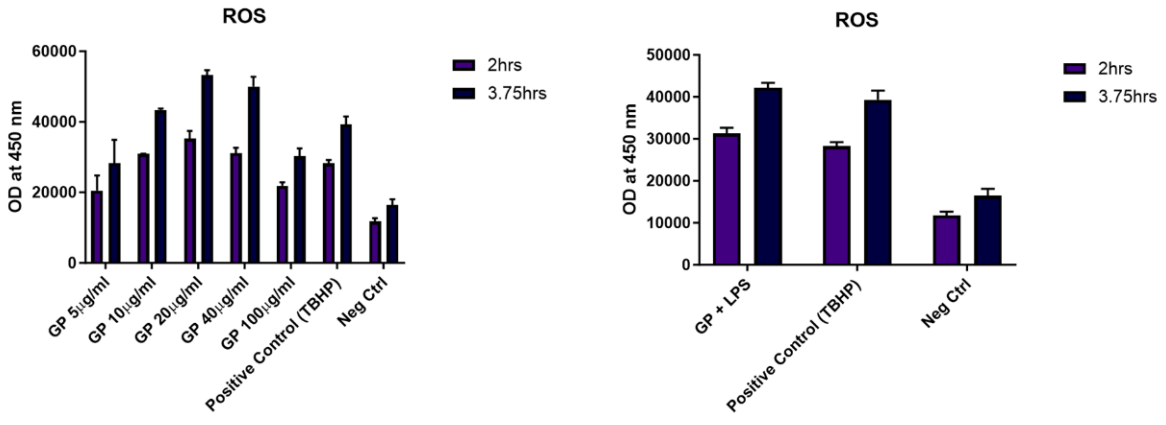

Figure 9: Quantitative Analysis of ROS.
*GP_L (189.3nm), GP: LPS - 10µg/mL/1µg/mL. TBHP - 110µM. Microfluorescence reading at Ex/Em = 490/520nm.

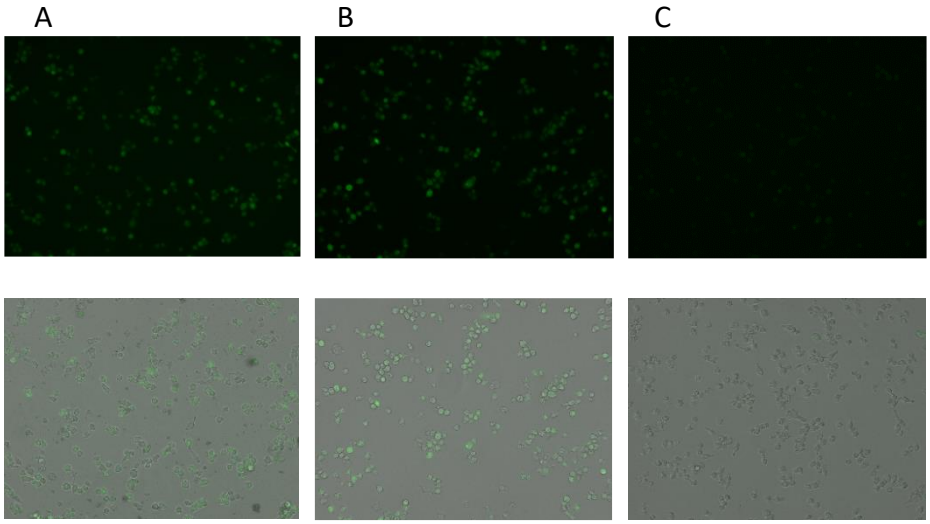

Figure 10: Qualitative Analysis of ROS.
A.) GO-PEI B.) TBHP (positive control) C.) Neg Ctrl. *GPL (189.3nm, 10µg/mL)

4. DISCUSSION

One mechanism through which adjuvants work is in the stimulating of inflammasomes which in turn, turns on the adaptive immune system. It has also been demonstrated that inflammasome activation may be a vital contributing factor to the development of biological responses induced by engineered nanomaterials [13]. Inflammasomes serve as a bridge between the innate and adaptive immune system. There are two ways in which inflammasomes have been found to bridge the gap between the innate and the adaptive immune systems. The first method involves them serving as a “direct bridge” when they cause the maturation of innate antigen-presenting cells which then initiate T-cell-mediated responses. The second method involves the secretion of cytokines and chemokines by the mature innate antigen-presenting cells that then induce specific immunological programs which then induce the adaptive immune system. This second method involves serving as an “indirect bridge” to adaptive immunity [1]. Inflammasome-induced activation of adaptive immunity is realized

through an “indirect route” via the secretion of inflammasome-dependent cytokines, IL-1β and IL-18 [1]. One of the outcomes of inflammasome activation is a form of cell death called pyroptosis and although this eventually terminates the secretion of cytokines, certain DAMPS and cytokines released by the pyroptotic cell prior to cell death help sustain the inflammation in the host [1].

With our *in-vitro* study, we found our GP nanoparticles were able to induce inflammasome activation in murine bone marrow-derived macrophage cells and THP-1 macrophage cells. Verification of inflammasome activation was done mainly through the identification of secretion of IL-1β cytokine and active caspase-1 protein. We found the use of adjuvants like LPS resulted in a more robust secretion of IL-1β cytokine and we believe it served as a priming signal for the induction of inflammasome activation. The method through which the inflammasomes then induce adaptive immunity (direct or indirect bridge) is yet to be studied and might involve investigation through an *in-vivo* study.

Some of the mechanisms that have been associated with the activation of NLRP3 inflammasome by engineered nanomaterials are lysosomal damage and cathepsin B release, TLR4 and NF-κB activation, ROS generation, plasma membrane perturbation, and potassium efflux [13]. With our study, we were able to verify some of the mechanisms involved in GP-induced inflammasome activation. We discovered cathepsin B release and reactive oxygen species generation were some of the mechanisms involved in GP-induced inflammasome activation. We were able to eliminate potassium efflux as a mechanism of GP-induced inflammasome activation.

The outcomes of our study serve as a step towards better understanding the adjuvant mechanism of GP nanoparticles. However, further studies such as imaging of the inflammasome protein complex, confirmation of gasdermin D cleavage and exploration of other mechanisms were needed to determine the exact mechanisms involved in the induction of inflammasome activation by engineered GP nanomaterials.

REFERENCES

1.) Reinke, S., Thakur, A., Gartlan, C., Bezbradica, J. S., & Milicic, A. (2020). Inflammasome-Mediated Immunogenicity of Clinical and Experimental Vaccine Adjuvants. Vaccines, 8(3). https://doi.org/10.3390/vaccines8030554

2.) Kheirollahpour Mehdi, Mehrabi Mohsen *, Dounighi Mohammadpour Naser *, Mohammadi Mohsen and Masoudi Alireza , Nanoparticles and Vaccine Development, Pharmaceutical Nanotechnology 2020; 8 (1) . https://dx.doi.org/10.2174/2211738507666191024162042

3.) Dong, C., Wang, Y., Gonzalez, G. X., Ma, Y., Song, Y., Wang, S., Kang, S., Compans, R. W., & Wang, B. (2021). Intranasal vaccination with influenza HA/GO-PEI nanoparticles provides immune protection against homo- and heterologous strains. Proceedings of the National Academy of Sciences, 118(19), e2024998118. https://doi.org/10.1073/pnas.2024998118

4.) Guo, H., Callaway, J. B., & Ting, Y. (2015). Inflammasomes: Mechanism of Action, Role in Disease, and Therapeutics. Nature Medicine, 21(7), 677. https://doi.org/10.1038/nm.3893

5.) Tweedell, R. E., Malireddi, R. K. S., & Kanneganti, T. D. (2020). A comprehensive guide to studying inflammasome activation and cell death. Nature protocols, 15(10), 3284–3333. https://doi.org/10.1038/s41596-020-0374-9

6.) Christgen, S., Place, D. E., & Kanneganti, T. (2020). Toward targeting inflammasomes: Insights into their regulation and activation. Cell Research, 30(4), 315-327. https://doi.org/10.1038/s41422-020-0295-8

7.) Kelley, N., Jeltema, D., Duan, Y., & He, Y. (2019). The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. International Journal of Molecular Sciences, 20(13). https://doi.org/10.3390/ijms20133328

8.) Jo, K., Kim, J. K., Shin, M., & Sasakawa, C. (2016). Molecular mechanisms regulating NLRP3 inflammasome activation. Cellular and Molecular Immunology, 13(2), 148-159. https://doi.org/10.1038/cmi.2015.95

9.) Rhazouani, A., Gamrani, H., El Achaby, M., Aziz, K., Gebrati, L., Uddin, M. S., & Aziz, F. (2021). Synthesis and Toxicity of Graphene Oxide Nanoparticles: A Literature Review of In Vitro and In Vivo Studies. BioMed research international, 2021, 5518999. https://doi.org/10.1155/2021/5518999

10.) Wegmann, F., Gartlan, K. H., Harandi, A. M., Brinckmann, S. A., Coccia, M., Hillson, W. R., Kok, W. L., Cole, S., Ho, L. P., Lambe, T., Puthia, M., Svanborg, C., Scherer, E. M., Krashias, G., Williams, A., Blattman, J. N., Greenberg, P. D., Flavell, R. A., Moghaddam, A. E., Sheppard, N. C., … Sattentau, Q. J. (2012). Polyethyleneimine is a potent mucosal adjuvant for viral glycoprotein antigens. Nature biotechnology, 30(9), 883–888. https://doi.org/10.1038/nbt.2344

11.) Wang, J., Chen, J., Hang, T., Yu, Y., Liu, G., He, G., Xiao, S., Yang, C., Liu, F., Tao, J., Wu, M. X., & Xie, X. (2018). Physical activation of innate immunity by spiky particles. Nature Nanotechnology, 13(11), 1078. https://doi.org/10.1038/s41565-018-0274-0

12.) Liang, Z., Yang, Y., Yu, G., Zhu, H., Xia, X., Chen, C., Fu, D., Li, M., Cheng, G., Xue, C., Shi, L., Zeng, H., & Sun, B. (2021). Engineering aluminum hydroxyphosphate nanoparticles with well-controlled surface property to enhance humoral immune responses as vaccine

adjuvants. Biomaterials, 275, 120960. https://doi.org/10.1016/j.biomaterials.2021.120960

13.) Liao, X., Liu, Y., Zheng, J., Zhao, X., Cui, L., Hu, S., Xia, T., & Si, S. (2022). Diverse Pathways of Engineered Nanoparticle-Induced NLRP3 Inflammasome Activation. Nanomaterials, 12(21). https://doi.org/10.3390/nano12213908

14.) O'Brien, W.T., Pham, L., Symons, G.F. et al. The NLRP3 inflammasome in traumatic brain injury: potential as a biomarker and therapeutic target. J Neuroinflammation 17, 104 (2020). https://doi.org/10.1186/s12974-020-01778-5

VITAE

IBINABO LILLY-TARIAH

Ibinabolillytariah2@gmail.com | https://www.linkedin.com/in/ibinabo-lilly-tariah-4530a7142/

➢ EXPERIENCE

PROFESSIONAL EXPERIENCE

JUNE 2021 – JUNE 2022
MEDICAL OFFICE ASSISTANT, MIND & BODY CHRISTIAN HEALTH GROUP

- Conducted routine tasks involving patient care.
- Experience assisting medical providers with patient care and procedures.
- Experience performing duties relating to that of a scribe.
- Experience performing administrative tasks, billing and coding, updating and managing team operation manuals.
- Management of patient charts, appointments and operations.

JANUARY 2020 – JUNE 2021
MEDICAL OFFICE STAFF ASSISTANT, MIND & BODY CHRISTIAN HEALTH GROUP

- Assisted the medical office assistant with routine patient and administrative tasks.
- Performed duties relating to that of a scribe.
- Experience with managing patient appointments and scheduling.

OCTOBER 2019 – DECEMBER 2020
OUTREACH AND PATIENT CARE ASSISTANT, MIND & BODY CHRISTIAN HEALTH GROUP

- Assisted with the coordination of outreach initiatives at Mind and Body Christian Health Group.
- Managed patient intake and satisfaction for the primary care office at Mind and Body Christian Health Group.

RESEARCH EXPERIENCE

SEPTEMBER 2022 – CURRENT
GRADUATE RESEARCH ASSISTANT, GEORGIA STATE UNIVERSITY

- Currently participating in research that involves the study of various nanoparticles used as a nanoplatform in the design of vaccines, particularly Influenza vaccines.

JANUARY 2018 – APRIL 2019
UNDERGRADUATE RESEARCH ASSISTANT, KENNESAW STATE UNIVERSITY

- Worked in an organic chemistry laboratory doing research that involved the development of new methods for the synthesis of various tosylate compounds and triflate compounds.

VOLUNTEER EXPERIENCE

AUGUST 2015 – MAY 2016
VOLUNTEER TUTOR, KENNESAW STATE UNIVERSITY
- Tutored in college algebra, precalculus, Biology 1 & 2, and General Chemistry 1 & 2
- Evaluated students' understanding of concepts like polynomial functions and logarithmic functions.
- Provided help to students in areas of concern in their courses.

JULY 2018 – DECEMBER 2018
INTERNATIONAL STUDENT PEER LEADER, INTERNATIONAL STUDENT ASSOCIATION KSU
- Assisted incoming international students with their registration and orientation.
- Informed them of upcoming events and workshops that could be of assistance to them.
- Organized student check-ins designed to offer assistance in areas they might be challenged with academic and non-academic.

MAY 2017 – AUGUST 2017
INTERNATIONAL STUDENT ASSOCIATION FESTIVAL PLANNING COMMITTEE, KSU
- Generated ideas and planned events for the ISA
- Coordinated and collaborated with other organizations.

JANUARY 2017 – APRIL 2017
INTERNATIONAL STUDENT ASSOCIATION PUBLICITY COMMITTEE, KSU
- Publicized events for the International Students Association.
- Assisted with childcare and youth instruction classes.

MAY 2016 – AUGUST 2016
INTERNATIONAL STUDENT ASSOCIATION FUNDRAISING COMMITTEE, KSU
- Brainstormed ideas for fundraising
- Coordinated with other committees for fundraising events

➢ EDUCATION

Georgia State University
Master of Interdisciplinary Studies (M.I.S) Biomedical Science and Enterprise
August 2022 – PRESENT

Kennesaw State University
Bachelor of Science (B.Sc.) Biology
August 2015 – May 2019

Chattahoochee Technical College
Health Science Major
January 2015 – August 2015

➢ SKILLS

- Western Blot
- Cytokine ELISA
- Fluorescent Microscopy
- Cell culture
- Thin Layer Chromatography (TLC)
- Column Chromatography
- Rotary Evaporator
- 400 Nuclear Magnetic Resonance Instrument (NMR)

➢ CERTIFICATIONS

- GHS Lab Certificate (2018)
- PPE Certificate (2018)
- RCRA Certificate (2018)
- Laboratory Safety Certificate (2018)
- Spill Prevention and Control Certificate (2018)
- Compressed Gas Safety Certificate (2018)
- RTK Bloodborne Pathogens Training (2022)
- RTK Global Harmonized System Training (2022)
- RTK Hazardous Waste Awareness Training (2022)
- CITI Basic Human Subjects Research Course (2022)
- Corporate Financial Statement Analysis, Linkedin (2023)
- Enterprise Design Thinking Practitioner, IBM (2023)

➢ INTERESTS

- Vaccine Therapy
- Clinical or Medical Research
- Biomedical Analysis and Diagnostics

➢ ORGANIZATIONS

- National Society of Leadership and Success (NSLS)
- International Student Association, KSU

- **CONFERENCES**

- Georgia International Leadership Conference - 2018