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THE STUDY OF THE EFFECTS OF 50 NANOMETER GOLD PARTICLES ON MOUSE MACROPHAGE AND SPLEEN CELLS

by

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A thesis

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Committee Approval

To the Graduate Faculty:

The members of the committee appointed to examine the thesis of Micah Kingston find it satisfactory and recommend that it be accepted.

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Dedication

This is dedicated to my family, whose patience and faith in me has allowed me to push this project forward to completion.

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I would like to acknowledge Dr. Rich Brey, who genuinely cares for his students and staff and provided a path to graduation. I sincerely thank you.

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List of Abbreviations

Bovine Serum Albumin **(BSA)**

Cytometric Bead Array **(CBA)**

Dischlorflurescein Diacetate **(DCFDA)**

Dynamic Light Scattering **(DLS)**

Enzyme-Linked Immunosorbent Assay **(ELISA)**

Gold nanoparticle (**AuNP)**

Gold nanoparticles **(AuNPs)**

Idaho State University **(ISU)**

Interferon-γ **(IFN-γ)**

Interleukin **(IL)**

Interleukin-2 **(IL-2)**

Interleukin-4 **(IL-4)**

Interleukin-6 **(IL-6)**

Interleukin-10 **(IL-10)**

Interleukin-17A **(IL-17A)**

Lipopolysaccharide **(LPS)**

Median Fluorescence Intensity **(MFI)**

Nanocomposix **(NC)**

Nanoparticle (**NP)**

Nanoparticles (**NPs)**

Phosphate Buffered Saline **(PBS)**

Poly(N-vinylpyrrolidone) or **(PVP)**

Reactive Oxygen Species **(ROS)**

T helper 1 **(Th1)**

T helper 2 **(Th2)**

T helper 17 **(Th17)**

Tetramaethylbenzidine **(TMB)**

Transmission electron microscopy (**TEM)**

Tuberculosis (**TB)**

Tumor Necrosis Factor Alpha **(TNF - α)**

Ultra-Violet Visual Spectroscopy (**UV-Vis)**

Glossary

Adaptive Immune System: A component of the immune system that is composed of highly specialized and systemic cells and processes that eliminate or prevent pathogen growth for specific threats to the body. After an initial response to a specific pathogen, any additional responses to the same pathogen will be greatly enhanced due to the antibodies developed in the initial response by the adaptive immune system.

Antigen: An entity that invokes an immune response.

B Cells: Lymphocyte (white blood cell) that contains a protein known as a B-cell receptor on its surface which allows a B cell to bind to a specific antigen. The B cell is originated in the bone marrow in mammals. The B cell's principal function is to make antibodies against antigens.

BioPure Gold NanoParticles: A product line of gold nanoparticles provided by NanoComposix that claimed to be monodisperse, unagglomerated, endotoxin free, and purified to remove residual reactants.

Bovine Serum Albumin (BSA): A protein derived from cows that is often used as a concentrated standard in lab experiments performed in biology.

Coulter Counter for Cell Viability Confirmation: A device that uses a non-optical method to measure the concentration and size of small objects such as particles or cells in

suspension. This technique uses electrical resistance to measure a volume of the objects that pass through an aperture. Because there is an electrical current is confined within the aperture, as the particles or cells are pulled through the aperture via vacuum they displace the conductive fluid equal to the volume of the cell or particle thereby changing the impedance in a measured pulse across an anode and cathode which allows the volume of the object to be measured.

Cytokines: Hormone like messenger molecules that cells use to communicate.

Cytokine Profile: A record of which cytokines are detected and how much is present within a sample.

Cytometric Bead Array (CBA): The Th1/Th2/Th17 cytometric bead array works by the same process for mouse splenocytes as the ELISA works with mouse macrophages with only two exceptions. The first exception is that there are beads used instead of a dependence of the antigen sticking to the surface of a 96 – well plate. The second exception is that the CBA not only tests for TNF - α, but it also tests for Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interferon-γ (IFN-γ), Interleukin-17A (IL-17A), and Interleukin-10 (IL-10) in the same sample.

Cytotoxic: The condition of being toxic to cells.

CyQuant Proliferation/Viability Assay: A patented process to analyze the viability of

cells that works on the principal of the CyQuant dye that undergoes fluorescence when it is bound to cellular nucleic acids. The solutions containing the cells are first frozen, and then thawed and a lysis buffer is added to complete the breakup of the cell membranes; then the CyQuant dye is added and bonds with the nucleus of the macrophage cells, fluoresces and is measured by a plate reader which will detect emitted fluorescence proportional to the amount of cells that were alive immediately before the freezing process.

Dentritic Cells: A starfish-shaped cell which, when activated, travels from the tissues to the secondary lymphoid organs to present antigen to un-activated T cells.

Dichlorofluorescein Diacetate. (DCFDA): A patented chemical that is non-fluorescent and is used to permeate mouse macrophage cells, then is subjected to esterases within the macrophage to strip off the acetate groups from the dichlorofluorescein (DCF). Then if oxidation within the mouse macrophage cells occurs, it reacts with the DCF and illuminates and becomes trapped within the cell. This fluorescence can be measured over time and is proportional to ROS.

Doppler Shift: A phenomenon where incoming light being reflected off a nanoparticle undergoes a change in wavelength that is proportional to the size of the nanoparticle.

Double Layer (DL): The structure that appears on the surface of an object when it is exposed to a fluid.

Dynamic Light Scattering (DLS): A process performed by shining a monochromatic light beam into a clear container that holds suspended particles such as spherical nanoparticles in a solution of water. The small particles undergo Brownian motion which causes a Doppler Shift when the incoming light hits the moving particle. The Doppler Shift changes the wavelength of the incoming light, which is proportional to the size of the particle. From there it is possible to back calculate the hydrodynamic diameter and Zeta potential of the suspended particles.

Econix Gold Nanoparticles: A product line of gold nanoparticles provided by NanoComposix that is claimed to be monodisperse and unagglomerated gold nanoparticles but are not guaranteed to be free of endotoxins or residual reactants.

Electron Paramagnetic Resonance Spectroscopy: An imaging technique used where the spins of electrons are excited and emit energy as they return to the ground state.

Electrophoretic Mobility: The migration of charged colloidal particles or molecules through a solution under the influence of an applied electric field usually provided by immersed electrodes. Also called electrophoresis.

Enzyme-Linked Immunosorbent Assay (ELISA): The sandwiched ELISA works by placing the TNF - α capture antibody in a plate, adding the sample which may or may not contain the antigen being screened, then adding detection antibody with an enzyme linked to it. Several washes occur and then a substrate for the enzyme is placed in the mix and the amount of TNF - α can be quantified because the substrate reacts with only

the enzyme that remained in the solution, which are connected to the TNF - α antibodies. Then the reaction is stopped and either read visually if testing whether or not any TNF - α is in the sample or is quantified using a plate reader.

Esterases: the process of a hydrolase enzyme that splits esters into acid and alcohol.

Flow Cytometry: Flow cytometry works by pumping the sample, consisting of the tissue broken up into single cells, through a flow chamber one cell at a time at approximately 500 cells per second. As the cells are pumped through the flow chamber they are hit by a laser, and the way in which the laser light emits from the cells gives information about the cell.

Fluorochrome: Fluorescent substance used in flow cytometry and fluorescence microscopy.

Gold Nanoparticle (AuNP): A nanoparticle consisting of gold, often spherical in shape and containing a stabilizing agent as a coating (necessary for many small nanoparticles).

Homeostasis: A property in a life system where internal conditions remain stable and relatively constant such as temperature regulation and balancing pH within the human body.

Innate Immune System: Known as the second line of defense (after physical barriers of

the body such as skin) that comprises the cells and mechanisms that defend the host from infection by other organisms in a non-specific manner. The innate immune system also sends the required signals necessary to activate the adaptive immune system when necessary.

Interleukins (IL): A protein (cytokine) that is used for communication between leukocytes.

Leukocytes: A generic term that includes all of the different kinds of white blood cells.

Light Microscopy: The use of a visible-light microscope to observe magnified objects such as cells.

Lipopolysaccharide (LPS): A large molecule of the outer membrane of Gram negative bacteria consisting of a lipid and a polysaccharide composed of O-antigen, with the outer core and inner core joined by a covalent bond and elicit strong immune responses in animals. Also known as endotoxins.

Lymphocytes: The generic term for the white blood cells within the body that can include natural killer cells, T cells, and B cells.

Lysis Buffer: A solution used to breakup cell membranes, usually containing some type of detergent, along with protease inhibitors to prevent protein degradation.

Macrophage Cells: Phagocyte able to engulf pathogens and debris. The macrophage is considered the "sentinel", or super-soldier of the innate immune system. The literal translation of macrophage is "Big Eater".

Nanoparticle (NP): A particle that has one or more dimension that is less than 100-nm.

Pathogenic response: A response that can produce disease.

Phagocytes: Cells such as macrophages and neutrophils that engulf (phagocytose) invaders.

Phosphate Buffered Saline (PBS): A water-based salt solution containing sodium phosphate and in some cases potassium chloride and potassium phosphate that match the osmolarity and ion concentration of the human body.

Poly(N-vinylpyrrolidone) or (PVP): A coating used in some biological applications and coatings for nanoparticles that is PVP is completely soluble in water and most conventional solvents and is non-irritant to the skin, eyes, and mucus membranes.

Raman Spectroscopy: Spectroscopy that is used to observe rotational, vibrational, and other low-frequency modes within a system and has been greatly enhanced by using AuNPs that were conjugated with antibodies.

Reactive Oxygen Species (ROS): Chemically reactive molecules containing oxygen that are a natural byproduct of the normal metabolism of oxygen and have significant roles in cell signaling and homeostasis. However, during time of stress, ROS levels can increase dramatically which can cause significant damage to cell structures.

T Cells: A lymphocyte (white blood cell) that originates from the thymus and has T-Cell receptors on its surface and plays a major role in the immune system and response to specific pathogens by sending signals to other cells to attack the pathogen.

T helper 1 (Th1) Response: The response that is optimal for dealing with viruses and cancer.

T helper 2 (Th2) Response: The response that is best for larger extracellular pathogens.

T helper 17 (Th17) Response: The response that is thought to be in several disease states with exaggerated immune responses such as autoimmune disease and hypersensitivities.

Th1/Th2 Balance: The condition where the IL's associated with Th1 and Th2 are in the optimal ratios with respect to each other which is believed to be present in the absence of deleterious health conditions such as cancer, viruses, and other pathogens.

Transmission electron microscopy (TEM): The technique in which a beam of electrons are transmitted through an ultra-thin sample, interacting with the sample as it passes through. An image is created and magnified from the interaction and focused onto a screen or a camera.

Tuberculosis (TB): A common and sometimes fatal disease caused by various strains of bacteria that typically attacks the lungs. Also known as consumption.

Tween-20: a nonionic polyoxyethylene surfactant that is an effective detergent for thorough removal of the excess protein in washing steps.

Ultra-Violet Visual Spectroscopy (UV-Vis): A process by which visible light or an ultra-violet source that is separated into its discrete wavelengths by either a diffraction grating or some type of dispersion device. Then each single wavelength beam is split into two beams that are equal in intensity. One beam is sent through a cuvette containing the sample while the other beam is passed through a reference cuvette containing a reference solvent. The intensities of both beams are measured by electronic detectors and compared.

Zeta Potential: the zeta potential is the electric potential in the interfacial double layer (DL) at the location of the slipping plane versus a point in the bulk fluid away from the interface. In other words, zeta potential is the potential difference between the dispersion mediumand the stationary layer of fluid attached to the dispersed particle.

Abstract

Nanoparticles are significant to multiple industrial processes, consumer products, and medical applications today. The health effects of many different types of nanoparticles, however, are largely unknown. The effects of exposing mouse macrophage and spleen cells with and without Lipopolysaccharide to 50-nm gold nanoparticles coated with Poly(N-vinylpyrrolidone) at multiple concentrations were studied. Gold nanoparticles had no effect on macrophage viability and produced an inhibitory response to macrophage inflammation observed by decreasing release of TNF – α with increasing gold nanoparticle concentration. The reactive oxygen species in exposed macrophages were reduced compared to untreated controls both with and without lipopolysaccharide. In splenocyte cultures, gold nanoparticles had no effect alone, but significantly reduced the release of IL-17, IL-10 and TNF – α triggered by lipopolysaccharide. These results suggest that gold nanoparticles used here are not cytotoxic to immune cells at these concentrations, but may affect cellular responses to infection or inflammation.

Introduction

A nanoparticle (NP) is defined as a particle that has at least one dimension that is between 1 and 100 nm, which is about 1/50,000th the width of a strand of human hair (Dwivedi & Paul 2013). Nanoparticles (NPs) can have unusual and useful properties because of their small volume to surface area ratio, and their surface structure, which affects the surface reactivity, solubility, shape, and aggregation. Because the small size has huge implications for reactivity, their creation must include controlling the materials' purity and composition. This technology is the driving force behind the creation of nanomaterials for many emerging nanotechnology industries today. The applications of nanotechnology are far reaching and will affect each of us through industrial, commercial, and even medical applications (Hussain et al 2013, Eissa et al 2013).

Nanoparticle Benefits

Some applications of nanotechnology in consumer products include the manufacture of more skid resistant tires, higher quality paints, nano-sized actuators, and nanocomposites that are lighter in weight but much stronger than the materials they will replace, such as a stain-resistant pair of khaki pants. Benefits of the uses of nanotechnology within industrial processes include increasingly smaller and more efficient electronics, and more effective polymers to be used in items such as packaging or automotive components.

Some of the areas within the medical industry affected by nanotechnology include improved drug transport, ailment detection, and more effective treatments for a variety of diseases by using multiple nanomaterials. It has been predicted that the economic impact of nanotechnology will surpass the effect of the industrial revolution of the early 20th

century, and nanotechnology is estimated to become a $1 trillion market by 2015 (Nel et al 2006).

Potential Health Risks of Nanoparticles

There are clearly tremendous benefits that are created by using NPs. However, the advancement of nanotechnology is growing so fast that research into the effects of these kinds of materials on the environment, safety of workers, and health of members of the public may not be able to keep pace. As nanotechnology is further incorporated into the various industrial processes, the potential for human exposure and inadvertent releases of these materials into the environment increases substantially. It would seem prudent to understand any deleterious consequences that may arise from exposure to these materials (Olson & Gurian 2012).

There have been several studies on the toxicology of NPs consisting of various types of materials, but it has become apparent that the properties and therefore the toxicity of these materials can change with the size, shape, coating, composition, surface structure, solubility, and their relative degree of aggregation. This complicates a definitive statement as to the health risks of any particular NP. Perhaps a specific health study will eventually be necessary for each case. Further studies may allow the prediction of health effects through knowledge of the critical physicochemical properties that impact reactivity with cells.

One possibility is the ability of NPs to induce Reactive Oxygen Species (ROS) in cells they encounter, since ROS can lead to not only damage to cell components and cell death, but also intracellular signaling that can impact downstream events. In addition, one must take into account the route of exposure and which cells will be exposed. Specific

types of NPs has been used to develop generalized health risk predictions, these are usually associated with what part of the body is exposed to the NPs. A summary of this kind of information is provided in Fig. 1.

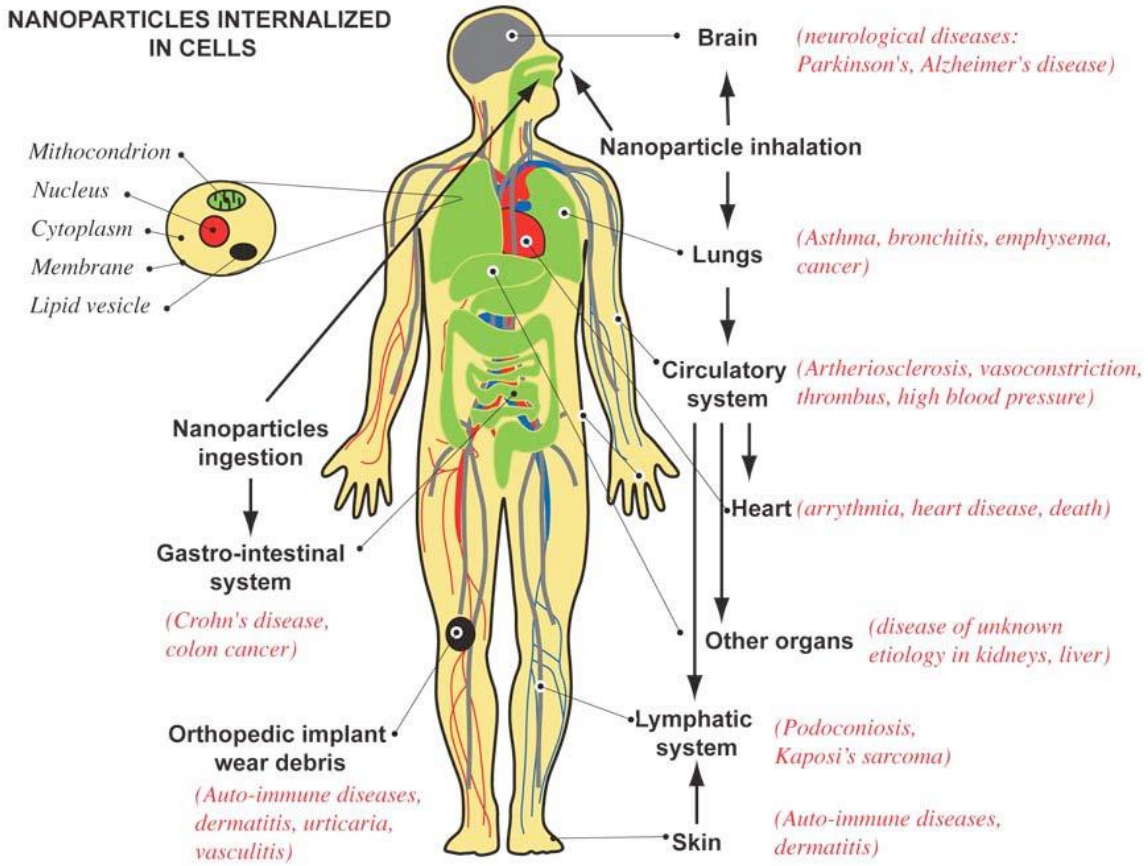

Fig. 1. Possible adverse health effects by exposure to NPs (Dwivedi & Paul 2013; Buzea et al 2007).

Nanoparticle Selection

While many studies focus purely on the cytotoxicity of NPs, which would lead to health effects simply due to tissue damage, some short and long-term effects can be induced through modulation of the immune response. Altering immune responses can lead to allergies, inflammation, and even cancer, making this a critical area of research.

For this study, a type of NP was selected that was readily available commercially and has a wide variety of potential applications. Gold has been used historically in the treatment of painful inflammatory conditions such as rheumatoid arthritis, but its effects at nanoscale levels have not been thoroughly investigated. AuNPs have been used in cell imaging, targeted drug delivery, cancer diagnostics and treatments.

Specific examples of the application of AuNPs in medicine have been reported in the literature. AuNPs at approximately 14 nm in diameter and attached to specific oligonucleotide probes have been shown to undergo a color change when interacting with target mycobacterial DNA both in pure mycobacterial cultures and in isolates from tuberculosis (TB) patients. This could lead to a rapid, low cost diagnostic tool (Hussain et al 2013). In another case, bladder cancer could be detected non-invasively by testing for a cancer-specific RNA in patient's urine with a solution containing magnetic 90-nm AuNPs which also underwent a colorimetric change upon interaction with the target sequence (Eissa et al 2013). There have also been applications of gold in the treatment of cancers and other ailments such as arthritis. These treatments are based on the NPs immune-modulating effects (Kim et al 2001). However, the effects of AuNPs on the immune system have not been systematically studied.

AuNP Quality Verification

AuNP Quality Verification by Visual Inspection Using the Plasmon Resonance Effect

To evaluate the quality of the particles the particle consistency, stability, size distribution, and agglomeration were evaluated. There are several common methods used in the evaluation of consistency and particle stability. First, one may perform a visual inspection of the solution containing the AuNPs realizing that due to the Plasmon

resonance effect the solution should be bright red in color (Ghosh & Pal 2007). A byproduct of the Plasmon resonance effect may be observed when the size of a particle becomes less than or equal to the wavelength of light that normally reflects off the macroscopic version of the material. If the AuNPs become unstable or large scale agglomeration occurs, the color of the solution will change from bright red to dark blue (Ghosh & Pal 2007). Measuring the wavelength of light reflected by the particles provides direct information about the particles' stability while in suspension. If the wavelength (i.e. the color) changes the particles are either dissolving in solution or undergoing agglomeration. A more quantitative approach is to perform UV-Vis.

AuNP Quality Verification Using UV – Visual Spectroscopy (UV-Vis)

When performing UV-Vis on 50-nm AuNP suspensions, the peak absorbance occurs at approximately 530 nm. If agglomeration begins, the size of the grouped particles increase causing the red light to be absorbed (from 650 nm to700 nm) and the blue light begins to be reflected. This color change does not occur until the peak absorbance of the suspension approaches 650 nm, which can happen over a few minutes to a few days. This allows one to confirm the quality of the AuNP solution with both a visual inspection of the color and with UV-Vis (Ghosh & Pal 2007).

UV-Vis uses light from either a visible light or ultra-violet source. The light is separated into its discrete wavelengths by either a diffraction grating or some other type of light splitting device. The subsequent monochromatic light beam is split into two beams of equal intensity. One beam is sent through a cuvette containing the sample while the other beam passes through a reference cuvette containing a reference solution. The intensities of both beams are compared. The reference beam's intensity (I_o) should

have had little or no light absorption, the intensity of the reference solution is simultaneously measured. If the sample does not absorb light of a particular wavelength then $I = I_o$, but if the sample does absorb light than I is less than I_o. This change can be plotted on a graph of absorbance versus wavelength where absorbance $A = log\left(\frac{I}{I_o}\right)$. A block diagram illustration of the setup of a typical UV – Vis spectrometer is provided in Fig. 2.

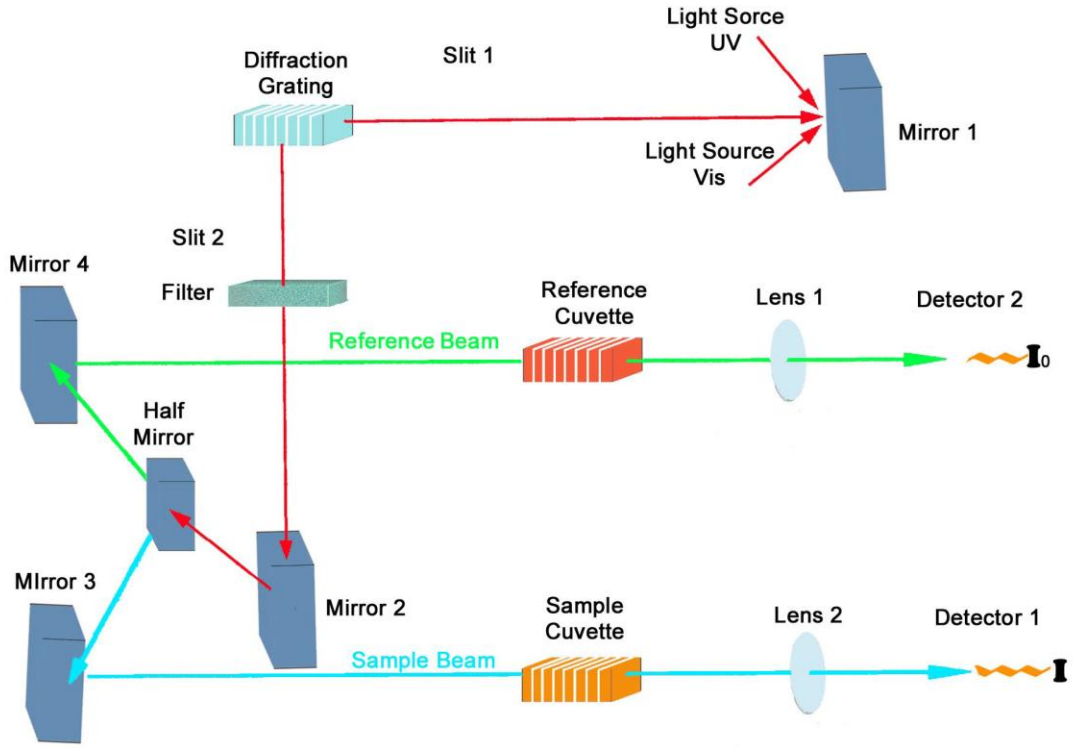

Fig 2. Illustration of UV- Vis spectroscopy.

AuNP Quality Verification Using a Transmission Electron Microscope (TEM)

The third method commonly used to evaluate the quality of particle shape, size distribution, and agglomeration involves using a TEM. The TEM allows one to observe the actual AuNPs to inspect their shape, size, size distribution, and to actually see if

agglomeration is occurring.

The TEM uses the thermionic agitation of a tungsten filament to produce free electrons that are accelerated through a potential gradient. Using magnetic lens configurations the electrons are organized into a beam within a vacuum chamber. Then the electron beam is accelerated through an electromagnetic field that focuses the beam until it is passed through the sample material. The sample must be very thin; less than 100 nm. The electrons that pass through the sample strike a phosphor screen, CCD, or film, to produce an image of the sample that may be in real time. Where the sample has less density, more electrons penetrate the sample and the image is brighter; a darker image is produced in areas where the sample is denser because fewer electrons pass through the sample to strike the imaging device.

AuNP Quality Verification Using Dynamic Light Scattering (DLS)

A third way to confirm the quality of the AuNPs is DLS which can measure the average hydrodynamic diameter and Zeta potential. The hydrodynamic diameter of an AuNP is the average diameter of AuNPs in the solution and includes all of the coatings that are on the nanoparticle to help to keep it stable. If agglomeration occurs this will quickly affect the hydrodynamic diameter and can be confirmed using DLS. The Zeta potential is proportional to, and therefore a measure of, the average surface charge density of the NP at the double layer.

DLS is performed by shining a monochromatic light beam into a clear container that holds the spherical NPs suspended in a solution of water. Since the NPs are moving because of Brownian motion, the incoming light being reflected off the particles will undergo a change in wavelength that is proportional to the size of the NP. The

hydrodynamic diameter is determined by correlating the magnitude of the Doppler shift to particle diameter. It is also possible to back calculate the hydrodynamic diameter and Zeta potential of the NP using theoretical models and the electrophoretic mobility measured by the DLS (Ware et al 1998, Gittings & Saville 1998). A diagram of how the DLS is performed is found in Fig. 3.

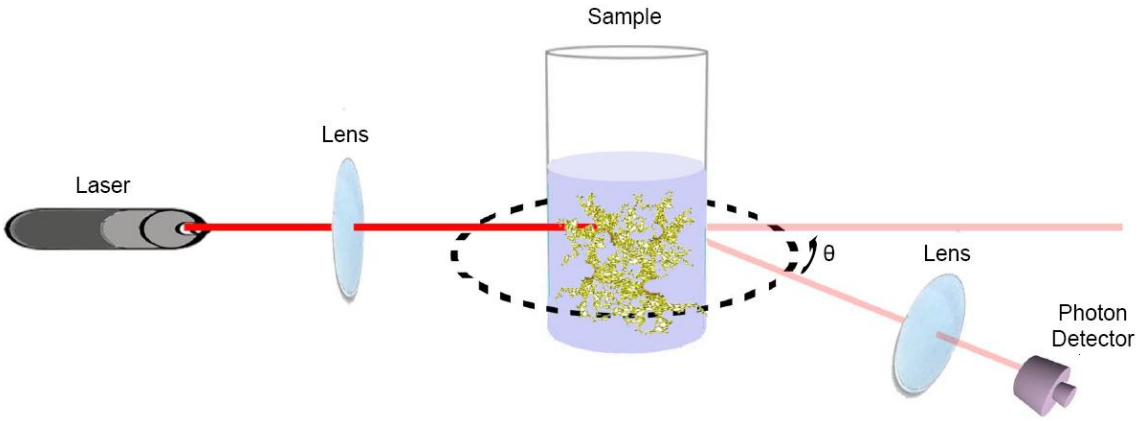

Fig. 3. Diagram of DLS process.

Determining the Size of the AuNP

There were two overriding factors governing the decision on particle size for this investigation: AuNP stability and cell uptake. When a NP gets small enough, its size distribution and therefore the stability of each individual NP can change in a process known as Ostwald ripening (Voorhees 1985).

Ostwald ripening is a phenomenon in which smaller particles in a solution will dissolve and deposit themselves on larger particles. It is theorized that Ostwald ripening is caused by multiple factors including a temperature gradient between particles, surface charge gradient, or even an increased curvature within the shape of the NP that can cause it to dissipate, travel through the media the NP is suspended in, and attach to a different

NP. It is thought that perhaps the primary cause of Ostwald ripening is thermodynamic instability of the atoms on the surface of the NPs which dissolve and reattach to larger NPs to become more stable (Voorhees 1985). Evidence suggests that Ostwald ripening occurs infrequently after the NP size reaches 15 nm and above (Wang et al 2011). An illustration of Ostwald ripening can be seen in Fig. 4.

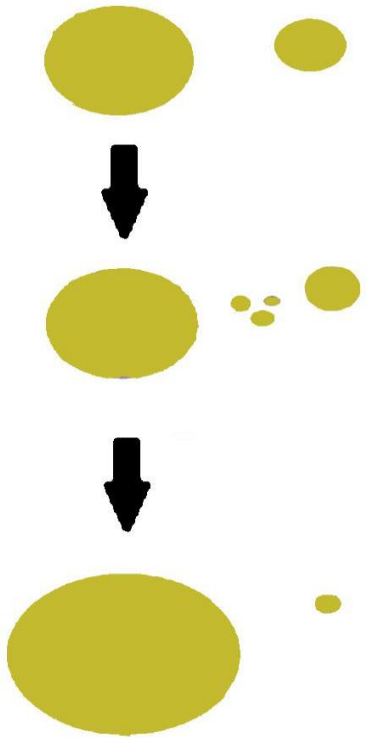

Fig. 4. Diagram of Ostwald Ripening.

AuNP particles large enough to avoid Ostwald ripening were selected for this investigation. Another issue to resolve was to find the size of AuNP that would have the most likelihood of being absorbed by mammalian cells. Chithrani et al investigated uptake of spherical AuNPs in mammalian cells ranging in sizes, from 14-nm through 100-nm. Their investigation concluded that 50-nm AuNPs had the most uptake of all

sizes tested (Chithranie et al 2006). Since 50-nm AuNPs had the best uptake in mammalian cells and was large enough to avoid significant Ostwald ripening, it was chosen for this investigation.

Confirming Cellular Uptake

The cellular uptake of the AuNPs can be verified using two methods; light microscopy and flow cytometry. The first method of verifying cellular uptake, light microscopy can be used to visualize the internalization of the AuNPs and their distribution in the cytoplasm with the appropriate magnification.

The second method of verifying cellular uptake is flow cytometry. Flow cytometry works by pumping the sample, consisting of the tissue broken up into single cells, through a flow chamber one cell at a time at approximately 500 cells per second. As the cells are pumped through the flow chamber they are hit by a laser, and the way in which the laser light bounces of the cells gives information about the cell. More specifically, if light is bounced off in small angles it is referred to as forward scatter, which gives the size of the cell, and if light is bounced off orthogonally it is referred to as side scatter which gives information of particle uptake. The light is then absorbed by a light detector which sends the information to a computer where a histogram is produced.

Each cell type within the immune system has a specific combination of forward and side scatter measurements making a histogram of the measurements. Cells can also be stained using fluorochromes which when struck by light, emit a specific color of light depending on the type of fluorochrome. The light emitted by the fluorochrome is then sent through a filter and is finally absorbed by another detector. Then that information is also sent to a computer and used to identify the fluorochromes that are labeling specific

components within the cell. A diagram of this can be seen in Fig. 5.

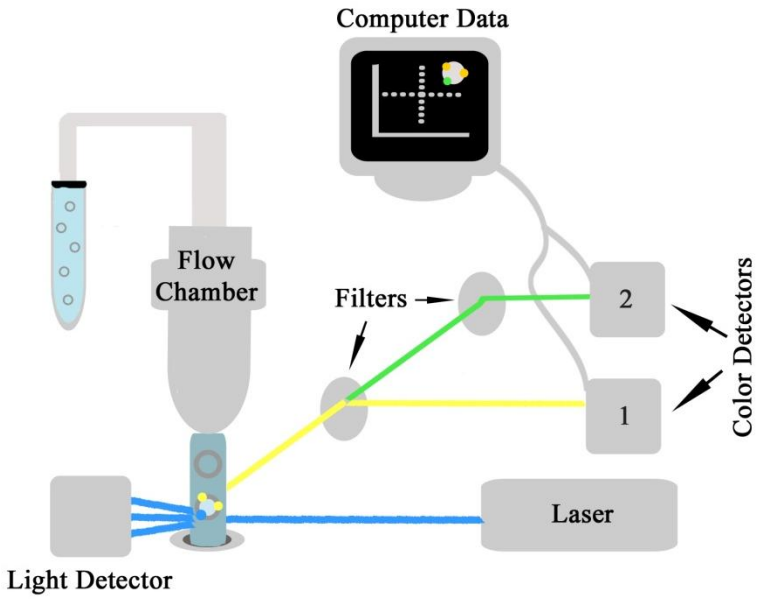

Fig. 5. Diagram of typical flow cytometry system.

Determining the AuNP Coating

NPs are coated during synthesis to decrease the likelihood of Ostwald Ripening. It is possible for such coating to confound the particle behavior especially if the behavior being evaluated is toxicity. It is possible that either the coating itself or some interaction of the particles and the coating can confound the interpretation of an investigation. The AuNPs were also coated in this investigation.

To minimize the potential for coating related confounding factors, particles coated with Poly(N-vinylpyrrolidone) (PVP) were used during the investigation. PVP is completely soluble in water and most conventional solvents. These are characteristics that have led to its wide use in the pharmaceutical industry (Liu et al 2013). PVP is a non-irritant to the skin, eyes, and mucus membranes. A failure of PVP as a particle coating is that may become detached from the AuNP after long periods of storage (Liu et

al 2013). The desirable properties of PVP including water solubility, chemical stability, biocompatibility, and general bio-inertness, justify its use as a coating for the AuNPs used in this investigation.

Viability Tests

A second approach to examine the possible toxicity of the AuNPs is to place the AuNPs at various concentrations with mouse macrophage cells and investigate cell death occurrence in the cells that are treated with the AuNPs as compared to control cell populations. In other words, to perform viability tests on the mouse macrophage cells treated with the AuNPs. There are two ways to perform viability tests; using a CyQuant proliferation/viability assay and to use a Coulter Counter to count the adhering cells present within the flask. Mouse macrophage cells must be alive in order to adhere to the surface of the flask. This allows the viability of the cells to be confirmed by washing away any dead cells, scraping the flask, and using the Coulter Counter to count the amount of living macrophage cells.

The CyQuant proliferation/viability assay works on the principal of the CyQuant dye that undergoes fluorescence when it is bound to cellular nucleic acids. The solution containing the cells are first frozen, and then thawed and a lysis buffer is added to complete the breakup of the cell membranes then the CyQuant dye is added and bonds with the nucleus of the macrophage cells, fluoresces and is measured by a plate reader.

The amount of fluorescence is proportional to the amount of cells that were alive immediately before the freezing process. The idea is that if a cell dies before such time, it will break apart and dissolve in the solution of cells. But if the cell is alive just before freezing, the cell and nucleus are intact, and the nucleus remains intact throughout the

freezing and lysis process to give the nucleic acid for the CyQuant dye to bind to and then fluoresce and therefore a measurement can be made of the amount of live cells. A diagram of this process can be seen in Fig. 6.

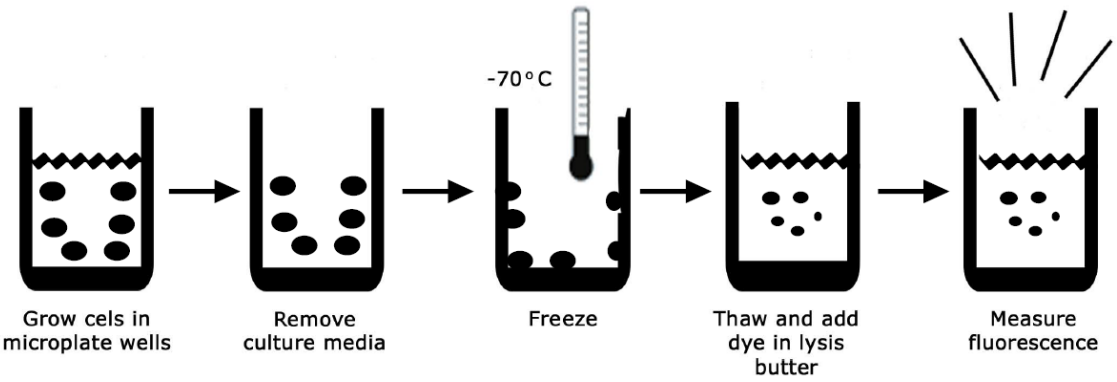

Fig. 6. Illustration of process of CyQuant proliferation/viability assay.

The Coulter Counter uses a non-optical method to measure the concentration and size of a solution containing small objects such as particles or cells. This technique uses electrical resistance to measure a volume of the objects that pass through an aperture. Because there is an electrical current confined within the aperture, as the particles or cells are pulled through the aperture via vacuum they displace the conductive fluid equal to the volume of the cell or particle thereby changing the impedance in a measured pulse across an anode and cathode which allows the volume of the object to be measured. An illustration of how a Coulter Counter works is shown in Fig. 7.

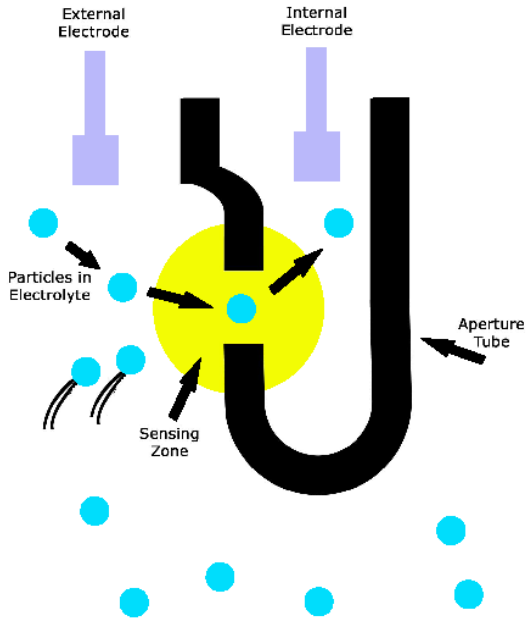

Fig. 7. Illustration of how a Coulter Counter works.

Immune Response

The innate immune system consists of phagocytic cells such as macrophages and dendritic cells, whose major functions are to remove foreign invaders and call in more immune cells to fight the infection. The process of "calling for help" consists of producing inter-cellular chemical signals called cytokines or interleukins. There are several cytokines produced by macrophages, but the earliest and most prevalent one is Tumor Necrosis Factor Alpha (TNF - α). Bacteria or bacterial components such as Lipopolysaccharide (LPS) trigger the production of large amounts of TNF - α from macrophages. For this study, a mouse macrophage cell line was used to test ROS, viability, uptake of AuNPs, and the production of TNF - α.

Another role for the innate immune system is to determine the type of infection (viral, bacterial, or fungal) and then to tailor the immune response appropriately using

different combinations of cytokines, which activate different components of the adaptive immune system. The adaptive immune system consists of T cells and B cells which respond to the cytokines with specific responses designed to destroy the invaders. The tailored responses have been grouped into three general response types, each with characteristic cytokine profiles: T helper 1 (Th1), T helper 2 (Th2) and T helper 17 (Th17). The Th1 response is optimal for dealing with viruses and cancer; the Th2 response is best for larger extracellular pathogens, and the Th17 response is described in several disease states with exaggerated immune responses such as autoimmune disease and hypersensitivities (Afzali et al 2007, Carballeda et al 2007). For this study, combined mouse splenocytes, including macrophages and T and B cells, were used to determine the effect of 50-nm AuNPs on the different cytokine profiles.

Villiers et al 2009 found that 10-nm AuNPs were not cytotoxic when exposed to mouse dentritic cells. The dentritic cells suppressed the release of interleukin-12. Interleukin-12 is implicated in inflammation and in auto-immune diseases (Kim et al 2001). Lin et al 2013 found that 15-nm AuNPs were effective in transporting the immune stimulant of CpG oligodeoxynucleotide. They also observed that injecting 30-nm tmCpG-AuNPs significantly inhibited tumor growth in Phosphate Buffered Saline (PBS) treated mice cells. The amount of TNF – α released from mouse macrophage and dendritic cells are also affected. TNF – α is a cytokine that is a primary mediator in the protective immune responses that induces the interleukin Th1, and if the Th1/Th2 balance is disrupted it can lead to disease (Kim et al 2001, Overocker et al 2012).

The immune response of the AuNPs can also be tested by adding a substance that is known to cause a negative (inflammatory) immune response and/or increased ROS and

investigating the cytokines and ROS released due to the added substance. Once such a substance is added, it can be determined if the AuNPs can reduce the harmful effects of the known cytotoxin thereby proving a positive effect. One such compound that can cause an inflammatory response is Lipopolysaccharide (LPS) (Pfau et al 2012). The effect that 50-nm AuNPs have on the inflammatory response of LPS on mouse macrophage and spleen cells was investigated.

Evaluating Particle Toxicity by Measuring Reactive Oxygen Species (ROS)

To evaluate toxicity, one may evaluate the creation of Reactive Oxygen Species (ROS) generated by the AuNPs. ROS can affect the functionality of proteins, lipids and nucleic acids within cells, leading to reduced function, tumorigenic mutations in DNA, and even cell death. In addition, they can also induce signaling through redox-sensitive transcription factors. ROS have been examined and found to have significantly increased due to asbestos exposure which later on led to considerable health problems such as reduced pulmonary function, enhanced autoimmune responses, and increased mortality from lung cancer (Blake et al 2007).

It is understood that many types of cancer cells produce an increased magnitude of ROS compared with normal cells. This implies that ROS can also be protumorigenic, or capable of causing tumors (Nogueira et al 2013). It is believed that there will be a difference in the measured quantity of ROS in cells that are exposed versus mouse macrophage and spleen cells that have not been exposed to AuNPs. It may also be possible to test the oxidative stress that is potentially caused by the AuNPs by using a Dichlorofluorescein Diacetate (DCFDA) assay which measured the oxidative stress of mouse macrophage cells throughout a time course (Overocker & Pfau 2012).

The chemical Dichlorofluorescein Diacetate (DCFDA) is non-fluorescent and is able to permeate the mouse macrophage cells. Then esterases, enzymes that split esters into acid and alcohol, occurs within the macrophage cells stripping off the acetate groups from the dichlorofluorescein (DCF). Then if oxidation within the mouse macrophage cells occurs, it reacts with the DCF and illuminates and becomes trapped within the cell. This fluorescence can be measured over time and is proportional to ROS which allows one to quantify the amount of ROS by the amount of fluorescence measured over time. An illustration of this process within a cell is shown in Fig. 8.

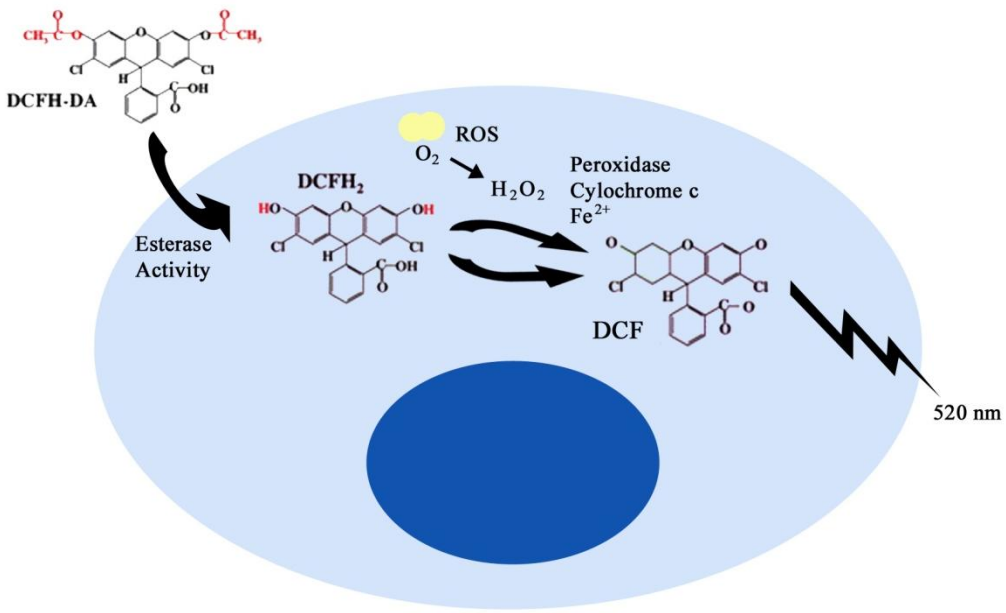

Fig. 8. Illustration of DCFDA process within a cell.

Using an Enzyme-Linked ImmunoSorbent Assay (ELISA) to Detect TNF – α

The sandwiched ELISA works by placing the TNF - α capture antibody in a plate, adding the sample which may or may not contain the antigen being screened. Then another capture antibody with an enzyme linked to it is added. Several washes occur and

then a substrate reagent is placed in the mix and the amount of TNF - α can be quantified because the reagent reacts with only the enzyme that remained in the solution which is connected to the TNF - α antibodies. Then the reaction is stopped and either read visually if testing whether or not any TNF - α is in the sample or is quantified using a plate reader. A diagram of this process can be seen in Fig. 9.

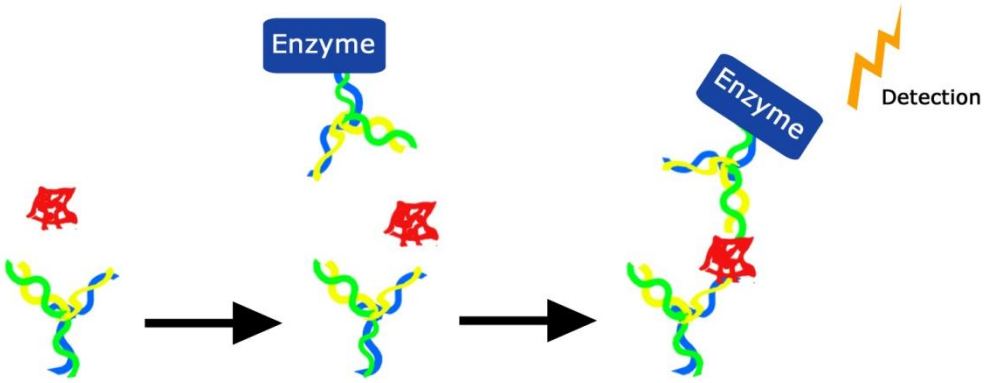

Fig. 9. Diagram of Sandwich ELISA Process.

Using a Th1/Th2/Th17 Cytometric Bead Array (CBA) to Develop a Cytokine Profile of Splenocytes

The Th1/Th2/Th17 cytometric bead array works by the same process for mouse splenocytes as the ELISA worked with mouse macrophages with only two exceptions. The first exception is that there are beads used instead of a dependence of the antigen sticking to the surface of a 96 – well plate. The second exception is that the CBA not only tests for TNF - α, but it also tests for Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interferon-γ (IFN-γ), Interleukin-17A (IL-17A), and Interleukin-10 (IL-10). An illustration is shown in Fig. 10.

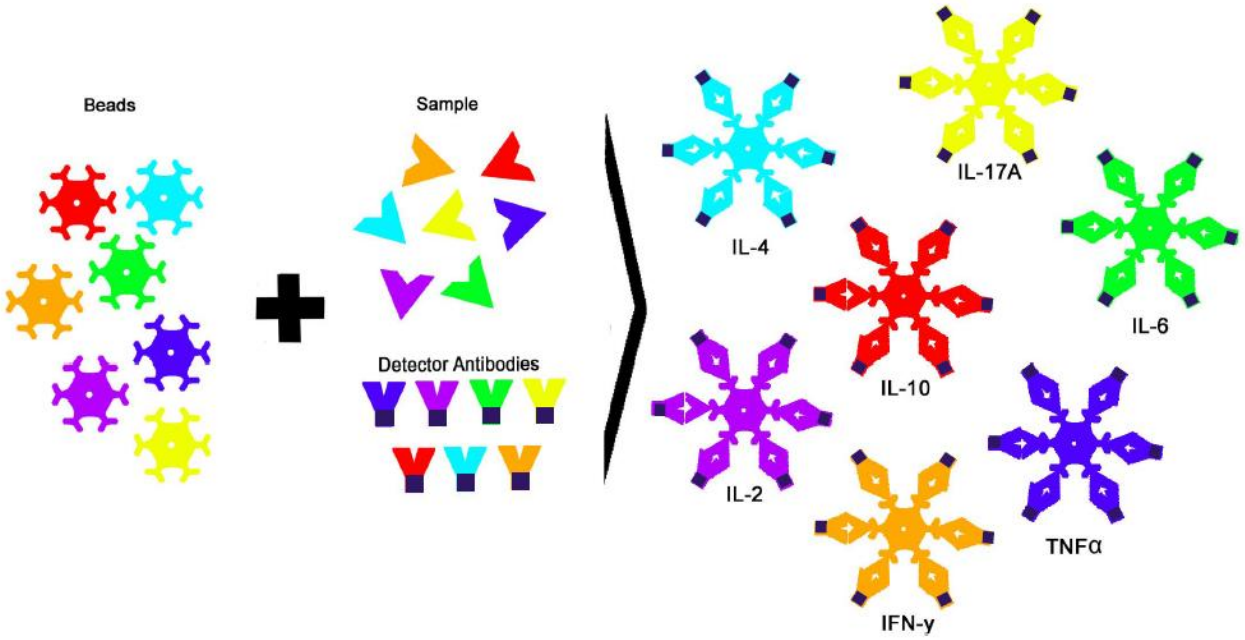

Fig. 10. Illustration of how the Cytokine Bead Array (CBA) Works.

Objective:

Both the innate and adaptive immune response of mouse macrophage and spleen cells (splenocytes) to various concentrations of 50-nm AuNPs with a coating of Poly(N-vinylprrolidine) (PVP), and treated with and without LPS, were investigated by measuring cell viability, ROS, and developing a cytokine profile. The cytokines involved in the profile are associated with ROS, inflammation, cancer, and immune disorders. Before the immune response could be investigated it was necessary to verify the quality of the AuNPs using visual inspection, UV-Vis, DLS, and TEM. Confirming that the macrophages and splenocytes consume the AuNPs was also necessary before any immune response could be investigated.

During the investigation there were two types of AuNPs used. The first type has been processed in a special way and is claimed to be free of endotoxins. This preparation is known as the BioPure product line provided by the company Nanocomposix (NC).

The second type had not been treated in such a stringent way and may have contained some endotoxin contamination, and this preparation is known as the Econix product line also provided by NC[1].

[1] Nanocomposix, Inc. 4878 Ronson Court, Suite K San Diego, CA 92111

Methods and Materials

To accomplish the stated objective there were five Null Hypotheses investigated using both mouse macrophage and spleen cells. The Hypotheses are described below:

Null Hypotheses:

1) 50-nm AuNPs will not be absorbed by mouse macrophage cells or spleen cells.
2) 50-nm AuNPs will have no effect on mouse macrophage cell viability.
3) 50-nm AuNPs will have no effect on ROS for mouse macrophage cells.
4) 50-nm AuNPs will produce no effect on the inflammatory response of mouse macrophage cells.
5) 50-nm AuNPs will produce no immune response on mouse spleen cells.

A summary of which type of cells used and what treatment was given is provided in Table 1 below. There were also some aspects of the investigation that were shared with most or all of the null hypotheses investigated and therefore will be discussed briefly before each specific process is discussed on what it took to investigate each null hypothesis. Then the processes specific to each null hypothesis will be briefly discussed.

Mouse Macrophages (Macs) and/or Spleen Cells (Spleen) Treated with 50-nm AuNPs											
		Null Hypothesis #1		Null Hypothesis #2		Null Hypothesis #3		Null Hypothesis #4		Null Hypothesis #5	
	Concentration [µg/mL]	With LPS	W/out LPS	With LPS	W/out LPS	With LPS	W/out LPS	With LPS	W/out LPS	With LPS	W/out LPS
BioPure	0			Macs	Macs	Macs	Macs	Macs	Macs	Spleen	Spleen
	5			Macs	Macs	Macs	Macs	Macs	Macs	Spleen	Spleen
	25			Macs	Macs	Macs	Macs	Macs	Macs	Spleen	Spleen
	50			Macs	Macs	Macs	Macs	Macs	Macs	Spleen	Spleen
Econix	0.01	Macs	Macs								
	0.05	Macs	Macs								
	0.11	Macs	Macs								
	0	Macs	Macs	Macs	Macs	Macs	Macs	Macs	Macs		
	5			Macs	Macs	Macs	Macs	Macs	Macs		
	25			Macs	Macs	Macs	Macs	Macs	Macs		
	50			Macs	Macs	Macs	Macs	Macs	Macs		

Table 1. Summary of Cells Used and Treatments Performed in Investigation.

To test the null hypotheses given, it was necessary to verify the overall quality and specifications of the 50-nm AuNPs provided by NC. To accomplish this, visual identification, UV-Vis., DLS, and TEM were performed on the AuNPs. The AuNPs were also coated by NC[1] with PVP to increase their stability (Liu et al 2013) and minimize Ostwald ripening (Voorhees 1985).

There was concern for the risk of bacteria contaminating the AuNP samples. So as a precaution, a special type of AuNP was ordered known as the BioPure line of products provided by NC[1] that claimed to be monodisperse, unagglomerated, endotoxin free, and purified to remove residual reactants. NC[1] also provided another line of products known as the Econix line of AuNPs which claim to provide monodisperse and unagglomerated AuNPs but do not guarantee to be free of endotoxins or residual reactants. Both product lines were purchased from NC[1] and used in the experiments.

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AuNP Quality Verification

AuNP Quality Verification Using Visual Inspection

The AuNPs had a specific reddish or pink color depending on their concentration in the pure water solution provided by NC[1] which was due to the Plasmon resonance effect (Kumar & Pal 2007). Any changes in color would indicate a change in the AuNPs material properties, such as degradation of the PVP coating, large scale agglomeration, Ostwald ripening, or instability. The reddish color was observed as the particles first arrived and the color of the AuNP solution remained consistent throughout the time the experiments were performed for both the BioPure and Econix product lines as is shown in Fig. 11.

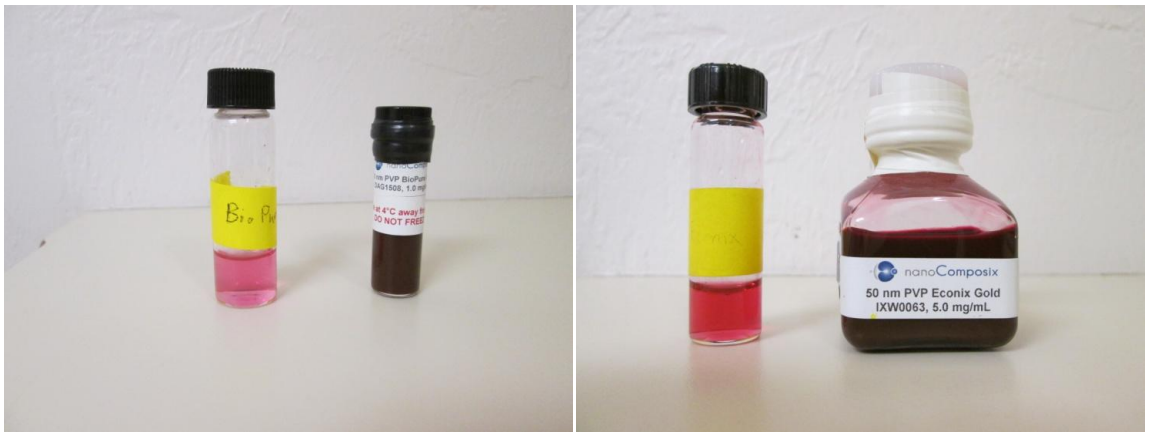

Fig. 11. Pictures of BioPure (left) and Econix (right) AuNPs taken after experiments completed.

AuNP Quality Verification Using UV-Visible Spectroscopy (UV-Vis)

UV-Vis was effective in confirming the stability of NPs (Haiss et al 2007). UV-Vis was done both by NC[1] and confirmed at Idaho State University (ISU). The BioPure AuNPs experienced their maximum absorbance at 532-nm at NC[1] and 534-nm at ISU.

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Their absorption spectra are displayed in Fig. 12. The Econix AuNPs experienced their maximum absorbance at 529-nm at NC[1] and 526-nm at ISU and is displayed in Fig. 13.

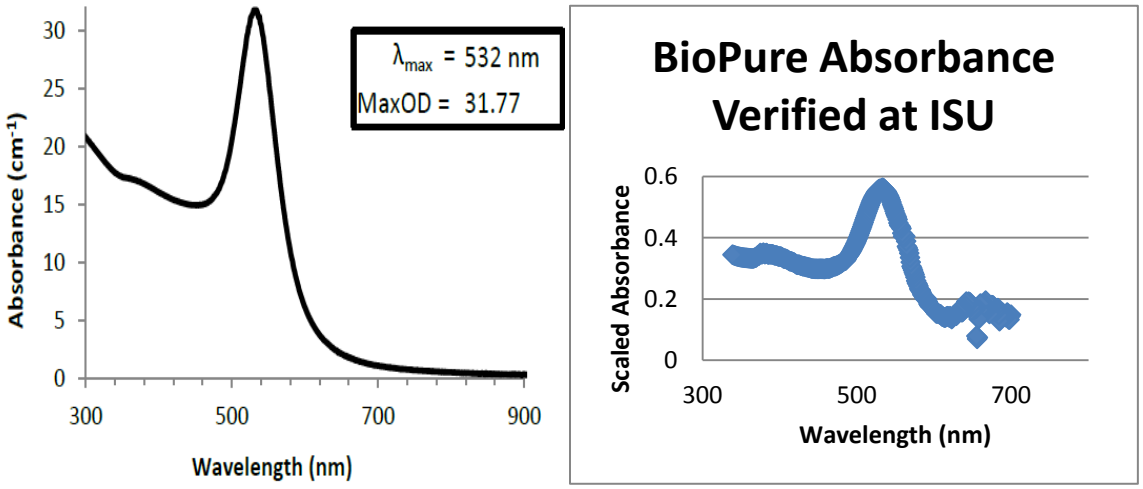

Fig. 12. UV-Vis performed by NC (left) and ISU (right) pre-experiments for BioPure AuNPs.

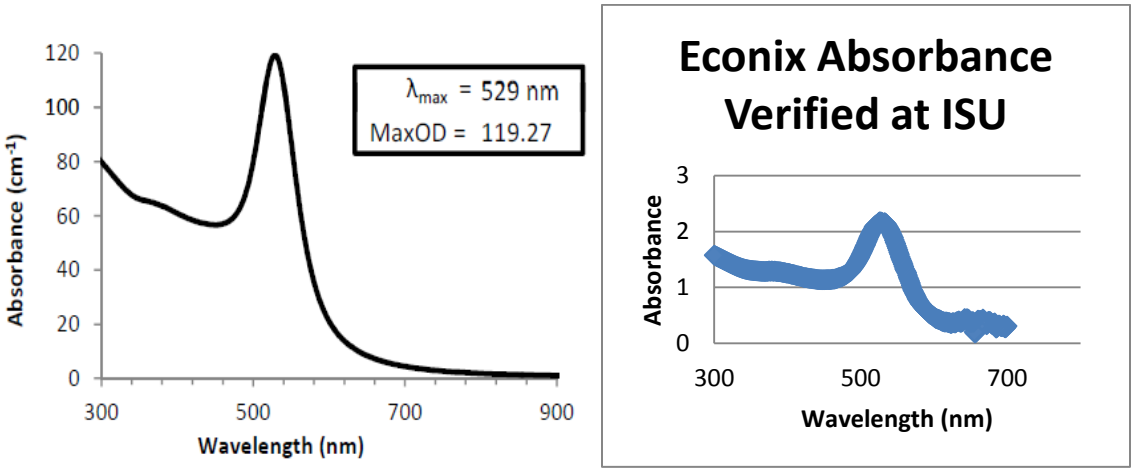

Fig. 13. UV-Vis performed by NC (left) and ISU (right) pre-experiments for Econix AuNPs.

It was also necessary to perform UV-Vis after the experiments were completed to

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verify the AuNPs stability throughout the experiments. The BioPure AuNPs experienced their maximum absorbance at 532-nm at ISU and the Econix AuNPs experienced their maximum absorbance at 530-nm. The UV-Vis results for the BioPure and Econix AuNPs can be seen in Fig. 14. The equipment used to perform the UV-Vis at NC[1] was the Agilent 8453 UV-Visible Spectrometer, while the equipment that was used at ISU was the PerkinsElmer Precisely Lambda 35 UV-Visible Spectrometer, which may explain the slight variation between the measurements although both measurements are within expected tolerances.

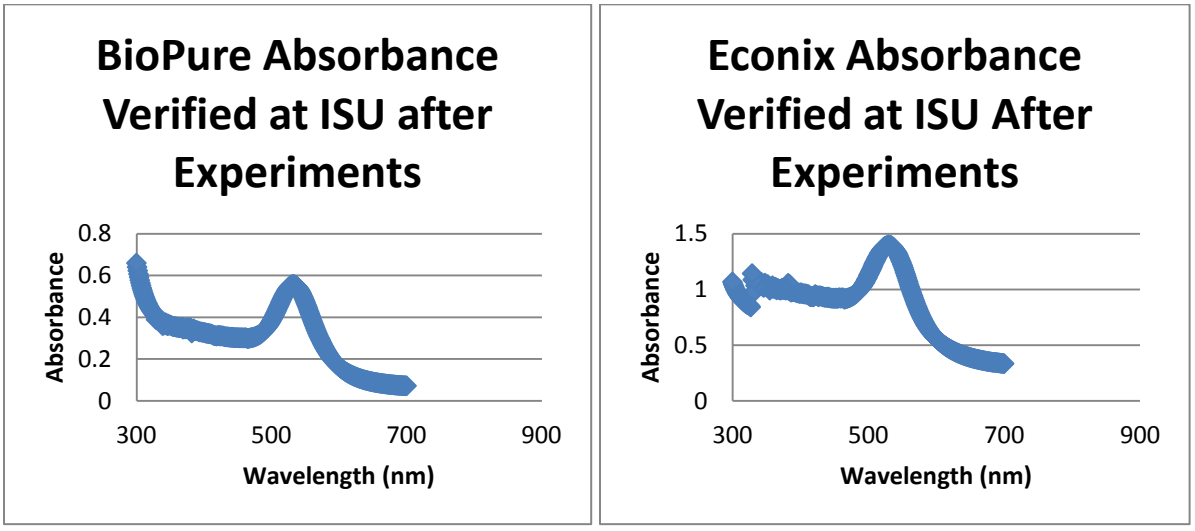

Fig. 14. UV-Vis performed by NC (left) and ISU (right) after experiments for BioPure and Econix AuNPs.

AuNP Quality Verification Using Dynamic Light Scattering (DLS)

DLS measures the hydrodynamic diameter and indirectly the Zeta potential of the AuNP. Measuring the hydrodynamic diameter is an effective way to measure agglomeration (Jans et al 2009) and the Zeta potential is proportional to the amount of

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average charge held by the AuNPs. ISU did not have access to a DLS measuring machine so the initial measurement was provided by NC[1]. Then after the experiments were completed, this researcher traveled to NC[1] to take another DLS measurement and verified the lack of agglomeration of the AuNPs provided. The initial and final measurements of the hydrodynamic diameter for the BioPure AuNPs were 72.5-nm and 77.3-nm respectively. The initial and final measurements of the hydrodynamic diameter for the Econix AuNPs were 82.1-nm and 80.4-nm. The initial and final Zeta potential for the BioPure was -25.7 mV and -25.7 mV respectively. The initial and final Zeta potential for the Econix was -25.7 mV and -32.8 mV respectively. The equipment that was used is a Malvern Zetasizer Nano ZS.

AuNP Quality Verification Using a Transmission Electron Microscope (TEM)

TEM was used to verify the shapes and the AuNP's size distributions. Using TEM it was confirmed that no agglomeration was occurring in the AuNPs provided by NC and to verify that Ostwald ripening had a minimal effect on the AuNPs. TEM shots were taken of the BioPure AuNPs by NC[1] before the experiments were done, then after the experiments were complete a portion of the unused BioPure and Econix AuNP solutions were sent to NC[1]. After experiments were completed, TEM shots were taken again to verify that the AuNP samples that were used in the experiments did not undergo agglomeration at any point during the experiments. The TEM shots produced the following pictures and size distributions before and after experiments that can be seen in Fig. 15 and Fig. 16 for the BioPure and Fig. 17 and Fig. 18 for the Econix product lines. Note: The scaling used by NC[1] is slightly different before and after the experiments, so it may appear as though the AuNPs are different sizes but they are actually the same. The

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scaling bar is seen in the lower right of each picture.

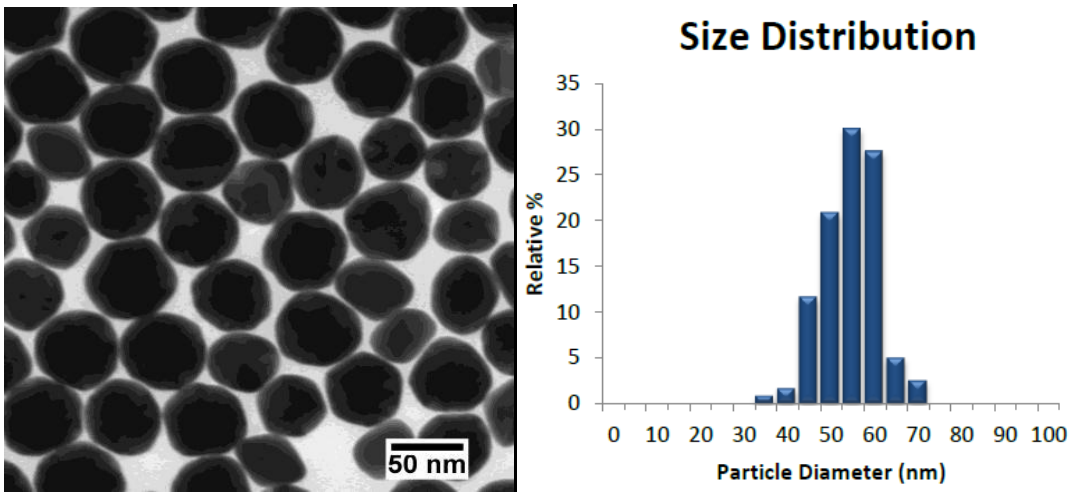

Fig. 15. TEMs taken by NC of BioPure AuNPs before experiments and their size distribution.

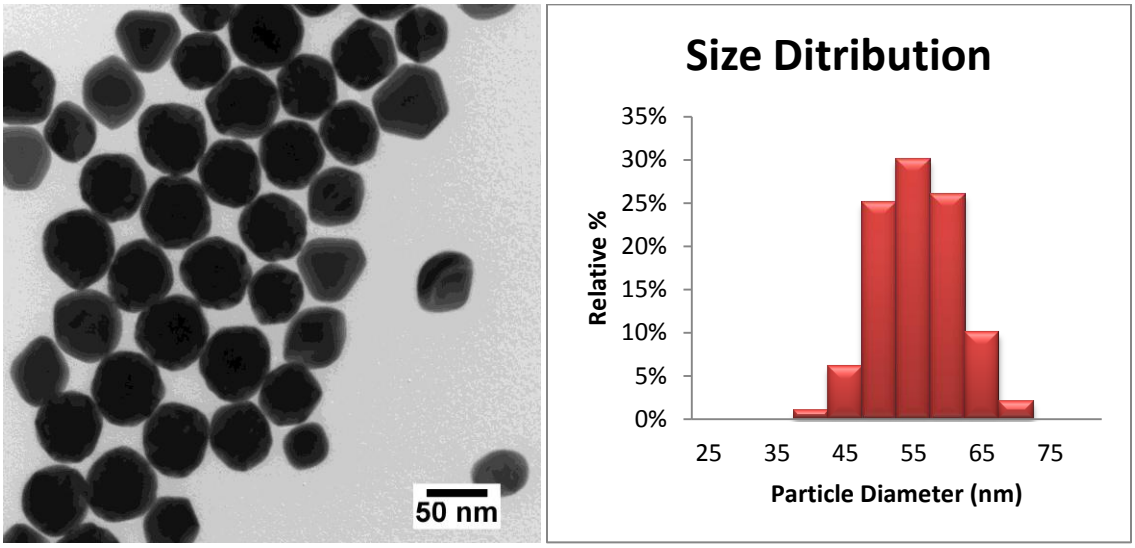

Fig. 16. TEMs taken by NC of BioPure AuNPs after experiments and their size distribution.

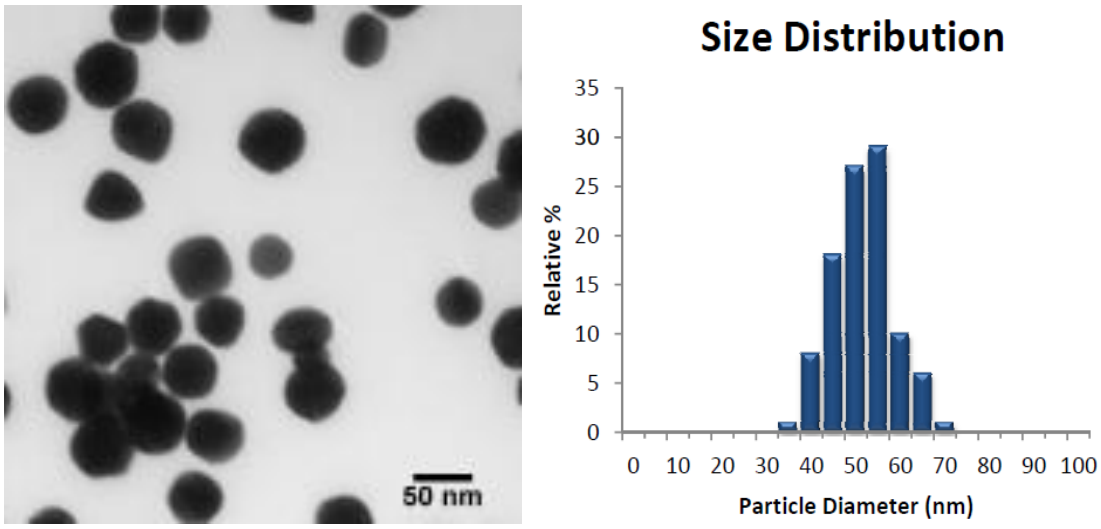

Fig. 17. TEMs taken by NC of Econix AuNPs before experiments and their size distribution.

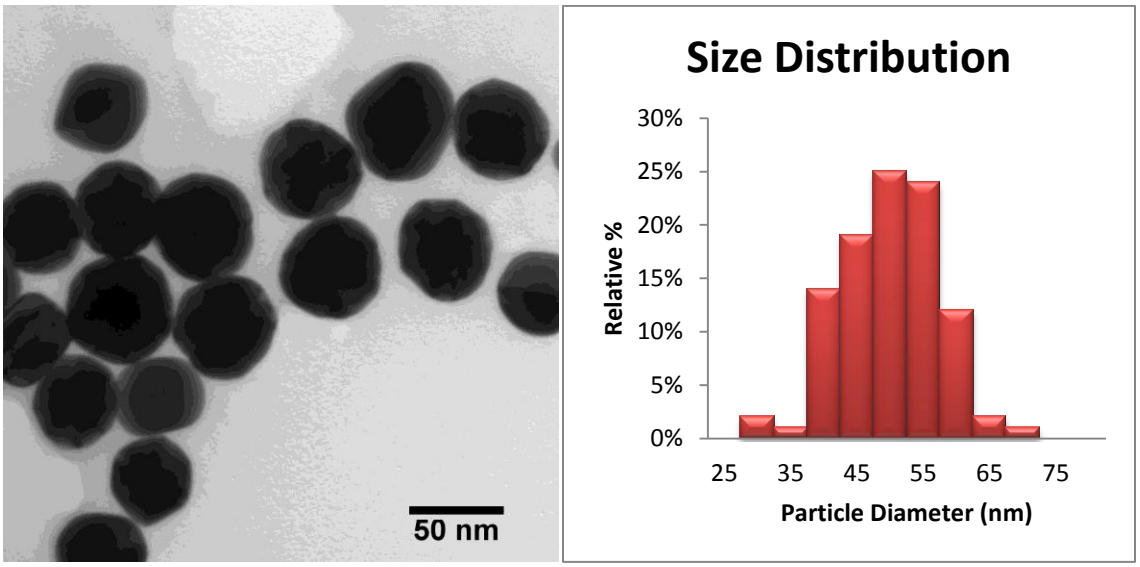

Fig. 18. TEMs taken by NC of Econix AuNPs after experiments and their size distribution.

Cells and Cell Culture Used for Macrophages

To investigate Null Hypotheses #1 - #3, RAW 264.7 macrophages were used. RAW 264.7 macrophages were obtained from *American Type Culture Collection* (ATCC, Rockville, MD, USA) and maintained at 37°C in RPMI media (CellGro Mediatech,

Manassas, VA, USA). The media contained l-glutamine, 100 U/mL penicillin/streptomycin, and 5% fetal calf serum (FBS, Atlanta Biologicals) in a humidified CO_2 incubator. The incubator was a NapCo Series 8000 water-jacketed CO_2 Incubator manufactured by Thermo Scientific and was kept at 5% CO_2.

Counting the Cells

To count the cells the Coulter Particle Counter (Beckman-Coulter, Z2 series) was used. The upper and lower limits were set to 17.00 µM and 5.00 µM respectively to ensure the detection of all particles within the expected size range. The dilution factor was set to $5\,x\,10^2$ and the resolution set to 256. The aperture was flushed with CoulterClenz detergent, and then flushed with diluent to remove any residual detergent. Then the sample was placed on the sample tray and analyzed. Once the analysis was complete the aperture was flushed once more in detergent and then stored within detergent to prevent contamination of the counter. It was determined that there were approximately 24 million RAW macrophage cells per flask of culture. It was necessary to count mouse macrophage cells to accomplish null hypotheses #1 - #3. To accomplish specific aim #4 mouse splenocytes were counted following the same settings described above.

For the 24 – Well plates that were used in null hypotheses #1 and #4, it was desirable to have approximately 1 million cells per well. Because each flask contained approximately 24 million cells (in 15 mL of media), the media was replaced with 24 mL of fresh media. Then the flask was scraped and the macrophages were mixed evenly and divided into 1 mL increments to fill the 24 – Well plate giving approximately 1 million cells per well. For null hypotheses #1 and #4, the AuNP solutions were added on top of

the 1 mL cell cultures and are described below.

For the 96 – Well plates that were used to investigate null hypotheses #2 and #3, it was desired to use approximately 50,000 cells per well. The experimental design required 60 wells of the 96 – Well plate to contain the macrophage cells. With 60 wells and approximately 50,000 cells per well it was found that approximately 3 million cells would be required for each 96 – Well plate. To accomplish this, the media was replaced and brought up to 24 mL, producing a cell concentration of 1 million cells per mL. Then the 3 mL was distributed evenly across the 60 wells by placing 50 µL of the cell-media mixture into each well.

Nanoparticle Solutions

In all experiments using the AuNPs it was important to maintain the monodispersion of the AuNP since surface properties and surface area are critical factors in the particles' interaction with cells. The materials from NC indicated that putting the water suspension of AuNP directly into a high salt media (isotonic with cells) could cause agglomeration. In order to avoid agglomeration of the AuNPs in all experiments, it was necessary to gradually "salt" the solution by adding 50 µL of media to the water suspensions every 10 minutes until a total of 200 µL of media was added to 200 µL of Econix or BioPure AuNPs (depending on the need of each experiment). The Econix AuNPs were used to investigate null hypotheses #1, #2, and #3. The BioPure AuNPs were used to investigate null hypotheses #2, #3, and #4.

For null hypotheses #1 a 24 – Well plate was used and it was desired to reach a point where cellular uptake was verified using light microscopy and flow cytometric side scatter without overwhelming the cells, immediately causing their death. It was also

important to take into account the limited amount of AuNP solution that was available. So there were three volumes of Econix AuNP solution (NanoComposix) that were added to each well in rows 2, 3, and 4 which were 2 µL, 10 µL, and 20 µL respectively to total 1 mL of AuNP solution and cell media per well. Starting with the equation of concentration (Eqn. 1) and using a scaling factor, Eqn. 2 was used and can be seen below. The concentrations of Econix AuNPs were determined and can be seen in Table 2. Once it was seen that the Econix AuNPs were taken up effectively and did not cause immediate cell death, it was decided to use greater concentrations of both the Econix and BioPure AuNPs in the remaining experiments.

$$C_1 V_1 = C_2 V_2 \qquad \text{(Eqn. 1)}$$

$$C_1 \left[\frac{\mu g}{mL}\right] = \frac{C_2\left[\frac{\mu g}{mL}\right] V_2\left[\frac{\mu L}{well}\right]\frac{1}{1000}\left[\frac{mL}{\mu L}\right]}{V_1[mL]} \qquad \text{(Eqn. 2)}$$

Where $C_1 = Initial\ Concentration$

$V_1 = Initial\ Volume$

$C_2 = Final\ Concentration$

$V_2 = Final\ Volume$

24-well Plate – Null Hypothesis #1	
Volume [µL]	Concentration of Stock of Econix AuNPs [µg/mL]
0	0
2	0.01
10	0.05
20	0.11

Table 2. Concentrations of Econix AuNPs solutions for Null Hypotheses #1.

A 96 – Well plate was used to investigate null hypotheses #2 and #3. Four concentrations of AuNPs were tested in each experiment using a 96 – Well plate for both the Biopure and the Econix AuNPs which were given in concentrations of $1.09\ mg\ mL^{-1}$ and $5.29\ mg\ mL^{-1}$ respectively. The four concentrations are $0\ \mu g\ mL^{-1}$ to establish a control, $5\ \mu g\ mL^{-1}$, $25\ \mu g\ mL^{-1}$, and $50\ \mu g\ mL^{-1}$. This was accomplished by getting six 1.5 mL vials and calculating how much fluid to pull from the un-diluted BioPure and Econix stock to get a total of 50 µL of the various AuNP solutions and allowing 50 µL for the cell prep solution to get a total of 100 µL per well of the combined AuNP and cellular solution. This was done by starting with the concentration equation (Eqn. 1) and adjusting it for the number of wells and scaling the units to get the equation shown below (Eqn. 3). Using Eqn. 3 the amounts of fluid from the Econix and BioPure stocks were determined and the values can be found in Table 3.

$$V_1[mL] = \frac{C_2\left[\frac{\mu g}{mL}\right]V_2\left[\frac{\mu L}{well}\right]6[well]\frac{1}{1000}\left[\frac{mL}{\mu L}\right]}{C_1\left[\frac{\mu g}{mL}\right]} \qquad \text{(Eqn. 3)}$$

96-well Plates – Null Hypotheses #2 & #3		
Concentration [µg/mL]	Total Volume of Stock of Econix AuNPs for 6 Wells [µL]	Total Volume of Stock of BioPure AuNPs for 6 Wells [µL]
0	0	0
5	0.57	2.75
25	2.84	13.76
50	5.67	27.52

Table 3. Volumes of AuNP stock used in experiments using the 96 –well plate.

For null hypothesis # 4 a 24 – Well plate was used. In order to avoid an endotoxin presence found to be in the Econics AuNP solution, only the BioPure AuNP solution was used. The equation of concentration (Eqn. 1) was used and was modified by a scaling factor to find the necessary volume to add to produce the same four concentrations of AuNP as were used for null hypotheses #2 and #3, but for 1 mL wells from the 24 – Well plate. The equation that was developed (Eqn. 4) can be seen below. The corresponding volumes can be seen in Table 4.

$$V_1[mL] = \frac{C_2\left[\frac{\mu g}{mL}\right]V_2\left[\frac{\mu L}{well}\right]\frac{1}{1000}\left[\frac{mL}{\mu L}\right]}{C_1\left[\frac{\mu g}{mL}\right]} \qquad \text{(Eqn. 4)}$$

24-well Plate – Null Hypothesis #4	
Concentration [µg/mL]	Volume of BioPure AuNP Stock used for each well [µL]
0	0
5	4.59
25	22.94
50	45.87

Table 4. Concentrations of BioPure AuNP solutions per well used for Null Hypothesis #4.

Adding Lipopolysaccharide (LPS)

In all experiments LPS was added to half of the cell wells in order to investigate the effects on the cells with and without LPS. To determine the volume of LPS stock to add to each well to give the desired LPS concentration in each experiment Eqn. 1 was used. To investigate null hypothesis #1, it was desired to have a final concentration of 1 $\mu g\ mL^{-1}$ of LPS, so 25 µL of 40 $\mu g\ mL^{-1}$ of LPS stock was added to each 1 mL well of the 24 – Well plate used. To investigate null hypotheses #2 and #3, it was desired to have a final concentration of 1 $\mu g\ mL^{-1}$ of LPS, so 2.5 µL of 40 $\mu g\ mL^{-1}$ of LPS stock was added to each 100 µL well of the 96 – Well plates that were used. To investigate null hypothesis #4, it was desired to have a final concentration of 0.2 $\mu g\ mL^{-1}$ of LPS, so 5 µL of 40 $\mu g\ mL^{-1}$ of LPS stock was added to each 1 mL well of the 24 – Well plate used.

Null Hypothesis #1: 50-nm AuNPs will not be absorbed by mouse macrophage cells or spleen cells.

Prepping the 24-well Plate

RAW 264.7 macrophages were counted and were found to contain 24 million mouse macrophage cells per flask. The media was brought up to 24 mL, the flask was scraped, then the macrophages and media were evenly divided and placed in a 24-well plate to get 1 million cells per well. Then the Econix AuNPs were added to create wells of four different concentrations of Econix AuNPs: 0 $\mu g\ mL^{-1}$, 0.01 $\mu g\ mL^{-1}$, 0.05 $\mu g\ mL^{-1}$, and 0.11 $\mu g\ mL^{-1}$. The methods of obtaining the various concentrations of the AuNPs are described above. To accomplish these concentrations from a

$40\ \mu g\ mL^{-1}$ stock 0 µL, 2 µL, 10 µL, and 20 µL of stock was added to the cell wells respectively. Then the remainder was filled with media to equal a total of 1 mL of media containing the cells and AuNPs. Then a concentration of approximately 1 µg μl^{-1} of LPS was inserted in half of the wells in the 24-well Plate by adding 25 µL of stock containing $40\ \mu g\ mL^{-1}$ of LPS to half of the cell wells in the 24-well Plate. This is displayed in Fig. 19.

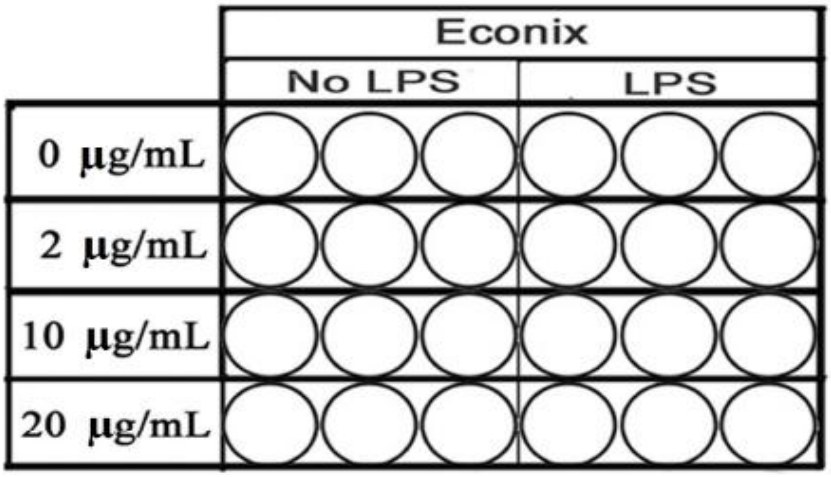

Fig. 19. 24-well Plate layout used to investigate null hypothesis #1.

Light Microscopy and Flow Cytometer Prep

The 24-well Plate was placed in the CO_2 incubator for one day and each plate was visually inspected using the Leica Microsystems DFC 295 DMIL camera attached to the Leica Microsystems CMS GMBH microscope. Then the 24-well plate was placed in the CO_2 incubator for 24 hours. 800 µL of media was removed from each well and saved for cytokine analysis. 500 µL of Phosphate Buffered Saline (PBS) was then added to each well to give a total volume of 700 µl per well. Then the cells were scraped and put into flow cytometry tubes that were already on ice. The tubes were then taken to the FACS

Calibur Flow Cytometer (BD Biosciences, San Jose CA).

Flow Cytometry

Flow cytometry was performed using a Becton Dickinson Biosciences FACS Calibur flow cytometer, and analyzed using CellQuest software (BD Biosciences, San Jose CA). Dead cells/debris and doublets were gated out of the analysis. A minimum of 10,000 gated (live, singlet) cells were analyzed for particle uptake using Forward and Side Scatter. Particle uptake by the cells resulted in vesicular and particle inclusions in the cells, resulting in increased Side Scatter (Suzuki, et al 2007). Data are presented as median fluorescence intensity.

Null Hypothesis #2: 50-nm AuNPs will have no effect on mouse macrophage cell viability

Part 1 – CyQuant Proliferation/Viability Assay

Prepping the 96 – Well Plate for the CyQuant Proliferation/Viability Assay

RAW 264.7 macrophages were used and approximately 50,000 cells were placed per 100 μL well in a 96-well plate using the method described previously. Then the AuNP solution was added to each well as described above with the exception that the concentration of the Econix NPs was 5 times more. The 96-well plate and corresponding concentrations are illustrated in Fig. 20 below.

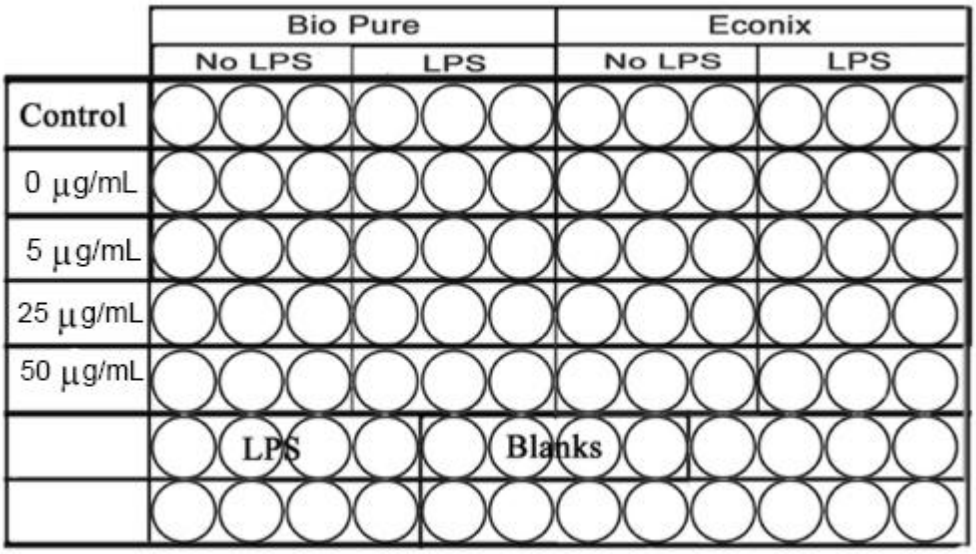

Figure 20. Illustration of experimental layout of 96-well plate for the CyQuant assay.

After 24 hours, pictures were taken under a microscope at 40 X magnification and the plate was incubated for an additional day at 37^o C. Then the fluid in the micro plate wells was gently removed, and placed in a 96-well container (to be tested for cytokines). The micro plate was then washed with PBS solution and placed in a freezer for approximately 1.5 hours at -80^o C in order the break the cell walls and allow easy access to the DNA.

Prepping the Reagent

On the day of the freezing and thawing, the Hanks Balanced Salt Solution (HBSS) buffer stock solution was diluted 20-fold by mixing 1 mL of the 20X stock with 19 mL of nuclease free distilled water. The CyQuant GR dye stock solution was diluted 400 – fold into the 1X HBSS. This was accomplished by mixing 22 µL of the CyQuant GR dye stock into the 11 mL of the HBSS.

Performing the Analysis

Then 100 μL of the CyQuant GR dye/HBSS was added to each sample well. The micro plate was then incubated for 2 – 5 minutes at room temperature while being protected from light. Then the micro plate was taken to the plate reader and set to read fluorescence using the filters for 480-nm for excitation and 520-nm for emission maxima. The dye fluoresces as it intercalates into the DNA, providing a method to measure the amount of DNA. As the cells die, the DNA degrades so it becomes undetectable creating a method to measure cell death.

Part 2 – Macrophage Cell Viability Confirmation Using the Coulter Counter

Using the Coulter Counter

Because the coloration and optical density of the AuNP interfered with methods depending on dye color absorption or fluorescence, viability was tested in the end simply by counting the cells on the Coulter Counter, as described above. Briefly, the macrophages were treated for 48 hrs in a 96-well plate exactly as for experiments, and then removed from the bottom of the wells using Trypsin/EDTA reagent and gentle scraping with a pipette tip. 20 μL of the cell suspensions were placed into 20 mL of counting Diluent and analyzed on the Coulter counter.

Null Hypothesis #3: 50-nm AuNPs will have no effect on ROS for mouse macrophage cells

Prepping the 96 – Well Plate for the DCFDA Assay

RAW 264.7 macrophages were used and approximately 50,000 cells were place

per 100 μL well in a 96-well plate using the method described previously. Then the plate was incubated for 2 days at 37^{o} C. The final 96-well plate layout of the AuNP concentrations, locations of the placement of LPS, adding the top row as a control and other controls are illustrated in Fig. 21.

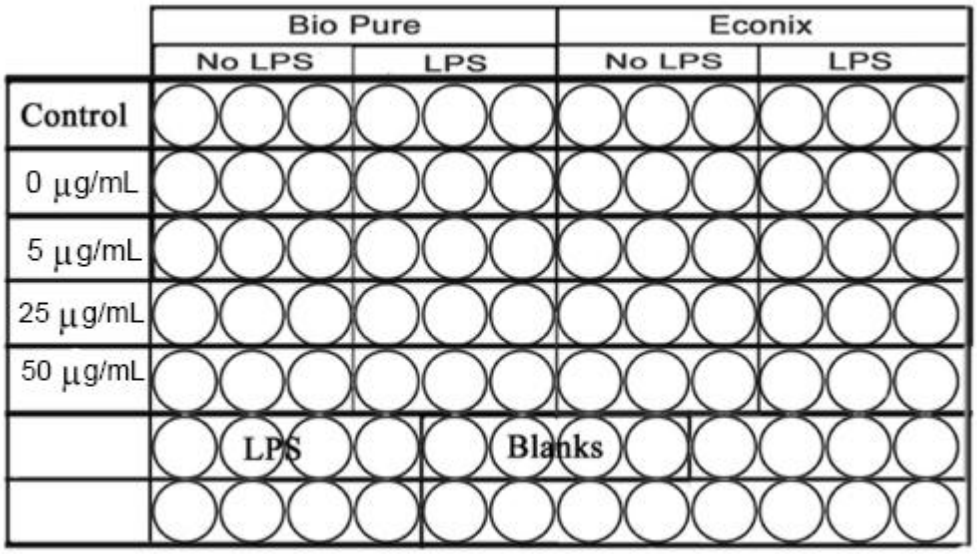

Figure 21. Illustration of layout of 96-well plate for the DCFDA toxicity assay.

DiChloroFluorescein Diacetate (DCFDA) Protocol

A 10 mL solution of 10 μM DCFDA was made by adding 2 μL of 50 mM of DCFDA stock into 10 mL of PBS. The fluid in the micro plate was then emptied out and 100 μL of the DCFDA/PBS solution was added to each well containing the cells (with the exception of a control section which contained no DCFDA/PBS solution). Then the solution was incubated in the dark for approximately 1 hour to load the dye into the cells. Then the dye solution was removed and the micro plate was washed gently using PBS. Then the AuNP and LPS solutions were added to each well as described and shown in

Fig. 21.

Then the micro plate was read from the plate reader a number of times throughout a day and a half. The 96-well plate was read for fluorescence and the excitation was set to 485-nm and the emission was set to 528-nm.

Null Hypothesis #4: 50-nm AuNPs will produce no effect on the inflammatory response of mouse macrophage cells

Standards Preparation

The standards solution contains lyophilized recombinant mouse TNF - α (BD Biosciences Pharmingen, San Diego CA) and was warmed to room temperature. Then it was reconstituted with 1.0 mL of de-ionized water to become the stock standard. It was then vortexed gently and allowed to equilibrate for 15 minutes. Then a $1000\ pg\ mL^{-1}$ standard was made from the stock standard and was vortexed thoroughly. Then 300 µL of Assay Diluent was added to 7 additional tubes. The tubes were labeled as $500\ pg\ mL^{-1}$, $250\ pg\ mL^{-1}$, $125\ pg\ mL^{-1}$, $62.5\ \mu g\ mL^{-1}$, $31.3\ pg\ mL^{-1}$, $15.6\ pg\ mL^{-1}$, and control ($0\ pg\ mL^{-1}$) respectively.

Next a serial dilution was performed on 6 of the tubes by transferring 300 µL of the $1000\ pg\ mL^{-1}$ stock solution to the tube labeled $500\ pg\ mL^{-1}$, then aspirating and releasing the Assay Diluent within the tube to ensure proper mixing. Then 300 µL of the mixture of stock solution and Assay Diluent was transferred from the tube labeled $5000\ pg\ mL^{-1}$ to the tube labeled $250\ pg\ mL^{-1}$. This tube was also aspirated and released to allow the proper mixing of the stock solution and the Assay Diluent. This process was repeated for the remaining tubes with the exception of the control tube as is

illustrated in Fig. 22.

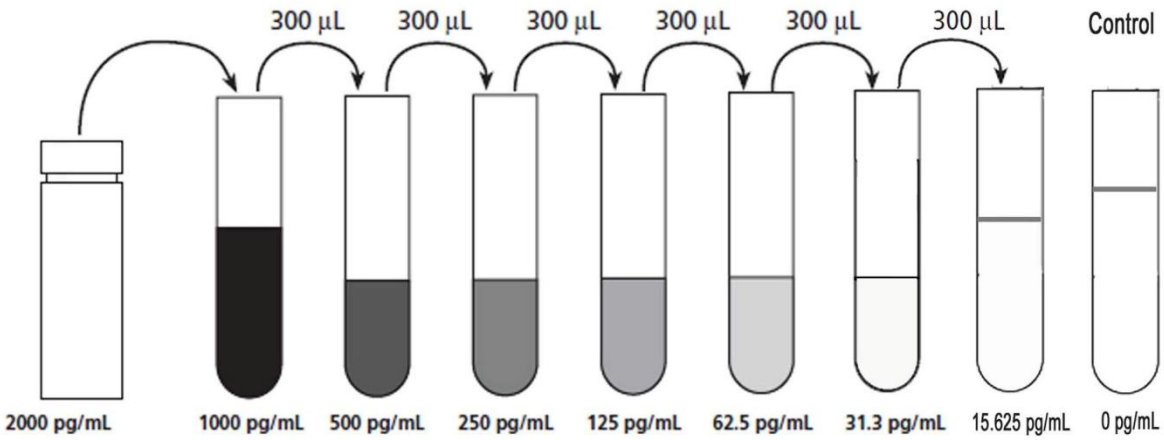

Figure 22. Illustration of the serial dilution used for the ELISA.

ELISA Assay Procedure

In order to perform the ELISA, The OptEIA (BD Biosciences Pharmingen, San Diego CA) set for mouse tumor necrosis factor (TNF - α) was used and contained all of the components necessary to perform the ELISA. The first step was to dilute the capture antibody, which was purified anti-Mouse TNF - α (250X) in coating buffer in a ratio of 1:250. The dilution was accomplished by adding 48 µL of the capture antibody into 12 ml of the coating buffer. The coating buffer contained 0.1 M Sodium Carbonate, pH 9.5, 8.40 g $NaHCO_3$, 3.65 g Na_2CO_3; q.s. to 1.0 L; pH to 9.5. Then the micro-wells were coated with 100 µL of diluted Capture Antibody per well, and the plate was sealed and incubated overnight.

The next step was to empty out all of the wells in the 96–well plate, and wash them by filling them completely with approximately 300 µL of wash buffer, then emptying them out again. This process was repeated 3 times. The wash buffer was a

PBS solution containing 0.05% Tween-20. Tween-20 is a nonionic polyoxyethylene surfactant that is an effective detergent for thorough removal of the excess protein.

The next step was to block the plates by adding 200 µL of Assay Diluent per well in order to prevent non-specific binding. Then the 96–well plate was incubated at room temperature for 1 hour. The Assay Diluent is PBS that contains 80.0 g NaCl, 11.6 g Na_2HPO_4, 2.0 g KH_2PO_4, 2.0 g KCl, q.s. to 10 L; pH to 7.0 with 10% FBS mixed in. Then the 96-well plate was washed 3 times using the PBS and Tween solution as described above.

The next step was to pipette 100 µL of each prepared standard, sample, and control into the appropriate wells in the 96-well plate. The plate was then sealed and allowed to incubate at room temperature for 2 hours. The placement of each of the wells within the 96-well plate can be seen in Fig. 23.

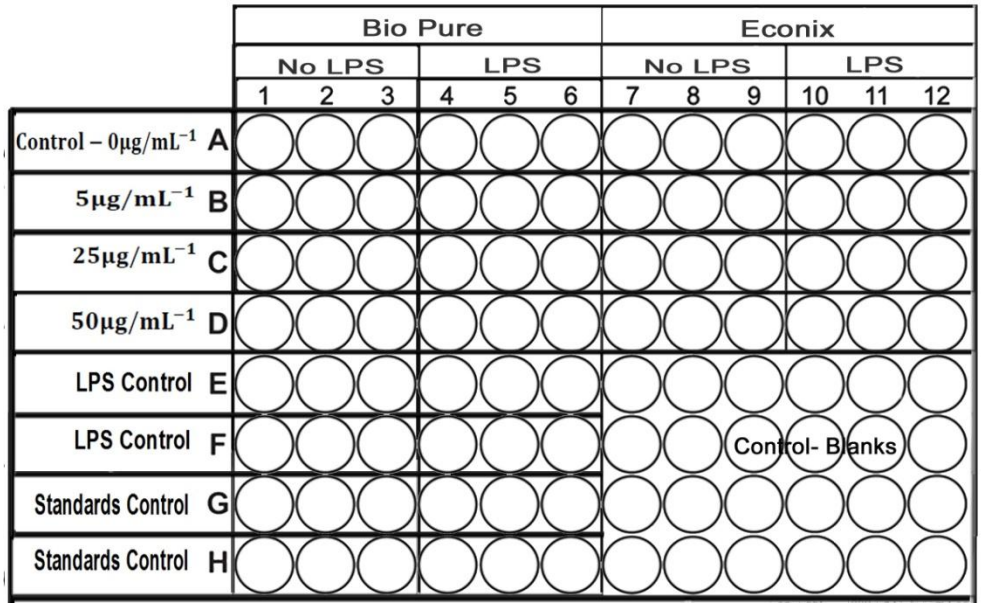

Figure 23. 96-well plate layout used to accomplish specific aim #2.

The next step was to aspirate and wash the 96-well plate as described above but with 5 total washes. Then the detection antibody was diluted by adding 48 μL of detection antibody, which was Biotin anti-Mouse TNF - α (250X) to 12 mL of Assay Diluent producing a ratio of 1:250. Then 100 μL of the diluted detection antibody was added to each well and then the 96-well plate was sealed and incubated in room temperature for 1 hour.

The next step was to aspirate and wash the 96-well plate 5 times as described earlier. Then the Enzyme Reagent, which is Streptavidin-horseradish peroxidase conjugate (SAv-HRP) (250X) was diluted in Assay Diluent at a ratio of 1:250 by adding 48 μL of the Enzyme Reagent to 12 mL of Assay Diluent. Then 100 μL of the diluted Enzyme Reagent was added to each of the wells in the 96-well plate. Then the plate was sealed and incubated at room temperature for 30 minutes.

The next step was to aspirate and wash the 96-well plate 7 times by filling each well with approximately 300 μL of wash buffer of the PBS and Tween-20 solution as described above and letting it sit for 1 minute, then emptying the 96-well plate out again. This process was repeated 7 times.

The next step was to add 100 μL of TMB substrate solution to each well of the 96-well plate. The TMB substrate solution contained Tetramethylbenzidine and Hydrogen Peroxide. It was recommended by the manufacturer to incubate at room temperature in the dark for 30 minutes, after which time the fluid is meant to turn blue which indicates that the reaction worked. Then 50 μL of the Stop Solution, which consisted of $2NH_2SO_4$ was added all of the wells within the 96-well plate and all of the solutions turned yellow. However, in the case of this experiment, the reaction occurred

and the solution turned blue in within seconds of adding the TMB substrate but was still incubated in the dark approximately 2 minutes, which saturated the solution and the experiment had to be repeated.

The next step was to measure the absorbance by using the Bio-TEK Synergy HT plate reader and the Gen 5 2.00 software. The plate reader was set to run at the normal speed, to measure the entire 96-well plate, and have the wavelength measurement set to 450-nm.

Analysis of ELISA Results

After the absorbance was read on the plate reader, the mean absorbance was calculated for each set of duplicate standards, controls, and samples. Then a curve describing the prepared standards was plotted using Excel with the absorbance on the x-axis and the TNF – α concentration on the y-axis. Polynomial curve fit was used and a polynomial equation was developed describing the relationship between Absorbance and TNF – α concentration. Using this polynomial, the absorbance values were used as the "X's", and the equation was solved for "Y" to get the corresponding TNF – α concentration. These values were then multiplied by a dilution factor of 4 and averaged over each duplicate sample.

Null Hypothesis #5: 50-nm AuNPs will produce no immune response on mouse spleen cells

Preparing the Mouse Spleen Cells (Splenocytes)

To investigate null hypothesis #4, mouse splenocytes were used by harvesting the spleen from 2 mice and placing each spleen in a vial containing 1 mL of sterile PBS. Then each vial containing the PBS solution and the spleen was poured into a Petri dish

and the spleens were examined. It was confirmed that the entire spleen was harvested. Then two glass slides were used to mince the organs by placing each spleen between the frosted ends of the glass slides and squeezing and rubbing them together until the tissue was translucent. This collection of cells and tissue was then washed back into the Petri dish containing 11 mL of PBS solution creating a total mixture of 12 mL of splenocytes and PBS solution.

The mixture was then pipetted up and down several times in order to break up the clumps. Then the entire 12 mL mixture was transferred to a 15 mL conical tube. This tube was then placed on ice and allowed to settle for approximately 20 minutes. After that, the top 11 mL of the mixture was transferred to clean conical tubes leaving approximately 0.5 mL of PBS solutions with clumps, which was discarded. The new conical tubes containing the 11 mL of PBS solution with splenocytes were then centrifuged for 5 min at 1500 rpm which caused a pellet of mice splenocytes to be formed at the bottom of the conical tube. With the pellet intact, the remaining solution was then removed.

Adding Lysis Buffer to the Splenocytes

The pellet was re-suspended in 5 mL of 1 X RBC Lysis Buffer (eBioscience, San Diego CA). Then the solution was incubated for approximately 5 min and was shaken periodically throughout the incubation. Then the reaction was stopped by diluting the Lysis Buffer with 10 mL of 1X PBS solution. Then the cells were centrifuged at 1500 rpm for 5 min and re-suspended. Then 40 μL of the cell suspension was transferred to new vials to be counted on a Coulter counter and was counted using the method described above. Then the cell suspension was plated in media in a 24 -well plate and

treated with AuNPs as described above for the macrophages. Then the plate containing the cell suspensions was incubated in the cell culture incubator for 48 hr.

After the incubation, the plate was inspected for evidence of bacterial contamination which would have been indicated by a change in the color of the media to either orange or yellow or may have become cloudy. After it was confirmed that there was no contamination of the plate, there were 2 sets of microfuge tubes (Fisher Brand Premium 1.5 mL MCT Graduated Mixed) prepared for the 24-well plate. Then the solution containing the mouse splenocytes from each of the 24 wells were transferred to its own individual microfuge tube. Then the microfuge tubes were centrifuged at 7000 rpm for approximately 3 min. Immediately after centrifugation, the media from each microfuge tube was gently poured into the second set of tubes. The first set of tubes containing the remaining pellet and the 24 – Well plate was then discarded. The microfuge tubes containing the culture media were then put on ice until they were needed.

Preparing Mouse Th1/Th2/Th17 Cytokine Standards

The lyophilized Mouse Th1/Th2/Th17 Cytokine Standards (BD BioSciences) were reconstituted and serially diluted immediately before mixing with the Capture Beads and the PE Detection Reagent. This was accomplished by placing the lyophilized Mouse Th1/Th2/Th17 Standards spheres to a 15 mL conical, polypropylene tube, then labeling the tube: "Top Standard". Then the standards were reconstituted with 2.0 mL of Assay Diluent and were allowed to equilibrate for approximately 15 minutes at room temperature while being gently mixed periodically using a pipette.

Then 10 tubes were set up (including 1 tube for the control) for the serial dilution

and were labeled: $5000\ pg\ mL^{-1}$, $2500\ pg\ mL^{-1}$, $1250\ pg\ mL^{-1}$, $625\ pg\ mL^{-1}$, $312.5\ pg\ mL^{-1}$, $156.3\ pg\ mL^{-1}$, $78.1\ pg\ mL^{-1}$, $39.1\ pg\ mL^{-1}$, $19.5\ pg\ mL^{-1}$, and $0\ pg\ mL^{-1}$ for control. Then 100 µL of Assay Diluent was added to each of the dilution tubes and the control. Then 100 µL of the $5000\ pg\ mL^{-1}$ standard was pipeted into the tube labeled $2500\ pg\ mL^{-1}$ and mixed creating a dilution ratio of 2:1. Then 100 µL of solution was taken from the tube labeled $2500\ pg\ mL^{-1}$ and pipeted into the tube labeled $625\ \mu g\ mL^{-1}$ and mixed creating a dilution ratio of 4:1. This process was repeated until reaching the tube labeled $19.5\ pg\ mL^{-1}$ attaining a dilution ratio of 1:256 and is illustrated in Fig. 24. Then the standards were set aside momentarily.

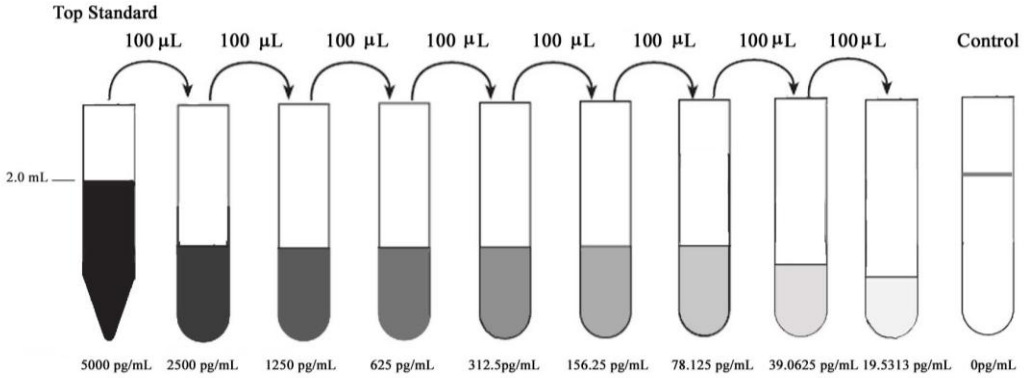

Figure 24. Serial dilution of the stock solution used in Th1/Th2/Th17 Cytokine Assay.

Mixing Mouse Th1/Th2/Th17 Cytokine Capture Beads

Mixing the mouse Th1/Th2/Th17 Cytokine Capture Beads (BD BioSciences, San Jose CA) was accomplished by first determining the number of assay tubes required for the experiment. To find this number, the number of standards, which were 10, was added to the number of samples, which were 24. Then this sum was multiplied by 10 and a small amount was added to account for surface tension of the solution, which was 10 µL.

This meant that 350 μL of each bead array was required. The bead arrays used were Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interferon-γ (IFN-γ), Tumor Necrosis Factor (TNF - α), Interleukin-17A (IL-17A), and Interleukin-10 (IL-10) which equaled a total volume of 2.45 mL of mixed bead arrays and were placed in a 5 mL conical tube. The Capture Beads solution was mixed gently via pipeting.

Performing the Mouse Th1/Th2/Th17 Cytokine Assay

To perform the Mouse Th1/Th2/Th17 Cytokine Assay 34 new microfuge tubes were used and labeled for the appropriate samples. 24 microfuge tubes were labeled for the samples treated with the various concentrations of AuNPs, with and without LPS, and 10 microfuge tubes were labeled for the 10 standards that were created as described above. Then 50 μL of the Capture Bead solution was added to all 34 microfuge tubes and were mixed gently to prevent settling. Then 50 μL of the culture media samples were added to 24 of the microfuge tubes and 50 μL of the Standards solutions were added to 10 of the microfuge tubes. Then 50 μL of mouse Th1/Th2/Th17 PE Detection Reagent (BD BioSciences, San Jose, CA) was added to all 34 microfuge tubes. Then all 34 microfuge tubes were allowed to incubate in the dark for 2 hours in the dark at room temperature.

Then 1 mL of wash buffer was added to all 34 microfuge tubes, which were then centrifuged at 200 g for 5 minutes. Then the fluid in each microfuge was discarded. Centrifuging each microfuge tube created an invisible pellet so great care was taken to orient the placement of the pellet in such a way that as the fluid was being poured out of the microfuge tube, the pellet would be at the upper-most portion of the microfuge tube and can be described in Fig. 25 below. This prevented the unknowing loss of the pellet. Then 300 μL of wash buffer was added to all tubes and the pellet was re-suspended.

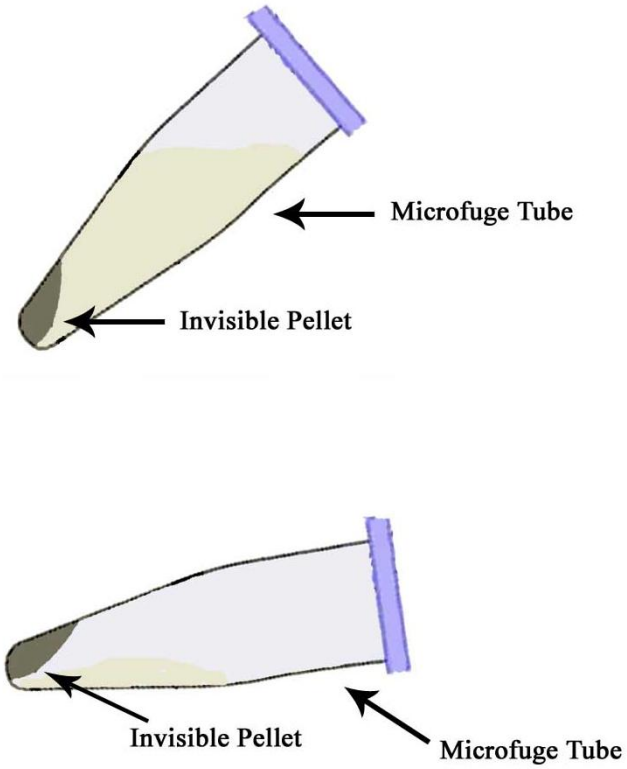

Fig. 25. Illustration of the method to discard fluid while preserving the invisible pellet.

The last step required before the samples were analyzed by the flow cytometer was to setup the Cytometric Bead Array (CBA) (BD BioSciences, San Jose, CA) to allow the flow cytometer to detect multiple proteins simultaneously. To accomplish this, three tubes were labeled: A, B, and C. Then 50 µL of Cytometer Set-Up beads (BD BioSciences, San Jose, CA) were added to all three tubes. Then 50 µL of PE positive control beads were added to tube B, and 50 µL of FITC positive control beads were added to tube C. Then all three tubes were incubated in the dark for approximately 30 minutes at room temperature. Then 450 µL of wash buffer was added tube A, and 400 µL of wash buffer was added to tubes B and C.

To perform the flow cytometry, the instrument was set to detect the different bead

values in one detector (FITC), and to quantify the amount of each cytokine using the PE detector, and the Cytometer Set-Up bead samples were used to optimize the settings for the PMT voltage for both colors, and to eliminate emission overlap using fluorescence compensation. Once optimized, all of the standards and samples were analyzed using the same settings. Standard curves for all of the cytokines were created using the Median Fluorescence Intensity (MFI) for each known concentration. The concentration of each cytokine for each sample was then calculated using the standard curves.

Statistical Methods

The cytokine data were analyzed by comparing means of the treated groups to the control using a 2-tailed, unpaired *t*-test (StatPlus Software, AnalystSoft, Inc.). Dose response data were analyzed using ANOVA with a Bonferroni post-test to compare between treatments (StatPlus). The mean values (± standard error of mean [SEM]) are reported as bars in the figures. Statistical significance was established as a two-tailed probability of type I error occurring at less than 5%.

Results

Null Hypothesis #1: 50-nm AuNPs will not be absorbed by mouse macrophage cells or spleen cells.

Light Microscopy

After confirming the quality of the AuNPs provided by NC[1], the first step was to test the cellular uptake of both the BioPure and Econix AuNPs by the mouse macrophage cells with and without LPS. The mouse macrophage cells consumed all AuNPs regardless of the concentration of AuNPs. This is shown in Fig. 26 for the BioPure AuNPs and in Fig. 27 for the Exonix AuNPs. Cells without LPS treatment were more homogeneous in size, and AuNPs appear to be dispersed throughout the cytoplasm. With LPS treatment, the macrophages become activated as seen by variations in size and cell division (black arrow), AuNPs became concentrated in lysosomes (red arrow), and cells are highly phagocytic with extended pseudopods (gray arrow).

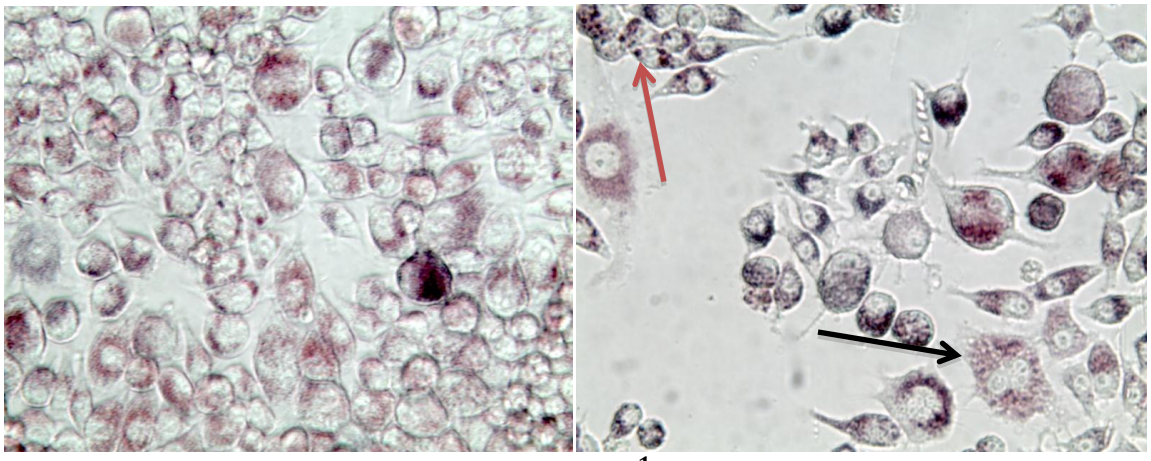

Fig. 26. Mouse macrophages with $25\ \mu g\ mL^{-1}$ of BioPure AuNPs without LPS (left) and with LPS (right). Images were taken with the 40X objective; total magnification = 400x.

[1] Nanocomposix, Inc. 4878 Ronson Court, Suite K San Diego, CA 92111

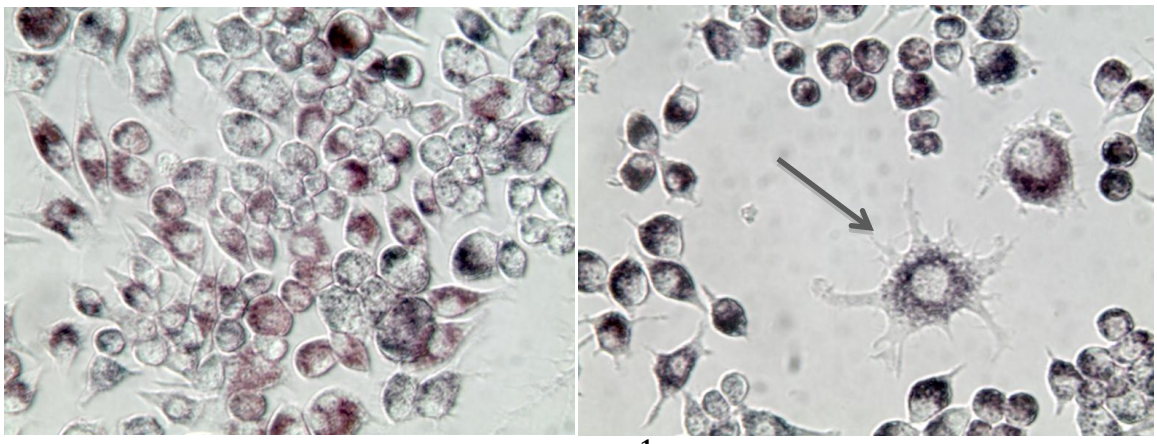

Fig. 27. Mouse macrophages with $50\ \mu g\ mL^{-1}$ of Econix AuNPs without LPS (left) and with LPS (right). Total magnification = 400x.

The cellular uptake of the splenocytes was inspected for the BioPure AuNPs with and without LPS. The Econix product line of AuNPs was not used to investigate null hypothesis #4 because of the potential presence of endotoxins. Though it was less visible than with the macrophages alone (due to the presence of many lymphocytes), the macrophages in the mixed cultures consumed all AuNPs regardless of the concentration. A picture of the splenocytes dosed with $25\ \mu g\ mL^{-1}$ of BioPure AuNPs is shown in Fig. 28.

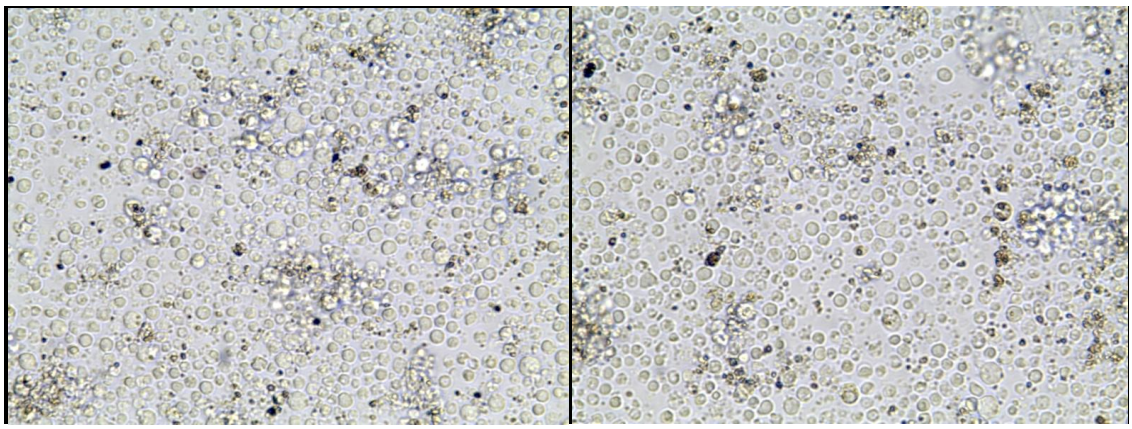

Fig. 28. Mouse splenocytes with $25\ \mu g\ mL^{-1}$ of BioPure AuNPs without LPS (left) and with LPS (right).

Flow Cytometry

The AuNP uptake by the mouse macrophage cells were also confirmed by using flow cytometry. As the concentration of AuNPs increased, side scatter increased verifying the uptake of the AuNPs and is shown in Fig. 29. Representative dot plots from the flow cytometry analysis are shown in Fig. 30 for macrophages treated with and without Econix AuNPs and LPS.

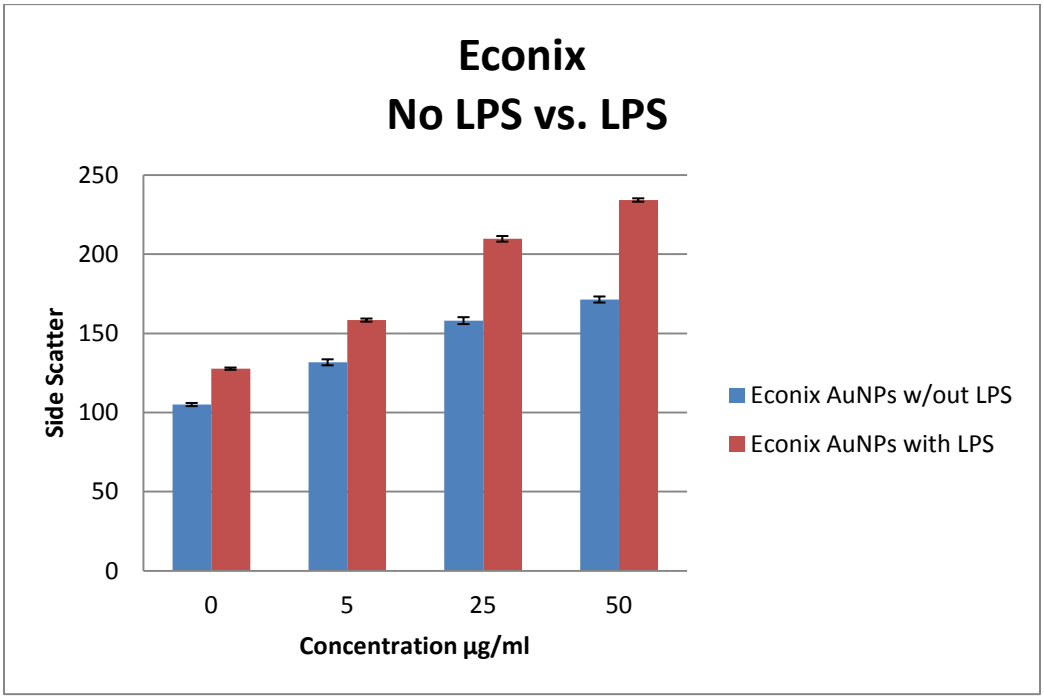

Fig. 29. Plots of Side Scatter of Econix AuNPs with and without LPS. The progression of side scatter between 0 $\mu g\ mL^{-1}$ to 5 $\mu g\ mL^{-1}$, 5 $\mu g\ mL^{-1}$ to 25 $\mu g\ mL^{-1}$, and 25 $\mu g\ mL^{-1}$ to 50 $\mu g\ mL^{-1}$ are statistically significant for all Econix AuNPs with LPS ($p<0.05$) using a one way ANOVA. The progression of side scatter between 0 $\mu g\ mL^{-1}$ to 5 $\mu g\ mL^{-1}$, and 5 $\mu g\ mL^{-1}$ to 25 $\mu g\ mL^{-1}$ are statistically significant for the Econix AuNPs without LPS ($p<0.05$) but the p-value for the side scatter from 25 $\mu g\ mL^{-1}$ to 50 $\mu g\ mL^{-1}$ was found to be < 0.05 using a one way ANOVA.

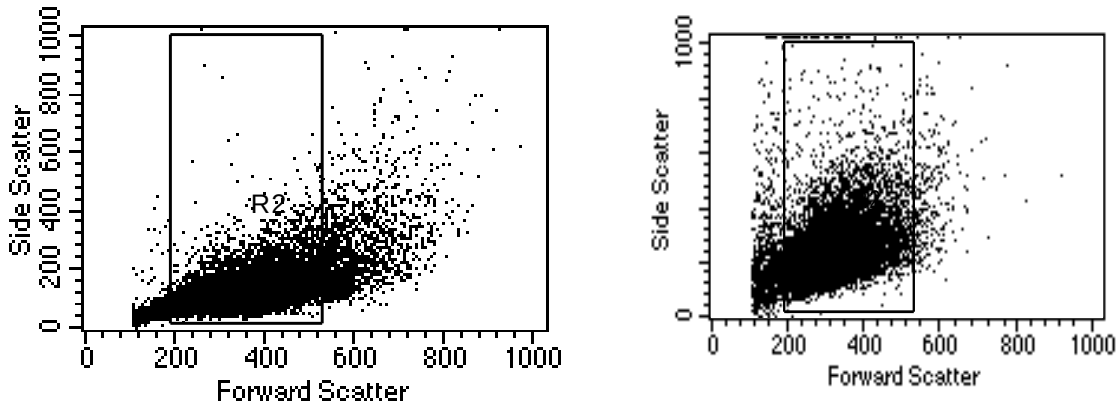

Fig 30. Plots of forward and side scatter of mouse macrophages untreated (left) and treated with 0.11 $\mu g\ mL^{-1}$ Econix AuNPs with LPS (right).

Null Hypothesis #2: 50-nm AuNPs will have no effect on mouse macrophage cell viability

Part 1 – CyQuant Proliferation/Viability Assay

The viability of the cells exposed to the BioPure and Econix AuNPs of the concentrations of 0 $\mu g\ mL^{-1}$, 5 $\mu g\ mL^{-1}$, 25 $\mu g\ mL^{-1}$, and 50 $\mu g\ mL^{-1}$ were measured using the CyQuant proliferation/viability assay with and without LPS. A comparison of fluorescence was produced by the CyQuant proliferation/viability assay of mouse macrophage cells exposed to the BioPure and Econix AuNPs with and without LPS and it was found that the fluorescence decreased as the concentration of the AuNPs increased as is shown in Fig 31 and Fig 32 respectively. A comparison was also made of the effect of the BioPure and Econix AuNPs exposed to the mouse macrophage cells without LPS and the same trend of decreasing fluorescence as the AuNP concentration increased occurred and is shown in Fig 33.

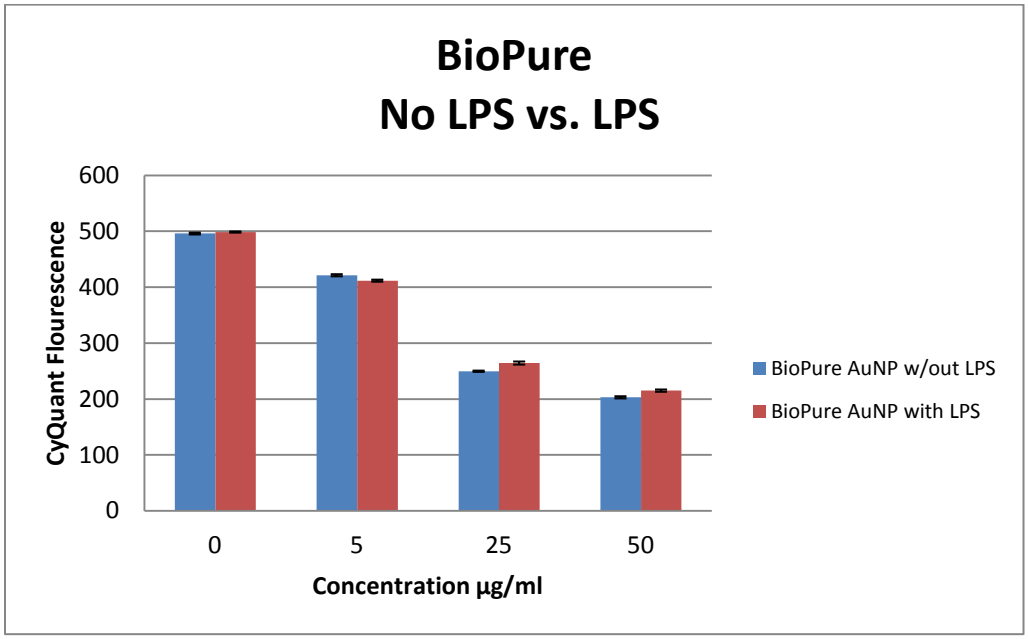

Fig. 31. CyQuant fluorescence for BioPure AuNPs with and without LPS. The progression of CyQuant fluorescence between 0 $\mu g\ mL^{-1}$ to 5 $\mu g\ mL^{-1}$, 5 $\mu g\ mL^{-1}$ to 25 $\mu g\ mL^{-1}$, and 25 $\mu g\ mL^{-1}$ to 50 $\mu g\ mL^{-1}$ are statistically significant for all BioPure AuNPs without LPS ($p<0.001$) using a one way ANOVA. The progression of CyQuant fluorescence between 0 $\mu g\ mL^{-1}$ to 5 $\mu g\ mL^{-1}$, and 5 $\mu g\ mL^{-1}$ to 25 $\mu g\ mL^{-1}$ are statistically significant for the BioPure AuNPs with LPS ($p<0.001$) but the p-value for the CyQuant fluorescence from 25 $\mu g\ mL^{-1}$ to 50 $\mu g\ mL^{-1}$ was found to be < 0.05.

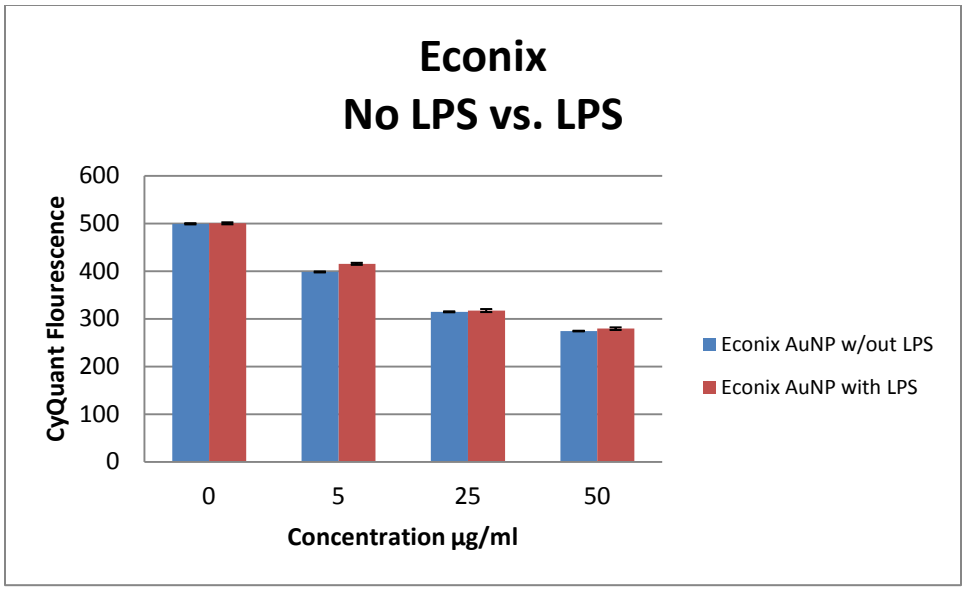

Fig. 32. CyQuant fluorescence for Econix AuNPs with and without LPS. The progression of CyQuant fluorescence between 0 $\mu g\ mL^{-1}$ to 5 $\mu g\ mL^{-1}$, 5 $\mu g\ mL^{-1}$ to 25 $\mu g\ mL^{-1}$, and 25 $\mu g\ mL^{-1}$ to 50 $\mu g\ mL^{-1}$ are statistically significant for all Econix AuNPs without LPS (p<0.001) using a one way ANOVA. The progression of CyQuant fluorescence between 0 $\mu g\ mL^{-1}$ to 5 $\mu g\ mL^{-1}$, and 5 $\mu g\ mL^{-1}$ to 25 $\mu g\ mL^{-1}$ are statistically significant for the Econix AuNPs with LPS (p<0.001) with the exception of the CyQuant fluorescence from 25 $\mu g\ mL^{-1}$ to 50 $\mu g\ mL^{-1}$, which was found to not be statistically significant.

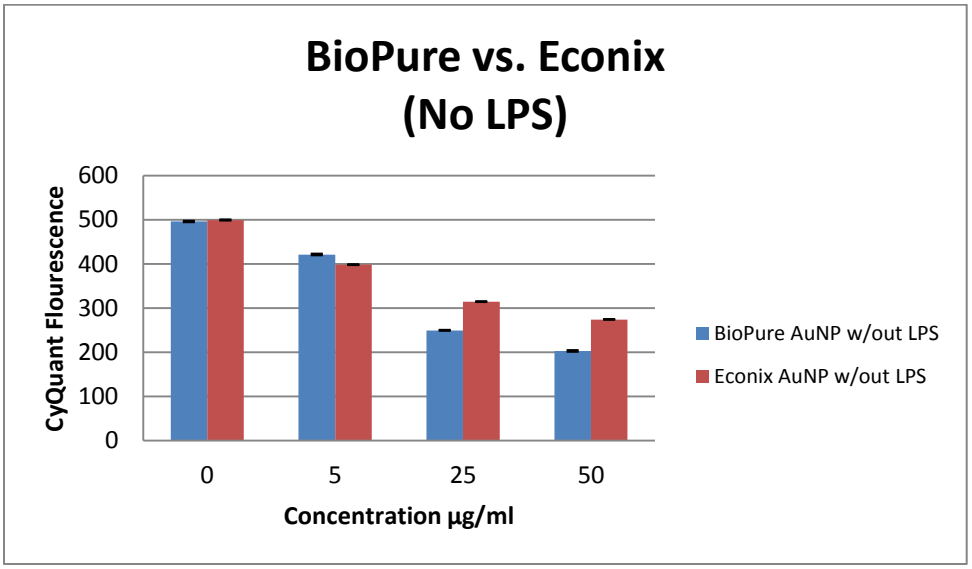

Fig. 33. CyQuant fluorescence for BioPure and Econix AuNPs without LPS. The progression of CyQuant fluorescence between 0 $\mu g\ mL^{-1}$ to 5 $\mu g\ mL^{-1}$, 5 $\mu g\ mL^{-1}$ to 25 $\mu g\ mL^{-1}$, and 25 $\mu g\ mL^{-1}$ to 50 $\mu g\ mL^{-1}$ are statistically significant for all BioPure and Econix AuNPs without LPS (p<0.001) using a one way ANOVA.

Although it appears as though there is a decrease in viability as the concentration of AuNP increases, follow-up experiments showed that the presence of the AuNP interfered with the plate reader's ability to detect the fluorescence from the Cy-Quant dye. This was further confirmed visually using a live-dead stain, trypan blue, but detection of dead cells with this dye was also obliterated by the presence of the particles. It was then shown by visual inspection that the same number of cells continued to adhere to the plate throughout the experiment, which indicated that the cells were alive. Therefore, a follow-up viability assay was performed by simply counting the adherent cells using a Coulter counter (Part 2).

Part 2 – Macrophage Cell Viability Confirmation Using the Coulter Counter

Using Coulter Counter to Confirm Viability

The effect of the various concentrations of the BioPure and Econix AuNPs with and without LPS was tested and it was found that there was no statistically significant change in the mouse macrophage cell viability. The results of the experiment for the BioPure and Econix AuNPs with and without LPS can be seen in Fig. 34 and Fig 35 respectively.

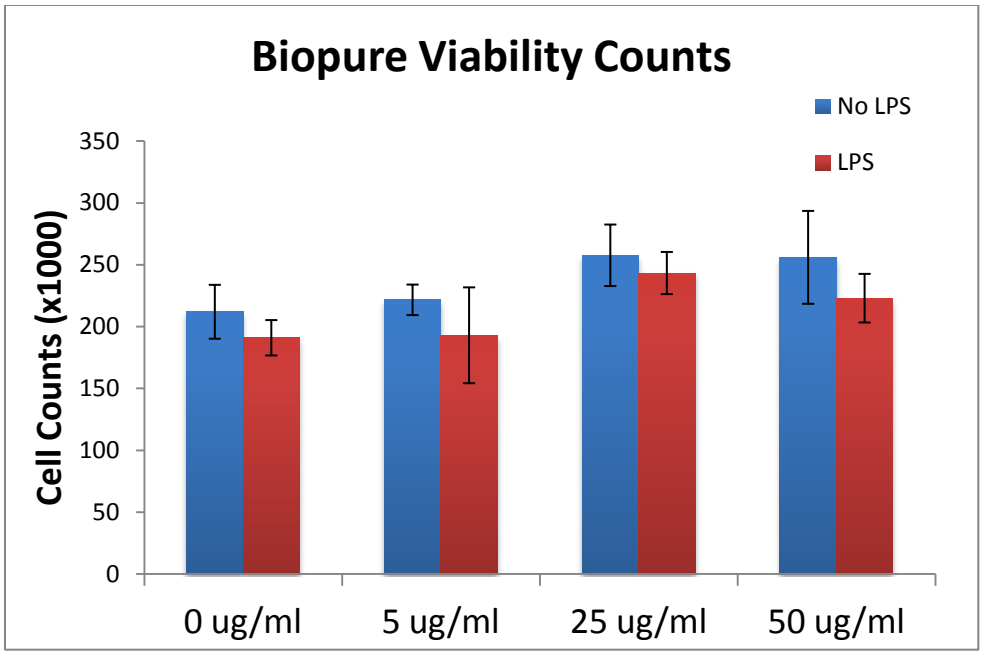

Fig. 34. Biopure Viability Counts with and without LPS. There were no statistically significant differences between a) with and without LPS, or b) any of the AuNP concentrations.

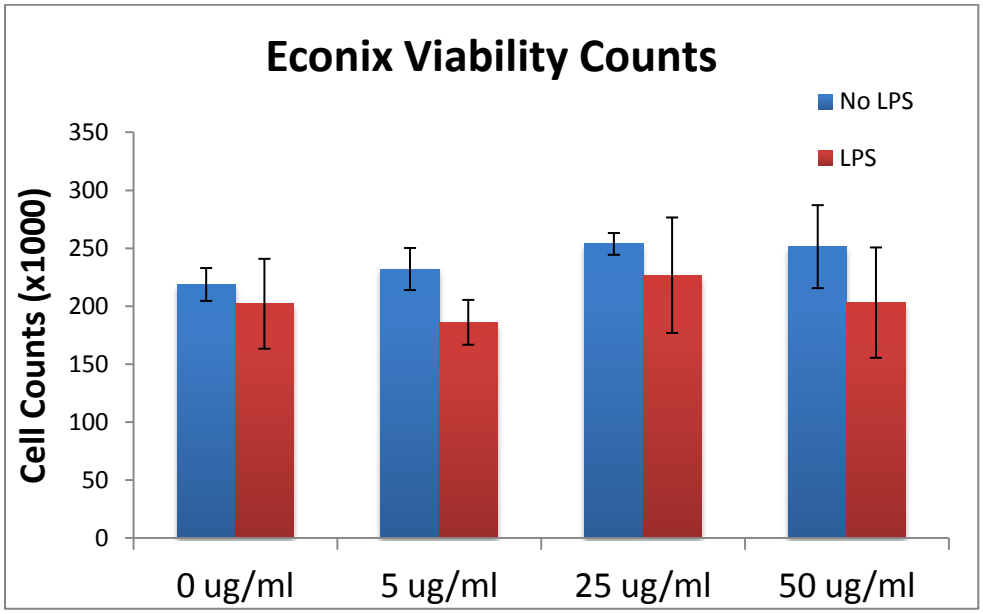

Fig. 35. Econix Viability Counts with and without LPS. There were no statistically significant differences between a) with and without LPS, or b) any of the AuNP concentrations.

Null Hypothesis #3: 50-nm AuNPs will have no effect on ROS for mouse macrophage cells

Using DCFDA to Test for ROS

The amount of reactive oxygen species present in the samples was tested using a DCFDA assay with the AuNP concentrations of $0\ \mu g\ mL^{-1}$, $5\ \mu g\ mL^{-1}$, $25\ \mu g\ mL^{-1}$, and $50\ \mu g\ mL^{-1}$ for both the BioPure and Econix product lines with and without LPS. A comparison was made of the DCFDA fluorescence 19 hours and 31 minutes after the first reading for the BioPure AuNPs with and without LPS and can be seen in Fig. 36. Using the 1 tailed T-test, the BioPure AuNPs with LPS was compared to the BioPure AuNPs without LPS and was found to be statistically significant for concentrations of $5\ \mu g\ mL^{-1}$, but was not statistically significant for the concentrations of $0\ \mu g\ mL^{-1}$, $25\ \mu g\ mL^{-1}$, and $50\ \mu g\ mL^{-1}$.

There was also a comparison of the DCFDA fluorescence made between the BioPure and Econix AuNPs (without LPS) at 19 hours and 31 minutes after the first reading and can be found in Fig. 37. Finally, there was also another comparison made of the DCFDA fluorescence for the Econix AuNPs with and without LPS which can be found in Fig. 38. Using the 1 tailed T-test The Econix AuNP with and without LPS was found to be statistically significant for concentrations of $0\ \mu g\ mL^{-1}$, $5\ \mu g\ mL^{-1}$, and $25\ \mu g\ mL^{-1}$ but was no longer statistically significant at $50\ \mu g\ mL^{-1}$.

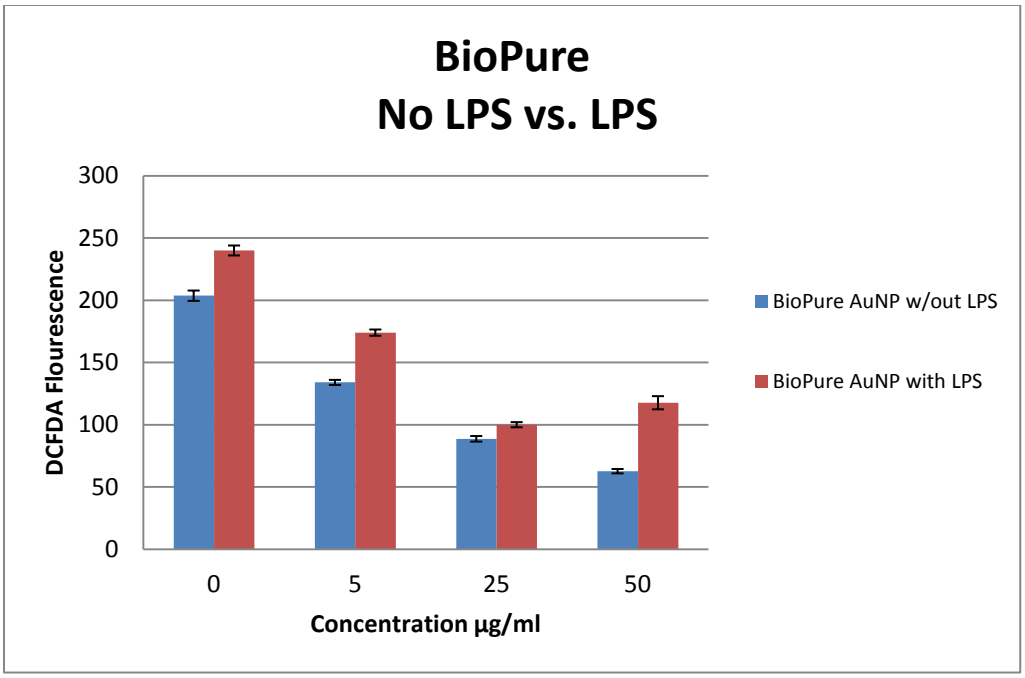

Fig. 36. DCFDA fluorescence for BioPure AuNPs with and without LPS. DCFDA (50 $\mu g\ mL^{-1}$) was loaded into cultured RAW macrophages, rinsed, and then treated with AuNP at concentrations shown for 19.5 hr. N = 4. Paired bars at each concentration were statistically different except at 25 $\mu g\ mL^{-1}$.

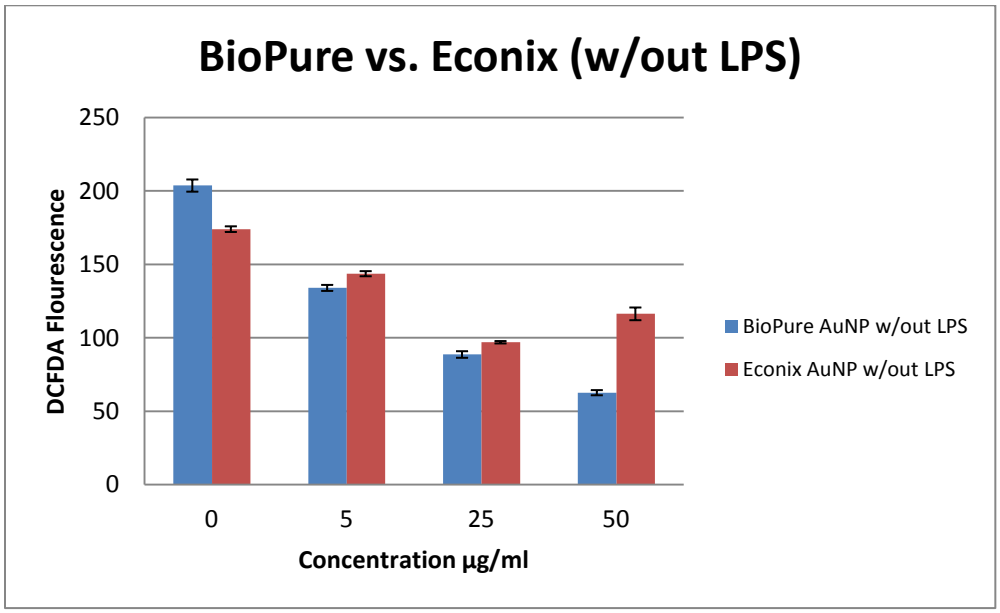

Fig. 37. DCFDA fluorescence for BioPure and Econix AuNPs without LPS. DCFDA (50 $\mu g\ mL^{-1}$) was loaded into cultured RAW macrophages, rinsed, and then treated with AuNP at concentrations shown for 19.5 hr. N = 4. Paired bars at concentrations 0 $\mu g\ mL^{-1}$ and 50 $\mu g\ mL^{-1}$ were statistically different. Paired bars at concentrations 5 $\mu g\ mL^{-1}$ and 25 $\mu g\ mL^{-1}$ were not statistically different.

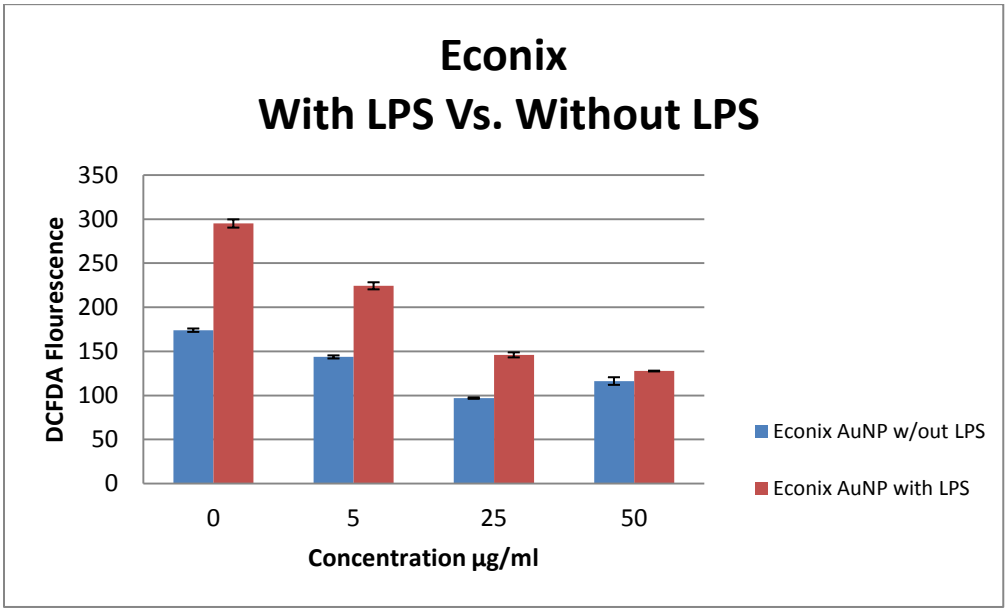

Fig. 38. DCFDA fluorescence for Econix AuNPs with and without LPS. DCFDA ($50\ \mu g\ mL^{-1}$) was loaded into cultured RAW macrophages, rinsed, and then treated with AuNP at concentrations shown for 19.5 hr. N = 4. Paired bars at each concentration were statistically different except at $50\ \mu g\ mL^{-1}$.

The readings of the DCFDA fluorescence for the BioPure and Econix AuNPs were taken for the different concentrations multiple times over a day and a half to present the changes in the DCFDA fluorescence for both the BioPure and Econix product lines with and without LPS. The DCFDA fluorescence over time for the BioPure AuNPs without LPS for the AuNP concentrations of $0\ \mu g\ mL^{-1}$, $5\ \mu g\ mL^{-1}$, $25\ \mu g\ mL^{-1}$, and $50\ \mu g\ mL^{-1}$ can be seen in Fig. 39 which shows that the AuNPs reduce the ROS produced in the DCFDA assay over time. Similar results were found with the BioPure AuNPs with LPS and the Econix AuNPs with and without LPS.

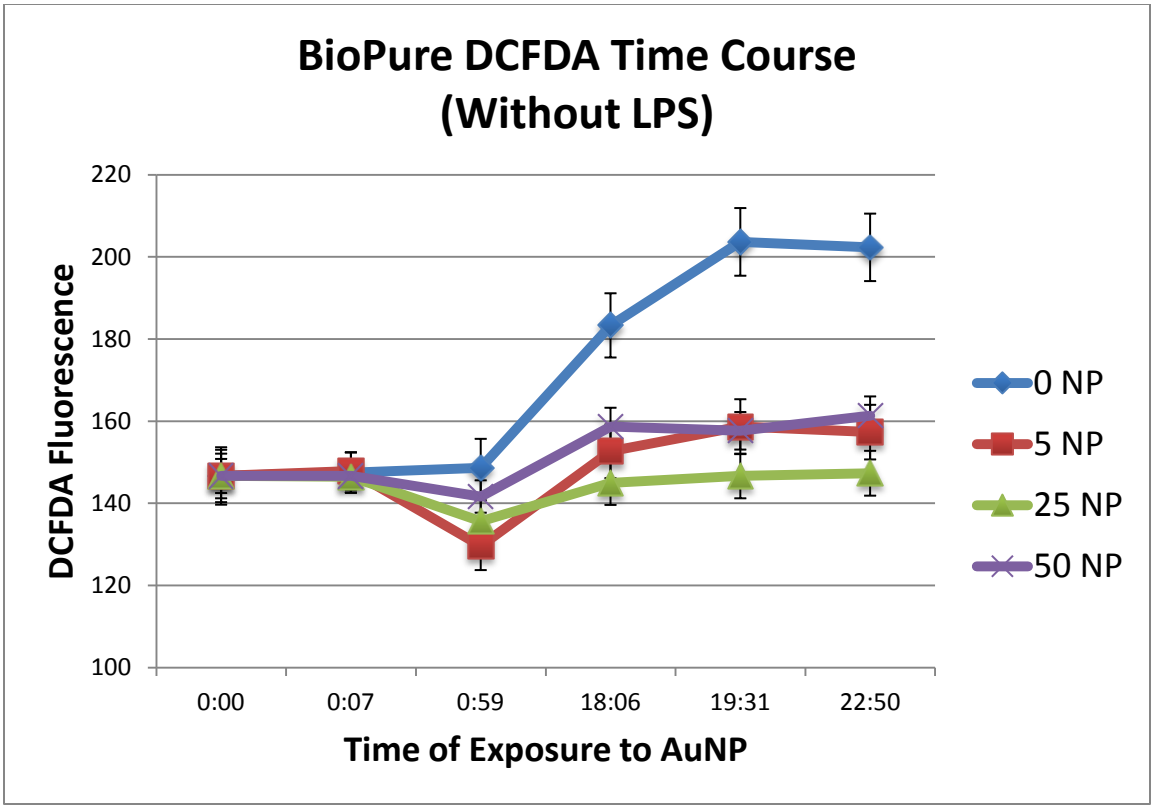

Fig. 39. DCFDA fluorescence over time for BioPure AuNPs without LPS. The DCFDA fluorescence over time between the 0 $\mu g\ mL^{-1}$ and 5 $\mu g\ mL^{-1}$, 25 $\mu g\ mL^{-1}$, and 50 $\mu g\ mL^{-1}$ for BioPure without LPS were statistically significant by 2 way ANOVA at p<0.001.

Null Hypothesis #4: 50-nm AuNPs will produce no effect on the inflammatory response of mouse macrophage cells

ELISA Results

The ELISA was used to test for TNF – α, which is a cytokine that is a primary mediator in protective immune responses, and the ability of the both the Econix and BioPure AuNPs to induce an inflammatory response in the mouse macrophages with and without LPS. When comparing the BioPure and the Econix samples without LPS, the same trend of a decreased response of TNF – α occurred as the AuNP concentration increased, though this was more prominent in the BioPure product line and is displayed

in Fig 40. When comparing the BioPure and Econix sample with LPS, both AuNP decreased TNF - α production by the macrophages in a dose-dependent manner as is shown in Fig. 41.

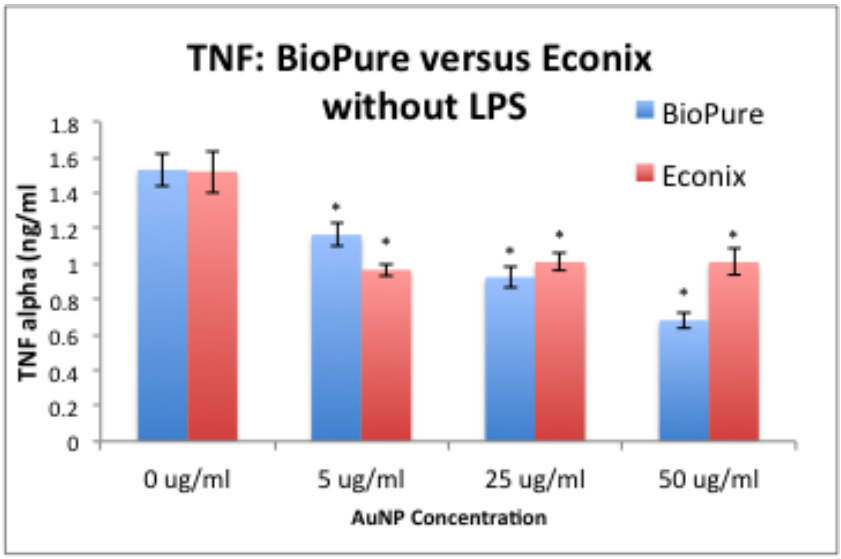

Fig. 40. TNF – α response to increasing AuNPs for BioPure and Econix without LPS. The dose response for both BioPure and Econix were statistically significant by ANOVA at p<0.01. * = p < 0.05 compared to control (0 $\mu g\ mL^{-1}$) in same series.

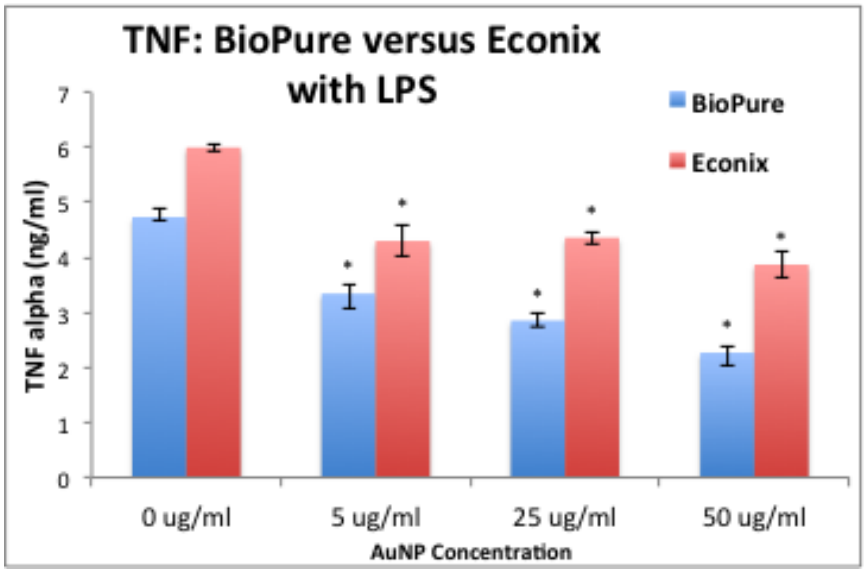

Fig. 41. TNF – α response to increasing AuNPs for BioPure and Econix with LPS. The dose response is statistically significant at p <0.01 for both particles. * = p < 0.05 compared to control (0 $\mu g\ mL^{-1}$) in same series.

The next two figures show the same data presented in an alternate way, comparing with and without LPS for each of the AuNP preparations. These graphs illustrate clearly a) the increase in TNF - α by LPS treatment, and b) the ability of the AuNP to inhibit TNF - α release in the absence or presence of LPS (Figures 42 and 43 respectively).

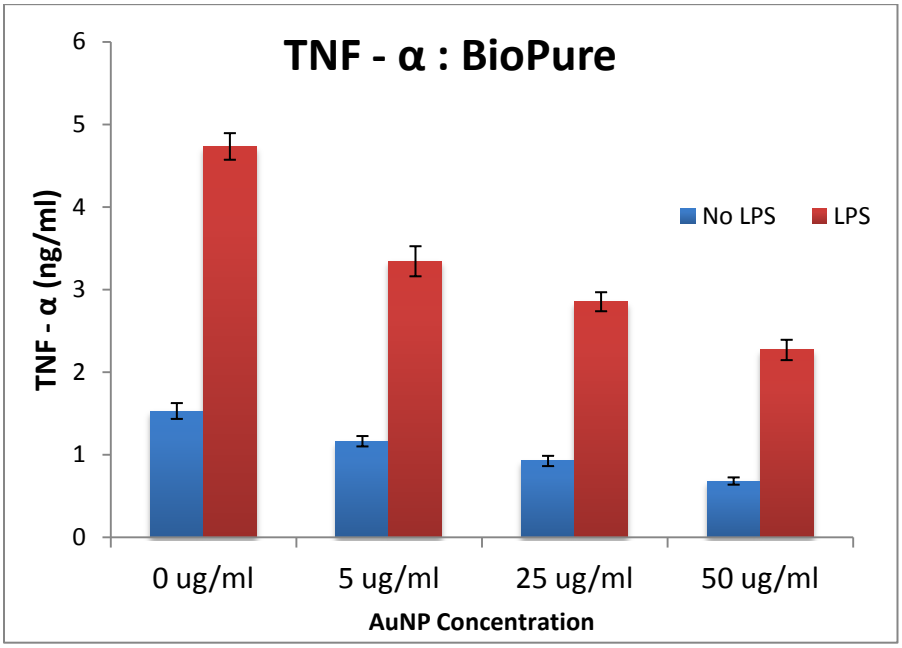

Fig. 42. TNF – α response to increasing AuNPs for BioPure with and without LPS. Responses with LPS were significantly elevated compared to no LPS at all concentrations of AuNP ($p < 0.001$). The dose response for both BioPure with and without LPS were statistically significant by ANOVA at $p<0.01$.

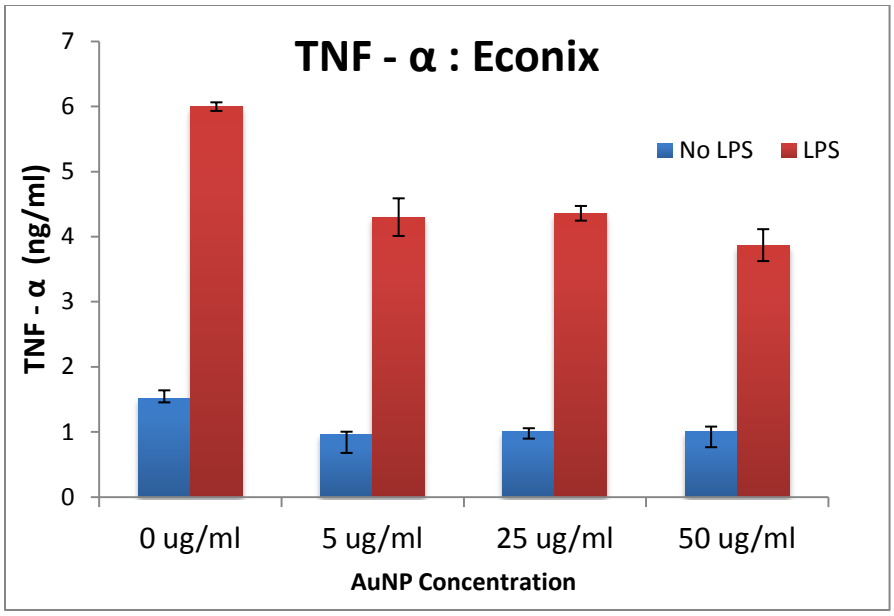

Fig. 43. TNF – α response to increasing AuNPs for Econix with and without LPS. Responses with LPS were significantly elevated compared to no LPS at all concentrations of AuNP ($p < 0.001$). The dose response for Econix both with and without LPS were statistically significant by ANOVA at $p<0.01$.

Null Hypothesis #5: 50-nm AuNPs will produce no immune response on mouse spleen cells

Using Flow Cytometry to Measure the Th1/Th2/Th17 Cytokines Bead Array (CBA)

The Th1/Th2/Th17 CBA Mouse Cytokine Kit (BD BioSciences, San Jose CA) was used to measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interferon-γ (IFN-γ), Tumor Necrosis Factor (TNF - α), Interleukin-17A (IL-17A), and Interleukin-10 (IL-10) protein levels within the media samples from the 24-well plate treated for 48 hours with BioPure AuNPs. In the absence of LPS treatment, there were no statistically significant changes in the amount of cytokine release by the splenocytes regardless of the AuNP concentration as is shown in Fig. 44 and Fig 45.

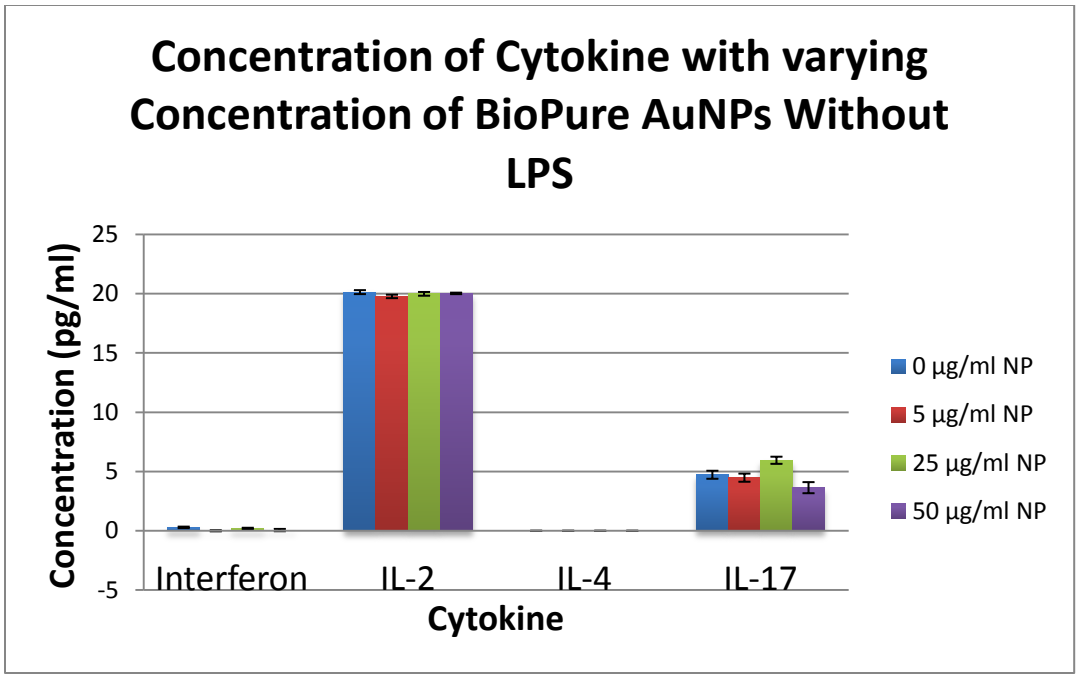

Fig. 44. Concentration of varying cytokines with changing concentrations of BioPure AuNPs without LPS. There were no statistically significant changes between concentrations for each cytokine.

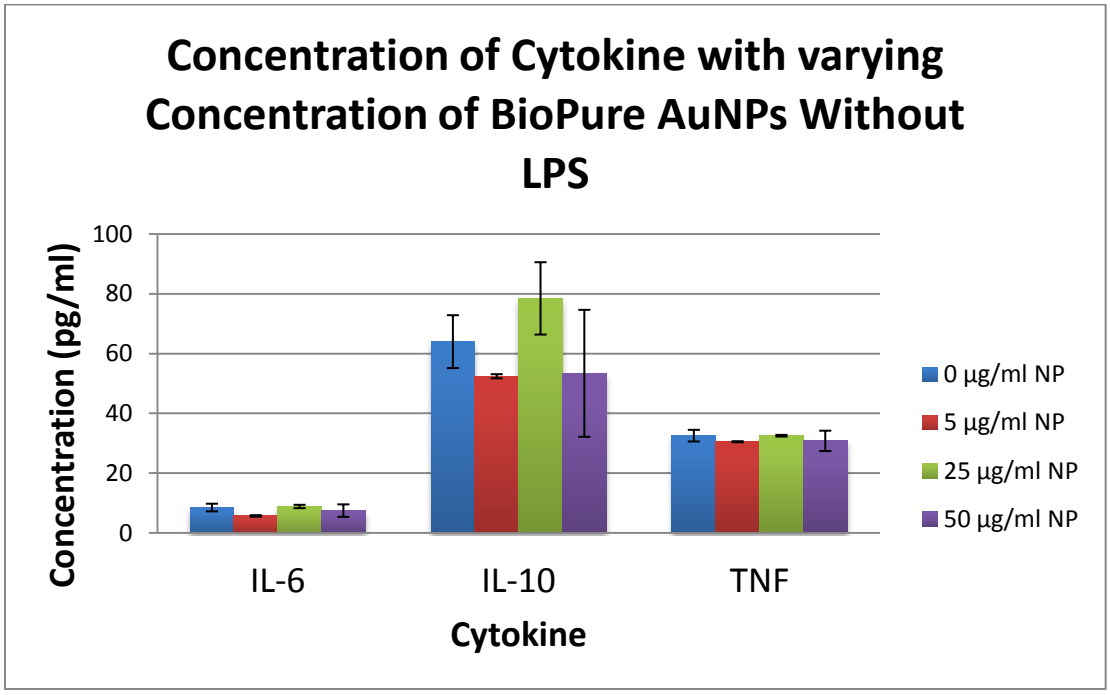

Fig. 45. Concentration of varying cytokines with changing concentrations of BioPure AuNPs without LPS. There were no statistically significant changes between concentrations for each cytokine.

For the samples containing the Th1/Th2/Th17 CBA and AuNP solution containing LPS, there were no statistically significant changes in the amount of cytokine release by the splenocytes until the AuNP concentration reached $50\ \mu g\ mL^{-1}$. Then for the cytokines IL-17, IL-10, and TNF - α the amount of cytokines released actually decreased. This implies that at $50\ \mu g\ mL^{-1}$ AuNPs actually reduce the inflammatory response of the splenocytes justifying the rejection of the null hypothesis #5. This is shown in Fig. 46 and Fig 47.

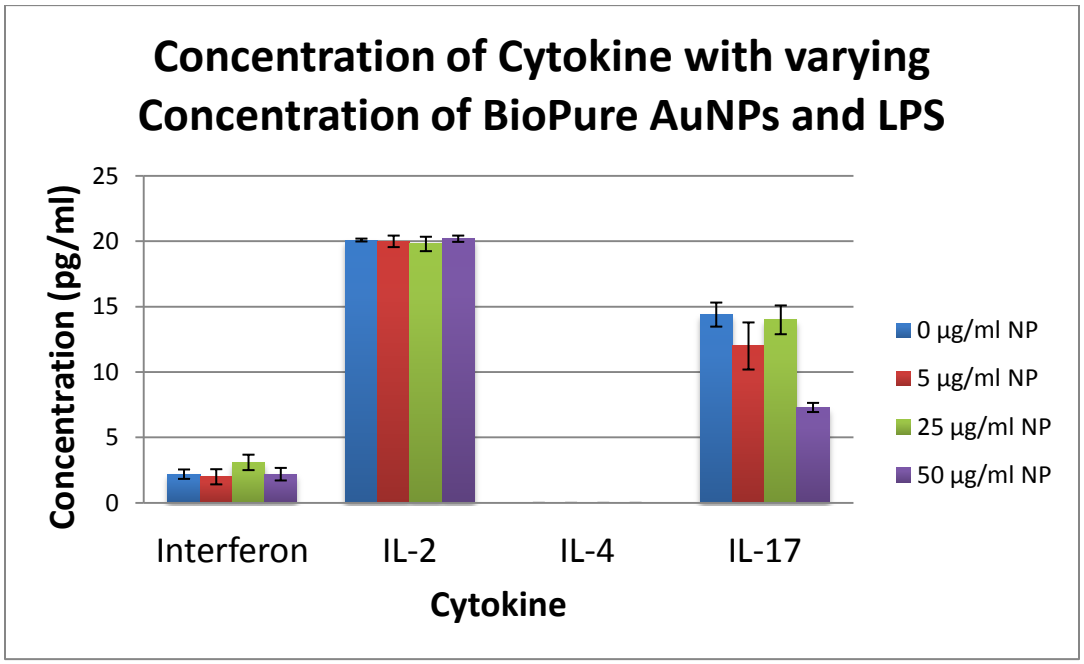

Fig. 46. Concentration of varying cytokines with changing concentrations of BioPure AuNPs with LPS. There were no statistically significant changes between concentrations for each cytokine except for IL-17 at the concentration of $50\ \mu g\ mL^{-1}$ at which point there was a statistically significant difference ($p<0.001$).

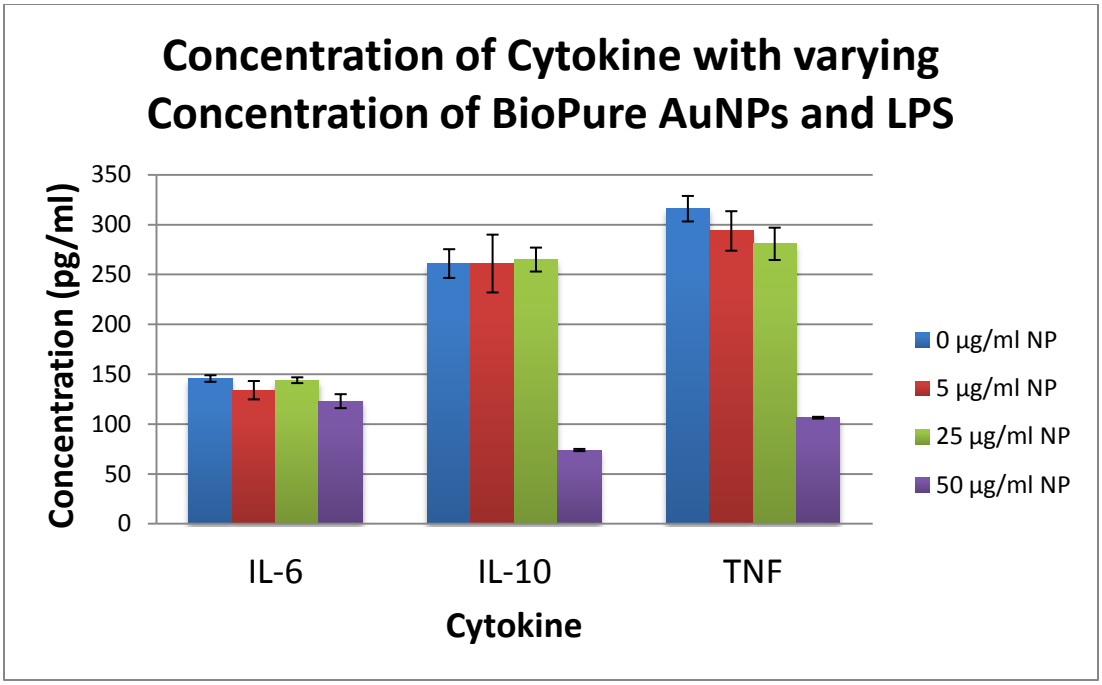

Fig. 47. Concentration of additional cytokines with changing concentrations of BioPure AuNPs with LPS. There were no statistically significant changes between concentrations for each cytokine except for IL-10 and TNF - α at the concentration of 50 $\mu g\ mL^{-1}$ at which point there was a statistically significant difference ($p<0.001$).

Summary

A simplified summary of the results for the Null Hypotheses #1 - #4 and Null Hypothesis #5 is given in Table 6 and Table 7 respectively.

Summary of Results for Null Hypotheses #1 - #4									
		Null Hypothesis #1: Testing Cellular Uptake		Null Hypothesis #2: Testing Cellular Viability		Null Hypothesis #3: Effect of AuNPs on ROS		Null Hypothesis #4: Effect of AuNP on Inflammation	
	Concentration [µg/mL]	With LPS	W/out LPS	With LPS	W/out LPS	With LPS	W/out LPS	With LPS	W/out LPS
BioPure	0			No Effect	No Effect	No Effect	No Effect	No Effect	No Effect
	5			No Effect	No Effect	Inhibitory	Inhibitory	Inhibitory	Inhibitory
	25			No Effect	No Effect	Inhibitory	Inhibitory	Inhibitory	Inhibitory
	50			No Effect	No Effect	Inhibitory	Inhibitory	Inhibitory	Inhibitory
Econix	0.01	Full Uptake	Full Uptake						
	0.05	Full Uptake	Full Uptake						
	0.11	Full Uptake	Full Uptake						
	0	Full Uptake	Full Uptake	No Effect	No Effect	No Effect	No Effect	No Effect	No Effect
	5			No Effect	No Effect	Inhibitory	Inhibitory	Inhibitory	Inhibitory
	25			No Effect	No Effect	Inhibitory	Inhibitory	Inhibitory	Inhibitory
	50			No Effect	No Effect	Inhibitory	Inhibitory	Inhibitory	Inhibitory

Table 5. Summary of Results for Null Hypotheses #1 - #4.

Null Hypothesis #5 Summary: Cytokine Response to 50 nm BioPure AuNP Treatment Different from Untreated Spleen Cells?														
	Interferon		IL-2		IL-4		IL-17		IL-6		IL-10		TNF	
Concentration [µg/mL]	With LPS	W/out LPS	With LPS	W/out LPS	With LPS	W/out LPS	With LPS	W/out LPS	With LPS	W/out LPS	With LPS	W/out LPS	With LPS	W/out LPS
0	No	No	No	No	No	No	No	No	No	No	No	No	No	No
5	No	No	No	No	No	No	No	No	No	No	No	No	No	No
25	No	No	No	No	No	No	No	No	No	No	No	No	No	No
50	No	No	No	No	No	No	Reduced Response	No	No	No	Reduced Response	No	Reduced Response	No

Table 6. Null Hypothesis #5 Summary.

Discussion

Because there are many potential biomedical applications for AuNPs, it is critical to understand the interaction of these materials with cells and tissues. Gold has toxicity to oxidative stress and inflammation (Thakor 2011, Gerber 2013). Specifically, very little is known about the effects of AuNP on the immune system. The immune system mediates not only local, short-term inflammatory responses, but also the ability to fight infection and control cancer. It also can become over-activated and lead to allergies, asthma, and autoimmune diseases. So any effect of nanomaterials on cells of the innate and adaptive immune system could have dramatic side effects when used for medical purposes.

Titanium dioxide NPs, for example, have been shown to induce pro-inflammatory cytokines and reactive oxygen species in macrophages (Kang et al 2008, Valles et al 2006), and this translates to pulmonary inflammation in vivo (Liu et al 2013). Silver and zinc oxide NPs also induce inflammatory responses (Liu et al 2013), but clearly the size and specific structure of the particles modifies the response (Scherbart et al 2011, Kang et al 2008), so that it is impossible to generalize. AuNPs, however, showed little cytotoxicity in vivo (Chen et al 2013, Downs et al 2012), but results in vitro have been mixed with several studies indicating at least some immuno-stimulation (Du et al 2012, Liu et al 2012) but dependent on coating and stabilization (Hashimoto et al 2013).

Importantly, effects on the immune system by AuNP are generally reported as minimal (Downs et al 2012, Villiers et al 2010). In fact, one study demonstrated a down-regulation of inflammatory cytokines induced by LPS (Villiers et al 2010). This could

have a negative impact if it limited the ability to respond to infection, but a positive impact if it limited pathogenic responses. Interestingly, in both the Villiers study and the results reported here, IL-6 was not affected by the presence of AuNP, while other highly inflammatory cytokines (IL-12, and TNF, respectively) were inhibited. This suggests that AuNP do not completely inhibit the ability to mount an inflammatory or immune response, but rather they selectively impact only a subset of cytokines. Because the results showed a specific inhibition of IL-17, a cytokine implicated in pathologic responses, the inhibition by AuNP may be protective. These results require follow-up in specific infection and disease models.

Interestingly, gold in the form of colloid (Auranofin) has long been used in the treatment of rheumatoid arthritis due to its ability to substantially decrease the inflammatory component of the disease (Madeira JM et al 2012). Therefore, the feasibility of using AuNPs for the inhibition of inflammatory diseases has also been considered (Sumbayev et al 2013). However, at this time, there are very few studies looking at different AuNP preparations in different models, so that there remains a tremendous need for research in this area.

Summary of Results in the Context of the Project's Hypotheses

This project set out to observe the immune response effects of 50 nm AuNPs with a coating of PVP on both the innate and adaptive immune system which was tested with two types of AuNPs known as the BioPure and Econix product lines provided by NC[1]. The immune response effect of both product lines was compared with and without the addition of LPS. The first step was to test the uptake of the AuNPs and then to measure the inflammatory response of the mouse macrophages by testing the production of TNF-

[1] Nanocomposix, Inc. 4878 Ronson Court, Suite K San Diego, CA 92111

α, which is the earliest and most prevalent cytokine in mouse macrophage cells. TNF-α is also triggered by bacteria or bacterial components like LPS, which was used in all experiments. The mouse macrophage cells were also used to test the ROS response to the BioPure and Econix products lines of AuNPs with and without LPS. Lastly, viability tests were also performed using the BioPure and Econix product lines of AuNPs with and without LPS.

To study the adaptive immune response, mouse splenocytes were used to evaluate three cytokine profiles indicative of different immune responses which are the following: the Th1 response which is optimal for dealing with viruses and cancer; the Th2 response which is best for large extracellular pathogens, and the Th17 response which is present in autoimmune diseases and hypersensitivities (Afzali et al 2007, Carballeda et al 2007). To accomplish said goals there were four null hypotheses tested and are the following:

Null Hypotheses:

1) 50-nm AuNPs will not be absorbed by mouse macrophage cells or spleen cells.
2) 50-nm AuNPs will have no effect on mouse macrophage cell viability.
3) 50-nm AuNPs will have no effect on ROS for mouse macrophage cells.
4) 50-nm AuNPs will produce no effect on the inflammatory response of mouse macrophage cells.
5) 50-nm AuNPs will produce no immune response on mouse spleen cells.

Null Hypothesis #1

The first null hypothesis was investigated by using light microscopy and flow cytometry to discover whether or not full uptake of the 50-nm BioPure and Econix AuNPs occurred in both mouse macrophage and spleen cells. In order to avoid wasting AuNPs the first experiment used a much smaller amount of AuNPs and then once it was shown that uptake was occurring, the subsequent experiments used higher concentration of AuNPs for both the BioPure and Econix product lines. All experiments showed full uptake occurred, leading to the rejection of the first null hypothesis.

Null Hypothesis #2

The second null hypothesis was investigated using a CyQuant proliferation/viability assay using the BioPure and Econix product lines of AuNPs provided by NC at varying concentrations with and without LPS. As the assay was performed, it appeared initially as though there was a decrease in viability as the concentration of AuNPs increased; but follow-up experiments showed that the presence of the AuNP actually interfered with the plate reader's ability to detect the fluorescence from the Cy-Quant dye. This was confirmed by visually observing that the number of mouse macrophage cells that adhered to the plate remained consistent throughout the experiment (a macrophage must be alive in order to adhere to the plate). To independently confirm the viability of the mouse macrophage cells there was also a follow-up viability assay performed using a Coulter counter and it was found that there was no statistically significant change in the mouse macrophage cell viability indicating that the second null hypothesis has not been rejected. Therefore, results of subsequent experiments were not affected by cell death at the concentrations used in this study.

Null Hypothesis #3

The third null hypothesis was investigated by using mouse macrophages and a DCFDA assay testing the effect of the BioPure and Econix product lines of AuNPs at varying concentrations with and without LPS on ROS for the mouse macrophages over time. It was found that increasing the concentration of AuNPs decreased the amount of ROS released. While LPS significantly increased oxidative stress in the macrophages, the addition of AuNPs did not alter the slope of the curve over time for both the BioPure and Econix AuNPs meaning the inhibitory effect of the AuNPs were semi-proportional to the amount (or concentration) of AuNPs present in the sample. From the results discovered in the experiments it can be said that the third null hypothesis is rejected.

This is consistent with the results of other studies that explored the effect of AuNPs on ROS using alternate means to measure (Electron Paramagnetic Resonance Spectroscopy). AuNPs of both 2.5-nm and 15-nm diameter had the ability to adsorb oxygen radicals as effectively as therapeutic anti-oxidants such as Tempo, Tempone, and Tempamine (Zhang et al 2003). The oxygen radical scavenging was increasingly effective as the concentration of the AuNPs increased which actually eliminated the oxygen radicals measured after a high enough concentration of AuNPs was reached (Zhang et al 2003).

It can be said that the AuNP products used in the previously described experiments may have behaved similarly; absorbing any ROS produced by the cells by natural oxidative processes, or induced by LPS, leading to a return to baseline ROS levels in the presence of the AuNP. Further study is clearly needed to determine the

physicochemical characteristics of the AuNP used here that led to what appears to be ROS scavenging from the cells. The observation is particularly important because a) different particles have unique effects on cellular anti-oxidant mechanisms, and b) inflammatory cytokines are known to be differentially regulated by redox conditions (Pfau et al, 2013; Overocker et al., 2012). Therefore, particles that reduce ROS in macrophages would be expected to inhibit production of ROS-regulated cytokines.

Null Hypothesis #4

The fourth null hypothesis was investigated by using mouse macrophages and an ELISA assay testing the effect of the BioPure and Econix product lines of AuNPs at varying concentrations with and without LPS on the release of TNF – α by the mouse macrophages. It was found that as the concentration of AuNPs increased, the release of TNF – α decreased for both the BioPure and Econix product lines with or without LPS. In all of the samples that contained LPS there was a statistically significant increase in the release of TNF – α compared to samples without LPS, which was expected as LPS is a bacterial component that induces the TNF – α response in mouse macrophage cells.

It was found that the Econix AuNPs did not inhibit the cytokines as effectively as the BioPure AuNPs, which was expected as the Econix AuNPs came with no guarantee to be endotoxin free while the BioPure AuNPs did carry such a guarantee. Given the results, null hypothesis #4 was found to be rejected from the point of view that although the particles did not induce the inflammatory cytokine, they did have an effect through the reduction of TNF - α released from these cells in the absence of particles, with or without LPS. This suggests an anti-inflammatory effect of the AuNPs consistent with other studies (Villiers et al 2010, Sumbayev et al 2013).

Null Hypothesis #5

The fifth null hypothesis was investigated using a Cytokine Bead Array (CBA) to discover the cytokine profiles of the Th1, Th2, and Th17 response using the BioPure and Econix product lines of AuNPs provided by NC[1] at varying concentrations with and without LPS. The cytokines that were analyzed were Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interferon-γ (IFN-γ), Tumor Necrosis Factor (TNF - α), Interleukin-17A (IL-17A), and Interleukin-10 (IL-10). It was found that in the absence of LPS, there were no statistically significant changes in the amount of cytokine release by the splenocytes regardless of the AuNP concentration. Therefore, the AuNPs used in this study induced no effects on the production of any of the T cell cytokines in the absence of any other stimulation. This is consistent with other studies (Villiers et al), and supports the premise that AuNP at these concentrations are neither overtly toxic, nor immunotoxic.

When LPS was present, there were no statistically significant changes in the amount of cytokine release by the splenocytes until the AuNP concentration reached $50\ \mu g\ mL^{-1}$, then for the cytokines IL-17, IL-10, and TNF – α the amount of cytokines released actually decreased which implies that the AuNPs reduced the immune response to LPS by the splenocytes proving that the null hypothesis #5 is to be rejected.

IL-17 and TNF - α are implicated in a Th17 response profile, which exists in disease states as described in the immune response section of the introduction. The fact that this reduction required the highest concentration of particles suggests that it may be due to the ability of the AuNP to reduce the oxidative stress in these cells when stimulated with LPS. TNF – α has been shown to be inducible via oxidative stress

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(Schreck et al 1992). Since the AuNP reduced the ROS, this may be the mechanism of reduced TNF – α from the macrophages. In turn, TNF – α is one of the key cytokines involved in induction of the Th-17 pathway and IL-17 production by T cells (Afzali et al 2007). Therefore, by reducing ROS, the AuNP may sequentially inhibit both TNF – α and IL-17 following LPS stimulation.

Implications of Results

The results of the experiments imply that at least for the short term, and regarding the immune cells of mice, there are not only no negative effects of the use of 50 nm AuNPs, but actually an inhibitory effect of the release of a cytokine profile associated with diseases and a reduction of oxidative stress. This means that there are implications for less risk when dealing with AuNPs in the various industries previously described with a particular benefit to the application of AuNPs in the medical industry.

One of the advancements in the medical industry regarding AuNPs that the results of this research have implications to benefit is the use of AuNPs for Raman spectroscopy that can be used in in-vivo imaging. Raman spectroscopy is used to observe rotational, vibrational, and other low-frequency modes within a system and has been greatly enhanced by using AuNPs that were conjugated with antibodies (Cai et al 2008). With the implication for less risk of the AuNPs it can be said that this process may become more common among the many methods of medical imaging available today.

Another important medical application of AuNPs that may be benefited by the implication of the results of this research is the use of AuNPs for drug delivery. One study was found where TNF-α was loaded onto AuNPs and used in conjunction with local heating was found to enhance the efficacy of the treatment used (Cai et al 2008).

Afzalie used AuNP shells that were filled with drugs such as insulin and were released by irradiation at 1064 nm (Afzali et al 2007).

Another implication of the benefits of the results found from this research is the implication of less risk and therefore the allowance of a more aggressive use of AuNPs in the treatments of cancer. For example, Cai found that adding 5000 AuNPs per cancer cell was adequate to kill the cell when it was exposed to laser emitting in the near infrared region (700 – 900 nm) (Cai et al 2008).

Future Work

The increased implications for possible health benefits of AuNPs would need to be further studied for different sizes of AuNPs as the surface interactions and therefore chemistry of NPs are known to change at least somewhat as the size of the NPs change, particularly as the AuNP diameter reaches 15 nm and below which is small enough for Ostwald ripening to be significant. It would also be useful to study the effects of using coatings other than PVP to fully realize how much of an effect the coating has on the AuNPs' toxicity and what interactions actually occur.

An example of this was found by Farkas where rainbow trout were exposed to AuNPs mostly in the ranges of 5-nm and 10-nm that were coated with sodium citrate that caused the release of ROS to increase threefold (Farkas et al 2010). However, Ionia found that AuNPs were able to both generate and scavenge short lived free radicals while the AuNPs were in the presence of oxygen (Ionita et al 2008).

In another case, Misawa found that ROS were generated when AuNPs were irradiated by either X-rays or UV light, which can be very useful for tumors that are more difficult to target directly with concentrated radiation (Misawa et al 2011), while at the

same time can be harmful if the AuNPs collect in healthy cells and are unintentionally irradiated (Misawa et al 2011).

Further study would also be required to understand the immune response of a human to the 50-nm AuNPs, but it can at least be implied that the same effect may happen in the immune response of a human as occurred with the mouse immune response, especially realizing that compounds containing gold are already being used as an anti-inflammatory agent (Kim et al 2001).

Conclusions

In conclusion, the effects of exposing mouse macrophage cells and splenocytes to the concentrations of $0\,\mu g\,mL^{-1}$, $5\,\mu g\,mL^{-1}$, $25\,\mu g\,mL^{-1}$, and $50\,\mu g\,mL^{-1}$ 50 nm AuNPs coated with PVP was studied using two types of AuNPs known as the BioPure and Econix product lines provided by NC[1] with and without the presence of LPS, a known endotoxin. The cellular uptake of the AuNPs was tested on the mouse macrophage and splenocytes and it was found that all AuNPs were consumed completely by the cells. Then the inflammatory response of the mouse macrophage cells were tested by measuring the release of TNF – α and it was found that as the concentration of the AuNPs that the macrophages were exposed to increased, the TNF - α decreased which indicates an inhibitory response of the AuNPs.

The mouse macrophage cells were also used to test the ROS and toxicity of the AuNPs. It was found that the AuNPs had no statistically significant effect on the release of ROS by the mouse macrophage cells on the short term, but that the AuNPs actually reduced the ROS released by the macrophages over time. It was also found that the AuNPs produced no statistically significant changes to the cell viability.

The cytokine response of mouse splenocytes exposed to the BioPure and Econix product lines of 50-nm AuNPs with and without LPS by using a Th1/Th2/Th17 cytokine bead array (CBA). The CBA measured the release of Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interferon-γ (IFN-γ), Tumor Necrosis Factor (TNF - α), Interleukin-17A (IL-17A), and Interleukin-10 (IL-10). It was found that in the absence of LPS treatment, there were no statistically significant changes in the amount of cytokine release by the splenocytes regardless of the AuNP concentration.

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For the samples that did contain LPS, there were no statistically significant changes in the amount of cytokine release by the splenocytes until the AuNP concentration reached $50\ \mu g\ mL^{-1}$. Then for the cytokines IL-17, IL-10, and TNF – α, the amount of cytokines released decreased. This implies that at $50\ \mu g\ mL^{-1}$ AuNPs reduced the inflammatory response of the splenocytes.

References

1. Chandraprakash Dwivedi, Swarnali Das Paul. Nanotechnology to Nanotoxicology: A Review Article. Journal of Scientific & Innovative Research. April 2013, vol 2, issue 2, Pgs. 458 – 470.
2. Marwa M. Hussain, Tamer M. Samir, Hassan M.E. Azzazy. Unmodified Gold Nanoparticles for Direct and Rapid Detection of Mycrobacterium Tuberculosis Complex. Clinical Biochemsitry. January 11 2013, Pgs. 633 – 637.
3. Sanaa Eissa, Sherif M. Shawky, Marwa Matboli, Shaymaa Mohamed, Hassan M.E. Azzazy. Direct Detection of Unamplified Hepatoma Upregulated Protein RNA in Urine Using Gold Nanoparticles for Bladder Cancer Diagnosis. Clinical Biochemistry. October 21 2013, Pgs. 3 – 5.
4. Andre Nel, Tian Xia, Lutz Madler, Ning Li. Toxic Potential of Materials at the Nanolevel. Science. 2006, Pgs. 622 – 627.
5. Mira S. Olson, Patrick L. Gurian. Risk Assessment Strategies as Nanomaterials Transition into Commercial Applications. J Nanopart Res. March 14 2012, Pg. 5.
6. Cristina Buzea, Ivan I. Pacheco Blandino, Kevin Robbie. Nanomaterials and Nanoparticles: Sources and Toxicity. Biointerphases, 2007 vol 2, issue 4, Pg. 19.
7. T.S. Kim, B.Y. Kang, M.H. Lee, Y.K. Choe, S.Y. Hwang. Inhibition of interleukin-12 production by auranofin, an anti-rheumatic gold compound,

deviates CD4+ T cells from the Th1 to the Th2 pathway. British Journal of Pharmacology. 2001, 134, p571-578.

8. Sujit Kumar Ghosh, Tarasankar Pal. Interparticle Coupling Effect on the Surface Resonance of Gold Nanoparticles: From Theory to Applications. Chem. Rev. 2007, Vol 107. Pgs. 4797 – 4862.
9. Carl F. Ware, Sybil Santee, Alison Glass. Tumor Necrosis Factor-Related Ligand and Receptors. The Cytokine Handbook, 3rd edition. Academic Press 1998. Pg 580.
10. M. R Gittings, D. A Saville. The determination of hydrodynamic size and zeta potential from electrophoretic mobility and light scattering measurements. Colloids and Surfaces A: Physicochemical and Engineering Aspects, Volume 141, Issue 1. 1998. Pgs. 111 – 117.
11. P.W. Voorhees. The Theory of Ostwald Ripening. Journal of Statistical Physics. January 1985, vol. 38, issue 1-2, Pg. 1.
12. Shuguang Wang, Rasheeda Lawson, Paresh C. Ray, Hongtao Yu. Toxic Effects of Gold Nanoparticles on Salmonella Typhimurium Bacteria. Toxicology and Industrial Health. 2011, Pgs. 547 – 554.
13. B. Devika Chithrani, Arezou A. Ghazani, Warren C. W. Chan. Determining the Size and Shape Dependence of Gold Nanoparticle Uptake into mammalian Cells. Nano Letters. 2006, vol. 6, no. 4, Pgs. 662 – 668.
14. Xiaoli Liu, Yajun Xu, Zhaoqiang Wu, Hong Chen. Poly(N-vinylpyrrolidone)-Modified Surfaces for Biomedical Applications. Macromolecular Journals. 2013, 13, Pgs. 147 – 154.

15. Afzali B, Lombardi G, Lechler RI, Lord GM. 2007. The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. Clin Exp Immunol 148: 32-46.
16. Furuzawa-Carballeda J, Vargas-Rojas MI, Cabral AR. 2007. Autoimmune inflammation from the Th17 perspective. Autoimmunity Rev 6: 169-175.
17. Christian L. Villiers, Heidi Freitas, Rachel Couderc, Marie-Bernadette Villiers, Patrice N. Marche. Analysis of the Toxicity of Gold Nano Particles on the Immune System: Effect on Dendritic Cell Functions. Special Focus: Safety of Nanoparticles. July 10 2009, Pgs. 55 – 60.
18. Adam Yuh Lin, Joao Paulo Mattos Almeida, Adham Bear, Nathan Liu, Laureen Luo, Aaron Edward Foster, Rebekah Anna Drezek. Gold Nanoparticle Delivery of Modified CpG Stimulates Macrophages and Inhibits Tumor Growth for Enhanced Immunotherapy. PLOS One. May 2013, vol. 8, issue 5, e63550, Pgs. 1 – 9.
19. Jason Overocker, Jean C. Pfau. Cytokine Production Modified by System Xc- After PM10 and Asbestos Exposure. Journal of Young Investigators. June 2012, vol. 23, issue 6, Pgs. 34 – 39.
20. Jean C. Pfau, Todd Seib, Jason J. Overocker, Jeremy Roe, Aaron S. Ferro. Functional Expression of System Xc is Upregulated by Asbestos but Not Crystalline Silica in Murine Macrophages. Inhalation Toxicology. 2012, Pgs. 1 – 10.
21. David J. Blake, Celeste M. Bolin, David P. Cox, Fernando Cardozo-Pelaez, Jean C. Pfau. Internalization of Libby Amphibole Asbestos and Induction of

Oxidative Stress in Murine Macrophages. Toxicological Sciences. June 19, 2007, Pgs. 277 – 288.

22. Veronique Nogueira, Nissim Hay. Molecular Pathways: Reactive Oxygen Species Homeostasis in Cancer Cells and Implications for Cancer Therapy. Clinical Cancer Research Journal. May 29 2013, vol. 19, p 4309 - 4314.
23. P.W. Voorhees. The theory of Ostwald ripening. Journal of Statistical Physics. January 1985, Volume 38, Issue 1-2, pp 231 – 252.
24. Wolfgang Haiss, Nguyen T. K. Thanh, Jenny Aveyard, David G. Fernig. Determination of Size and Concentration of Gold Nanoparticles from UV-Vis Spectra. Anal Chem. 2007, Pgs. 4215 – 4221.
25. Hilde Jans, Xiong Liu, Lauren Austin, Guido Maes, Qun Huo. Dynamic Light Scattering as a Powerful Tool for Gold Nanoparticle Bioconjugation and Biomolecular Binding Studies. Anal Chem. 2009, Pgs. 9425 – 9432.
26. Hiroshi Suzuki, Tatsushi Toyooka, and Yuko Ibuki. Simple and Easy Method to Evaluate Uptake Potential of Nanoparticles in Mammalian Cells Using a Flow Cytometric Light Scatter Analysis. Environ. Sci. Technol., 2007 41 (8), pp 3018-3024.
27. A.S. Thakor, J. Joerst, C. Zavaleta, T.F. Massoud, and S.S. Gambhir. Gold Nanoparticles: A Revival in Precious Metal Administration to Patients. Nano Letters, 2011, 11 (10), pp 4029-4036.
28. Hans-Peter Gerber, Vishva Dixit and Napoleone Ferrara. Vascular Endothelial Growth Factor Induces Expression of the Antioapoptotic Proteins

Bcl-2 and A1 in Vascular Endothelial Cells. J. Biol. Chem. 1998, 273:13313-13316.

29. Kang JL, Moon C, Lee HS, Lee HW, Park EM, Kim HS, Castranova V. Comparison of the biological activity between ultrafine titanium dioxide particles in RAW 264.7 cells associated with oxidative stress. Journal of Toxicology and Environmental Health. 2008;71(8). Pgs. 478 – 485.
30. Gema Valles, Pablo Gonzalez-Melendi, Jose L. Gonzalez-Carrasco, Laura Saldana, Elena Sanchez-Sabate, Luis Munuera, Nuria Vilaboa. Differential Inflammatory Macrophase Respose to Rutile and Titanium Particles. Science Direct. June 21 2006, Pgs. 5199 - 5211.
31. Huanliang Liu, Danfeng Yang, Honglian Yang, Huashan Zhang, Wei Zhang, YanJun Fang, Zhiqing Lin, Lei Tian, Bencheng Lin, Jun Yan, Zhuge Xi. Comparative Study of Respiratory Tract Immune Toxicity Induced by Three Sterilisation Nanoparticles: Silver, Zinc Oxide and Titanium Dioxide. Journal of Hazardous Materials. January 28, 2013, Pgs. 478 – 486.
32. Agnes M Scherbart, Julia Lnger, Alexey Bushmelev, Damiem van Berlo, Petra Haberzetti, Frederik-Jan van Schooten, Annette M Schmidt, Christine R Rose, Roel PF Schins, Catrin Albrecht. Contrasting Macrophage Activation by Fine and Ultrafine Titanium Dioxide Particles is Associated with Different Uptake Mechanisms. Particle and Fibre Toxicology. 2011, Pgs. 1 – 19.
33. Hui Chen, Alisha Dorrigan, Sonia Saad, Dominic J. Hare, Michael B. Cortie, Stella M. Valenzuela. In Vivo Study of Spherical Gold Nanoparticles:

Inflammatory Effects and Distribution in Mice. PLOS One. February 28, 2013, Pgs. 1 – 8.

34. Thomas R. Downs, Meredith E. Crosby, Ting Hu, Shyam Kumar, Ashley Sullivan, Katherine Sarlo, Bob Reeder, Matt Lynch, Matthew Wagner, Tim Mills, Stefan Pfuhler. Silca Nanoparticles Administered at the Maximum Tolearted Dose Induce Genotoxic Effects Through an Inflammatory Reaction While Gold Nanoparticles Do Not. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. April 6, 2012, Pgs. 38 – 50.
35. Libo Du, Xiaoxiang Miao, Hongying Jia, Yanli Gao, Ke Liu, Xueji Zhang, Yang Liu. Detection of Nitric Oxide in Macrophage Cells for the Assessment of the Cytotoxicity of Gold Nanoparticles. Talanta. 2012, Pgs. 11 – 16.
36. Zhimin Liu, Wenqing Li, Feng Wang, Chunyang Sun, Lu Wang, Jun Wang, Fei Sun. Enhancement of Lipopolysaccharide-Induced Nitric Oxide and Interleukin-6 Production by PEGylated Gold Nanoparticles in RAW264.7 Cells. Nanoscale. September 2012, Pgs. 7135 – 7142.
37. Masanori Hashimoto, Hirokazu Toshima, Tetsu Yonezawa, Koji Kawai, Takashi Narushima, Masauki Kaga, Kazuhiko Endo. Responses of RAW264.7 Macrophages to Water-Dispersible Gold and Silver Nanoparticles Stabilized by Metal-Carbon б-Bonds. Society for Biomaterials. June 12, 2013, Pgs. 1 – 12.
38. Christian L. Villiers, Heidi Freitas, Rachel Couderc, Marie-Bernadette Villiers, Patrice N. Marche. Analysis of the toxicity of gold nanoparticles on

the immune system: effect on dendritic cell functions. J Nanopart Res (2010). 12, Pgs. 55 – 60.

39. Madeira JM, Gibson DL, Kean WF, Klegeris A. The biological activity of auranofin: implications for novel treatment of diseases. Infammopharmacology. 2012 Dec;20(6). Pgs. 297 – 306.
40. Vadim V. Sumbayev, Inna M. Yasinska, Cesar Pascual Garcia, Douglas Gilliland, Gurprit S. Lall, Bernhard F. Gibbs, David R. Bonsall, Luca Varani, Francois Rossi, Luigi Calzolai. Gold Nanoparticles Downregulate Interleukin-1β-Induced Pro-Inflammatory Responses. Gold Nanoparticles. 2013, 472 – 477.
41. Pfau, J.C., T. Seib, J. Overocker, J. Roe, A. Ferro. 2012. Functional expression of System xc- is upregulated by asbestos but not crystalline silica in murine macrophages. *Inhalation Toxicology*, 24(8):476-85.
42. Overocker, J. and J.C. Pfau. 2012. Cytokine Production Modified by System X_c- After PM10 and Asbestos Exposure. *Journal of Young Investigators* 23(6):34-39.
43. Zhenyuan Zhang, Alexander Berg, Haim Levanon, Richard W. Fessenden, and Dan Meisel. On the Interactions of Free Radicals with Gold Nanoparticles. JACS articles, 6/6/2003. Pgs. 7959 – 7963.
44. Ralf Schreck, Kaj Albermann, and Patrick A. Baeuerle. Nuclear Factor Kb: An Oxidative Stress-Responsive Transcription Factor of Eukaryotic Cells (A Review). J. Free Radical Research. 1992, Vol. 17, No. 4, Pages 221-237.
45. Weibo Cai, Ting Gao, Hao Hong, Jiangtao Sun. Applications of gold nanoparticles in cancer nanotechnology. Department of Radiology and Medical Physics, University of Wisconsin. 2008. Pg. 17 - 32.

46. Julia Farkas, Paul Christian, Julian Alberto Gallego Urrea, Norber Roos, Martin Hasselov, Knut Erik Tellefsen, Kevin V. Thomas. Effects of silver and gold nanoparticles on rainbow trout (Oncorhynchus mykiss) hepatocytes. Aquatic Toxicology 96 (2010). Pg 44 – 52.
47. Petre Ionita, Florica Spafiu, Corneliu Ghica. Dual behavior of gold nanoparticles, as generator and scavengers for free radicals. Springer J Mater Sci (2008) vol 43. Pgs. 6571 – 6574.
48. Masaki Misawa, Junko Takahashi. Generation of reactive oxygen species induced by gold nanoparticles under x-ray and UV Irradiations. Nanomedicine: Nanotechnology, Biology, and Medicine. Vol 7 (2011). Pgs. 604 – 614.