Design and Validation of a Live Cell Reporter for Macrophage Activation

by
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A thesis presented to the Honors College of Middle Tennessee State University in partial
fulfillment of the requirements for graduation from the University Honors College

Fall 2018

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Abstract

Macrophages are phagocytic cells of the innate immune system, which defend the body against invading pathogens. These immune cells are capable of transiently adopting different polarization states suited to different phases of an infection. They exist in a naïve (M0) state in the absence of appropriate stimulus but adopt a classical (M1) activation state, a pro-inflammatory, highly microbicidal phenotype in response to microbial ligands and certain cytokines to combat current infections. Macrophages can repolarize to an alternative (M2) activation state, a largely anti-inflammatory phenotype, after an infection has been resolved. A plethora of microbial and plant-derived compounds have the potential to interfere with normal macrophage polarization and thereby compromise or enhance the innate immune response to infection. In order to study this process in live macrophages, a fluorescent reporter for M1 activation has been designed, engineered, and validated. The reporter has been successfully validated through a series of experiments where murine macrophages that have been stably transduced with the reporter construct were exposed to lipopolysaccharide (LPS) from gram-negative bacteria and the cytokine interferon-gamma (IFN-γ), which are known inducers of macrophage M1 polarization. It is demonstrated that the reporter expression is concurrent with that of the endogenous gene. Furthermore, data also suggest that the reporter is a suitable tool for investigating the immunomodulatory properties of plant-derived compounds, such as polysaccharides extracted from American ginseng.

Acknowledgements

First, I would like to thank Dr. Nelson for allowing me to do undergraduate research in his lab almost three years ago, as a Freshman. I would like to thank all of the graduate students in the Nelson lab for their help in training, troubleshooting, and math when I struggled. I would like to thank the URC for sponsoring portions of this project with two URECA Silver Scholar awards. Finally, I would like to thank my parents for the support, my friends for the encouragement, my boyfriend, Jacob, for the love, and God for the strength. Without all of these factors, I would not have been able to complete my thesis today.

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Introduction

Macrophages are among the first line of defense against invading pathogens in mammals. These tissue-resident innate immune cells exist in a naïve (M0) state until activated by stimulation with pathogen-derived ligands or specific cytokines produced by other immune cells. These stimuli radically change both gene expression and the biological properties of these cells. The macrophages begin to secrete a range of pro- or anti-inflammatory signaling molecules that regulate the activity of other immune cells, and to a certain extent, also act as autocrine signals. Generally, activation increases the ability of macrophages to phagocytose – literally 'ingest'- and then destroy pathogens (1).

Macrophage activation, which is also referred to as polarization, is a continuum of differing and often contrasting phenotypes. However, it is generally described as two opposing states: the classical (M1) and alternative (M2). Different signals and ligands, both endogenous and exogenous in origin, can stimulate macrophages to adopt one state or the other or indeed switch from an M1 to an M2 state or vice versa. For example, lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall, and interferon-gamma (IFN-γ), an endogenous compound produced by other immune cells, promote M1 activation, increasing the anti-microbial activity of macrophages (1). This anti-microbial activity is partially driven by the expression of *Nos2*, a gene that encodes the enzyme inducible nitric oxide synthase (iNOS) (2). This enzyme utilizes L-arginine as a substrate, producing nitric oxide (NO). While NO has a variety of important and diverse biological functions, in M1 macrophages, it is used to generate highly toxic

reactive nitrogen species, such as peroxynitrite (2). These compounds chemically alter DNA, proteins, and lipids of phagosomal microbes, resulting in the antimicrobial activity seen within macrophages (3).

Microbe-derived ligands, often referred to as pathogen-associated microbial patterns (PAMPs) are recognized by a variety of macrophage pattern recognition receptors (PRRs), including the toll like receptors (TLRs). Engagement of TLRs with the appropriate PAMPs stimulates expression of *Nos2* at a transcriptional level through activation of the nuclear factor-kappaB (NF-κB) signaling pathway. Engagement of TLR4 receptors by LPS stimulates p65-containing dimeric NF-κB transcription factors to translocate from the cytosol to the nucleus where they are able to bind to cis-regulatory elements in the *Nos2* promoter to increase transcription of the gene (4, Fig. 1).

The NF-κB pathway has been

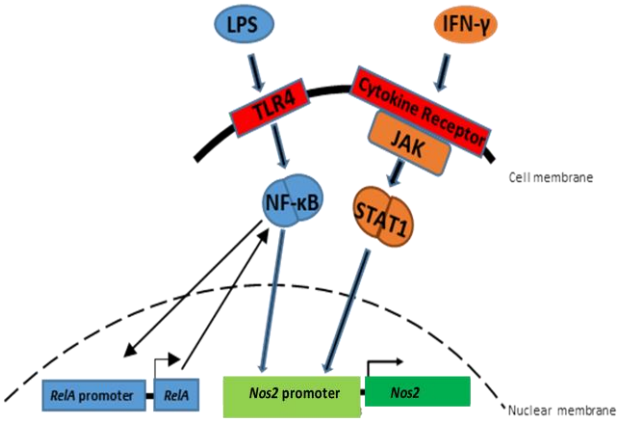

Figure 1: Schematic of signaling pathways in RAW 264.7 murine macrophages via LPS and Interferon-γ stimulation to induce *Nos2* reporter transcription and mCherry expression. LPS and IFN-γ cytokines bind with receptors on the cell membrane, stimulating the activation of signaling pathways. LPS activates TLR4, stimulating the NF-κB transcription factors, including p65, to translocate to the nucleus and bind to cis-regulatory elements in the proximal (-48 to -209 bp) and distal (-913 to -1029 bp) binding sites on the *Nos2* promoter region.NF-κB transcription factors also bind to the *RelA* promoter, where RelA is transcribed and prevents inhibitory proteins from binding to NF-κB factors, therefore creating a positive feedback loop within the NF-κB signaling pathway (13). IFN-γ also activates cytokine receptors to stimulate the JAK phosphorylation of STAT1 transcription factors to translocate into the nucleus, binding to cis-regulatory elements in the promoter.

shown to exhibit hysteresis. In biology, hysteresis is highly similar to bistability and is observed in systems where a threshold concentration of an inducer must be exceeded in order to shift the activity of a particular genetic circuit or system from one state to another. This concentration will be higher than that required to maintain the system in this state. Systems that exhibit this property often incorporate positive feedback loops (13).

The expression of *Nos2* is also increased by IFN-γ via activation of the Janus Kinase/signal transducers and activators of transcription 1 (JAK/STAT) pathway using a similar mechanism. Here, IFN-γ stimulation induces nuclear translocation of STAT1 transcription factors, which also bind cis-regulatory elements in the *Nos2* promoter to increase transcription (2, Fig.1). These pathways can work synergistically, substantially increasing *Nos2* expression when both LPS and IFN-γ are present (4).

Unlike M1 polarized macrophages, M2 macrophages do not typically exhibit enhanced antibacterial properties when compared to naïve macrophages. Instead, they function to attenuate the activity of certain immune cells and reduce inflammatory signaling. Macrophages can be M2 activated by cytokines such as interleukin-4 (IL-4), which increases the expression of *Arg1*, a gene that encodes the enzyme arginase-1 (Arg-1; 5). Arginase-1 converts L-arginine into urea and ornithine, thereby decreasing the L-arginine available for NO production by iNOS. This effectively reduces the antimicrobial activity in macrophages (1). Additionally, M2 polarized macrophages also produce a range of other proteins and compounds, including anti-inflammatory cytokines that suppress inflammation and promote tissue repair (1).

Macrophage polarization is highly plastic, with cells able to reversibly transition between the naïve, M1, and M2 states (Fig. 2), and this is vital to a normal immune response. Macrophages are able to temporarily adopt an M1 phenotype to fight infections efficiently and then re-polarize to the M2 state to attenuate the inflammatory response and participate in tissue repair. When the M1 state persists long after an infection had been successfully cleared, because of the intrinsic pro-inflammatory and cytotoxic nature of M1 macrophages, the continued M1 polarization state promotes tissue damage and chronic inflammation (6). Inappropriate M1 and M2 polarization contributes to many human diseases, including macrophage activation syndrome, which is characterized by a low mature blood cell count, abnormal liver function, and an inability to properly coagulate blood (7).

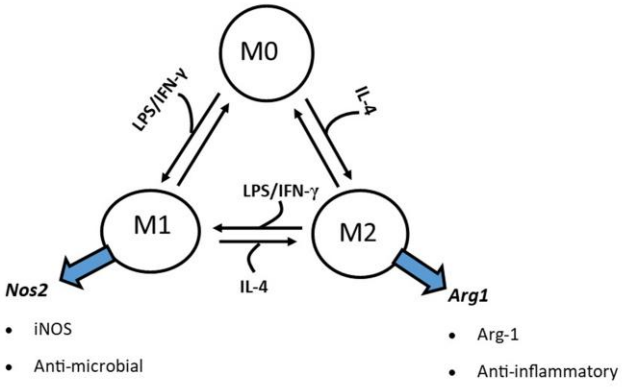

Figure 2. Diagram depicting macrophage activation plasticity. Macrophage activation can move between naïve (M0), M1 and M2 polarization depending on the stimulus.

Various pathogens are known to interfere with normal macrophage polarization by either blocking M1 activation or promoting M2 activation. By promoting M2 activation, some pathogens are able to survive within the macrophages and escape destruction. For example, the intracellular bacterium *Coxiella burnetti* disrupts normal M1 polarization to prevent NO production, causing a chronic infection of Q fever in immunocompromised patients, pregnant women, and patients with valve disease. *C. burnetti* is able to

upregulate production of the M2-associated enzyme, Arg-1, thereby decreasing NO production enabling the bacterium to survive and replicate within host macrophages (reviewed in (8)).

In addition to microbe-derived immunomodulatory compounds, a number of plant-derived products, including aurones and polysaccharides, have also been shown to affect macrophage activation and *Nos2* expression (9). Polysaccharides derived from *Panax quinquefolius* (American ginseng) activate TLR4 and CD14, the same cell surface receptors that bind bacterial LPS in macrophages and activate the NF-κB signaling pathway (9). While many of the plant-derived polysaccharides that have been examined so far are able to induce NF-κB activity, the induction of *Nos2* expression is generally modest when compared to LPS. This suggests that they are either poor agonists of these receptors or function by slightly different mechanisms. Furthermore, Lui *et al* have shown paradoxical influences of these polysaccharides on macrophage activation, causing both immuno-stimulatory and immunosuppressant responses (10). However, further study of these compounds could prove them to be useful, enabling modulation of macrophage polarization for therapeutic purposes.

It is therefore desirable to be able to monitor polarization state by developing a model system to quickly and easily measure the effects of pathogens and/or immunomodulatory drugs on macrophage polarization state. This could be accomplished through the use of live cell microscopy of cultured macrophages. Unlike most biochemical assays, which involve the bulk analysis of cell lysates, live cell imaging provides quantitative information about the responses of cells to a particular agent, pathogen, or drug with high

temporal resolution (11). Live cell imaging can provide information about the kinetics of a cell's response – put simply, it can show how quickly a pathogen or a compound influences the expression of specific genes in real time.

I have constructed a fluorescent reporter of *Nos2* promoter trans-activation that serves as a marker for M1 macrophage activation. This was done by cloning a 1,200 bp fragment of the *Nos2* promoter immediately upstream of the *Nos2* gene (-1200 to +1) from the murine macrophage-like cell line, RAW264.7. This portion of the *Nos2* promoter was selected as it is known to contain both the proximal and distal regulatory regions that contain STAT1 and NF-κB transcription factor binding sites, which have been shown to be important for *Nos2* expression in response to LPS and IFNγ (4; Fig 2). The promoter was recombined into a lentiviral vector upstream of the gene encoding mCherryPEST, a destabilized version of the red fluorescent protein, mCherry. Lentiviral particles containing the construct were packaged in HEK293T cells and these were transduced into RAW 246.7 murine macrophage cells.

In the presence of M1-inducing stimuli that promote *Nos2* transactivation, mCherryPEST was expressed as detected by fluorescence microscopy. The amount of mCherry fluorescence can be quantified to monitor the polarization state of the cells. The reporter line can also be used with other experimental methods, such as flow cytometry and fluorometry, for rapid analysis and high through put analyses as well as multiplexed with other fluorescent markers to measure multiple parameters simultaneously. In this sense, it is a highly versatile tool for basic research on macrophage biology and for screening of immunomodulatory compounds.

Thesis Statement

Macrophage activation is crucial to an appropriate immune response; therefore, it is important to be able to monitor macrophage activation when investigating the effects of microbial pathogens and potential immunomodulatory drugs on macrophages. During this project, two outcomes were completed using the M1 reporter I have already created; the first outcome was to complete the validation work on the reporter to ensure that the expression of the mCherry fluorescent reporter faithfully recapitulates the endogenous *Nos2* responses when macrophages are stimulated with known M1 polarizing stimuli. To do this, *Nos2* reporter response to the activation of the JAK/STAT pathway through IFN-γ stimulus was quantified using live cell imaging and immunoblotting. Both the reporter and the endogenous *Nos2* gene are predicted to show a dose-dependent response to IFN-γ, increasing in expression with increasing concentrations of IFN-γ, but dropping rapidly when the concentration is reduced or if IFN-γ is withdrawn entirely. The second outcome identified and characterized the effects of putative immunomodulatory polysaccharide extracts derived from American ginseng on macrophage polarization. As similar plant-derived polysaccharides have been shown to induce M1 polarization these compounds are predicted to have a similar effect. However, as plant polysaccharides are typically poor TLR4 agonists, it was predicted that these would elicit a more modest response from the *Nos2* reporter (and endogenous gene) than equimolar or even lower concentrations of LPS.

Methods

Construction of Nos2 reporter cell line and preliminary experiments

In collaboration with graduate student J. Logan Bowling, genomic DNA was extracted from RAW 264.7 murine macrophages using a DNeasy Blood and Tissue Kit (Qiagen, USA) and used as a template to amplify the *Nos2* core promoter region by polymerase chain reaction (PCR) (Forward primer sequence 5' to 3' AGA TCC AGT TTA TCG ATG ACT TTG ATA TGC TGA AAT CC, Reverse primer sequence 5' to 3': GCC CTT GCT CAC CAT GAC TAG GCT ACT CCG TGG). Custom primers were designed by Dr. Nelson in order to amplify a portion of the *Nos2* promoter corresponding with position -1200 to position +1 with respect to the transcriptional start site. The cDNA encoding mCherryPEST red fluorescent protein was also amplified using PCR (Forward primer sequence (5' to 3') ATG GTG AGC AAG GGC GAG and reverse primer sequence (5' to 3') TCT AGA GTC GCG GCC GCC CAT AGA GCC CAC CGC AT) from an existing reporter vector, pFUW-p6SP-GFPp65-mTNFaP-mCherry-PEST, which has been previously described (12). The annealing temperature was 55ºC. A lentiviral vector, pLVX-EF1α-AcEGFP-N1, was linearized using *Not*1 and *Cla*1 enzymes and the EF1α-AcEGFP promoter and gene were removed. Then, both the amplified *Nos2* promoter and mCherryPEST cDNA were recombined into the linearized vector using the Infusion DNA recombinase cloning kit (Takara, USA), creating the pLVX-pNos2-mCherryPEST lentivirus (Fig.2). Restriction digest mapping of the vector using *Not*1 and *Cla*1 enzymes ensured that recombination was successful. The lentiviral vector was transfected into a HEK293T-based LentiX packaging cell line to produce lentiviral

particles. These were then transduced into RAW264.7 macrophage-like cells and stably infected macrophages were selected by growth in the presence of 0.5 μg/mL puromycin antibiotic, which killed all macrophages not infected with the pLVX-pNos2-mCherryPEST lentivirus.

Validation of stably transduced reporter cells by live cell imaging

Stably transduced reporter cells were then tested to ensure that the responses of the exogenous reporter to M1-polarizing agents matched that of the endogenous gene. Live cell imaging experiments were performed by treating with 100 ng/mL Salmonella enterica serotype typhimurium LPS (Sigma Aldrich, USA) and 100 U/mL IFN-γ both independently and together for 24 hours. This was done as the endogenous gene has been short to respond to both signals independently but will exhibit a synergistic response to cotreatment with LPS and IFN-γ. RAW264.7 reporter macrophages were plated in 35 mm glass-bottom dishes, at a density of $2x10^5$ per dish in 2 mL medium a day prior to the imaging experiment. Cells were imaged using a Nikon Ti-Eclipse inverted microscope equipped with a 60x objective, low-light digital camera, heated stage insert, and full environmental enclosure with 5% CO_2 and humidified atmosphere. The microscope was controlled by computer using Elements (Nikon, USA) software. Fields were selected using Brightfield imaging. Once the stage positions for each of the fields were recorded in the control software, cells were stimulated with LPS and/or IFN-γ and mCherry fluorescence was imaged every 30 minutes over a period of 24 hours. Quantification of

mCherry fluorescence was performed using Fiji image analysis software, a modified version of Image J (NIH).

Clonal Line Creation

Once the mixed population of stably transduced reporter cells were shown to exhibit the expected responses to stimuli in the live cell imaging experiments, clonal lines were created for use in subsequent experiments. In order to do this, the cells were suspended at a concentration of $2x10^4$ cells/mL into the first well of a 96 well plate. The cells were then serially diluted 1:2 between wells moving both vertically and horizontally across the plate. After a few days, wells were identified that contained an individual colony arising from a single cell. These were then moved to a 24 well plate, a 6 well plate, and finally a 10 cm plate as these clonal lines were expanded. Because each individual cell likely contained different numbers of the reporter construct integrated into different regions of the genome, they may exhibit differing responses to stimuli. In order to select a clonal line for subsequent work, protein lysates before and after 100 ng/mL LPS and 100 U/mL IFN-γ treatments from each line were tested by western blot using an anti-iNOS primary antibody (#13120; Cell Signaling, USA) at a 1:1000 concentration and anti-mCherry primary antibody (#NBP196752SS; EMD Millipore, USA) at a 1:1000 concentration, and then incubated in either mouse or rabbit HRP secondary antibodies (Santa Cruz, USA) to compare the expression of mCherry to iNOS after treatment with LPS and IFN-γ.

Test for hysteretic response of the reporter to LPS stimulation

The *Nos2* reporter cell line was tested for hysteresis to determine whether the positive feedback mechanism in the NF-κB signaling pathway was indeed being induced when treated with LPS or other NF-κB activating compounds. *Nos2* reporter cells were treated with either a high dose (10 ng/mL) or a low dose (0.5 ng/mL) of LPS or untreated control media, and incubated for 16 hours. After the 16 hours, the media was changed, and the reporter cells were restimulated with either 10 ng/mL LPS, 0.5 ng/mL LPS or vehicle and incubated for another 4 hours before being lysed for western blotting. An eighth dish received a high dose for 16 hours, and then was incubated untreated for 8 hours as a protein degradation control. Protein was isolated and a western blot was performed to probe for mCherry using anti-mCherry antibody at a 1:1000 concentration to visualize reporter expression.

Test for hysteretic response of the reporter to IFN-γ stimulation

To determine if the reporter was dose responsive to IFN-γ, the reporter cell line was treated with different doses of IFN-γ: 0 U/mL, 0.3 U/mL, 1 U/mL, 3 U/mL, 10 U/mL, 30 U/mL, 100 U/mL, and 300 U/mL for 24 hours. After 24 hours, cell lysate was collected for a western blot probing for iNOS and mCherry proteins using anti-iNOS and anti-mCherry antibodies at 1:1000 concentrations. The expression of both proteins was quantified by densitometry using Fiji image processing software and a dose-response curve was plotted in Microsoft Excel in order to identify the appropriate concentrations for “high” and “low” doses to test for hysteresis. The low dose was chosen as it was able

to stimulate *Nos2* expression, but at a low level. The high dose was selected as the minimum concentration required to achieve maximum *Nos2* expression. A concentration of 1 U/mL was chosen as the "low" dose, and a concentration of 100 U/mL was selected as the "high" dose.

Nos2 reporter cells were treated with either the high dose or low dose of IFN-γ or vehicle control, and incubated for 16 hours. After the 16 hours, the media was changed, and the reporter cells either received vehicle control, a high dose IFN-γ treatment, or a low IFN-γ treatment and incubated for another 4 hours before being lysed for western blotting. An eighth dish received a high dose for 16 hours, and then was incubated untreated for 8 hours as a protein degradation control. A western blot using the anti-mCherry primary antibody was performed to confirm reporter expression.

Ginseng compound testing

Four compounds extracted from American ginseng were provided by the Farone lab for testing. Using the Zeiss confocal microscope, clonal *Nos2* reporter cells were treated with one of the four compounds at a concentration of 25 μg/mL, a 1 μg/mL LPS control, a PBS vehicle control, or a control with no treatment and were imaged to measure pre-treatment, and again at 24 hours post treatment. The amount of mCherry fluorescence was measured, and the fold change of mCherry fluorescence was calculated for each treatment compared to the no treatment control. Compound designated AGC1 elicited the largest response of the polysaccharides.

AGC1-treated reporter cells were then imaged on the Nikon widefield microscope for 24 hours, with images taken every 30 minutes to determine the dynamics of reporter expression. An LPS control was also imaged. A previously constructed NF-κB fluorescent live cell reporter line with a p65 promoter and eGFP fluorescent protein (Sung et. al) already owned by the Nelson lab was also used to test whether the ginseng compound stimulated mCherry and iNOS expression in an NF-κB-dependent manner. The NF-κB reporter cell line was treated with 25 μg/mL concentration of AGC1, and imaged every 3 minutes for 6 hours to observe p65 nuclear localization through eGFP protein fluorescence. A positive control of 1 ng/mL of LPS was also imaged every 3 minutes for 6 hours with the NF-κB reporter line.

BCA and Western Blotting

The Bicinchoninic acid Assay Kit (Pierce, USA) was used as directed by the manufacturer to determine the protein concentration in cell lysates prior to western blot analysis. Samples were incubated in working reagent for 30 minutes before spectrophotometry at 562 nm. Protein standards were used to generate a standard curve in order to calculate the protein concentration of the samples.

Equal quantities of protein for each sample were separated on a 10% SDS-PAGE gel together with Spectra ™ Multicolor Broad Range Protein Ladder (ThermoFisher). Separated proteins were transferred to nitrocellulose membranes using semi-dry transfer tanks (Bio-Rad, USA). After transfer, the membranes were blocked in 5% non-fat milk in PBS/5% Tween for 1 hour to prevent non-specific antibody binding. Membranes were

subsequently incubated in anti-iNOS and anti-mCherry primary antibodies at a concentration of 1:1000 and β-actin primary antibodies at a concentration of 1:5000 overnight, washed with PBS/T and incubated in appropriate horse radish peroxidase-conjugated secondary antibodies at a concentration of 1:5000 for an hour before ECL-treated membranes were imaged using a low-light camera-equipped gel-documentation station. Densitometry was performed using Fiji processing package software.

Cell Culture

The RAW264.7 macrophage cell line was maintained in Dulbecco′s Modified Eagle′s Medium (DMEM) with HEPES, and L-glutamine, penicillin/streptomycin, Fetal Bovine Serum (FBS), and gentamycin additives and 0.5 ug/mL puromycin used for cell selection. Cell lines were incubated at 37ºC in a humidified 5% CO_2 atmosphere. When cells were 75-80% confluent in a 10 cm dish, they were passaged by washing off media with PBS, and then treating with 0.05% trypsin EDTA solution (Gibco, USA) to detach cells from the bottom of the dish. Approximately 25 % of the cells were then replated into a new dish with 10 mL of medium. Alternatively, the required number of cells were plated into the appropriate tissue culture dishes or plates for experiments.

Statistical Analysis of Data

Data collected from image or western blot analysis were subjected to statistical tests to determine statistical significance and difference. First, if there were three or more treatments, a Shapiro-Wilks test was performed to assess normal distribution of each set

of data. If data were normally distributed, an ANOVA was performed to determine statistical difference. If any data set was not normally distributed, a Kruskal-Wallis was performed to determine statistical difference as a non-parametric analysis of variance. Once one of the two analysis of variance tests is performed, a Dunn's multiple comparison test is performed to determine which data sets are statistically different from each other.

If only two data sets were present, a Shapiro-Wilks test was performed to determine if the data sets were normally distributed. If data were normally distributed, an independent t-test was performed to determine if there was a significant difference between the two means of the data sets. If the data were not normally distributed, a Mann Whitney U test was performed, as a non-parametric version of the independent t-test. Statistical analysis was done using either GraphPad Prism software or IBM's SPSS software.

Results

Amplified mCherry cDNA and *Nos2* promoter sequences were successfully linearized and recombined into a lentiviral vector, creating the pLVX-pNOS2-mCherryPEST lentivirus, and then verified by restriction digest mapping (Fig. 3).

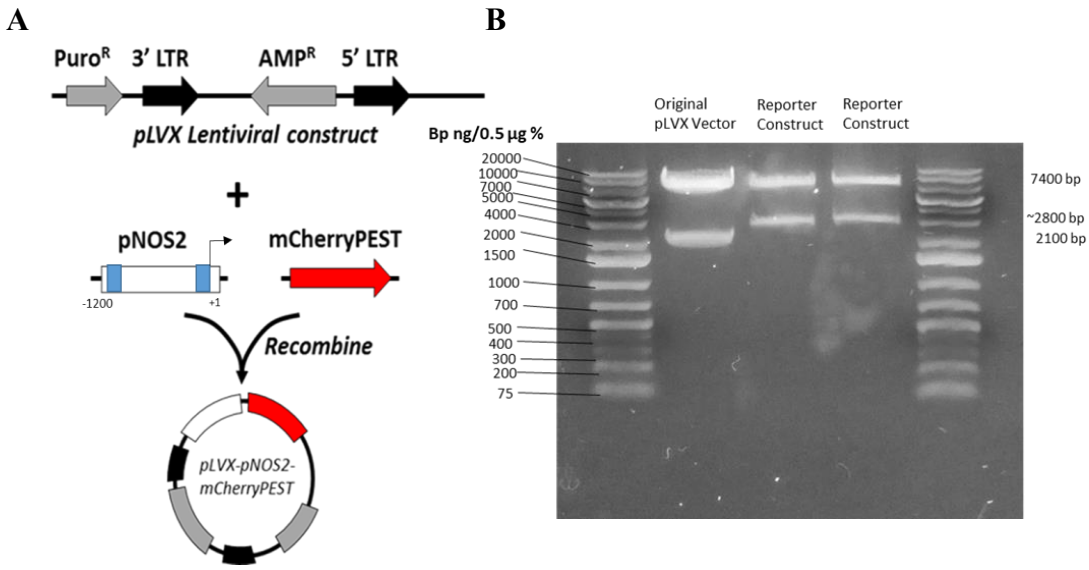

Figure 3. Diagram of recombination of pLVX-pNOS2-mCherryPEST vector (A) and Restriction Digest Mapping (B). The pLVX lentiviral construct containing puromycin and ampicillin resistance genes was combined with amplified *Nos2* gene promoter, with NF-κB binding sites at the distal (-913 to -1029bp) and proximal (-48 to -209bp) regions proximal, and mCherryPEST CDNA to create the pLX-pNOS2-mCherryPEST lentiviral vector **(**A**).** Control plasmid, pLVX-EF1alpha-AcGFP and two putative pLVX-pNOS2-mCherryPEST clones generated by a recombination reaction were subject to restriction digest with Not1 and Cla1 enzymes. Upper band (7.4 kb) represents the pLVX vector backbone. Band at 2.1 kb represents EF1alpha-AcGFP from the original parent vector. Band at 2.8 kb represents the pNOS2-mCherryPEST reporter cassette (B).

Once the lentivirus was verified to contain the *Nos2* promoter and mCherryPEST sequence and lentiviral particles were transfected into RAW 264.7 macrophages, a validation imaging experiment was performed, treating with 100 ng/mL LPS and imaging 24 hours post-treatment to determine if a stable fluorescent reporter line was created and fluorescing appropriately according to the applied stimulus. Expression of mCherry was measured and fold change from time 0 was calculated (Fig. 4). The imaging experiment demonstrated that the reporter was responsive to LPS-stimulation, as was expected.

Live cell imaging over 24 hours with 100 ng/mL LPS, 100 U/mL IFN-γ, independently and a co-treatment of both showed a synergistic response from the *Nos2* reporter, as expected. This confirmed that the exogenous mCherry reporter exhibited similar responses as the endogenous gene (Fig. 5). A Kruskal-Wallis/Dunn comparison test was performed on the 24-hour post treatment time point, which found that the LPS and co-treatments were significantly different from IFN-γ treatments. Greater fold change in the expression of the co-treated

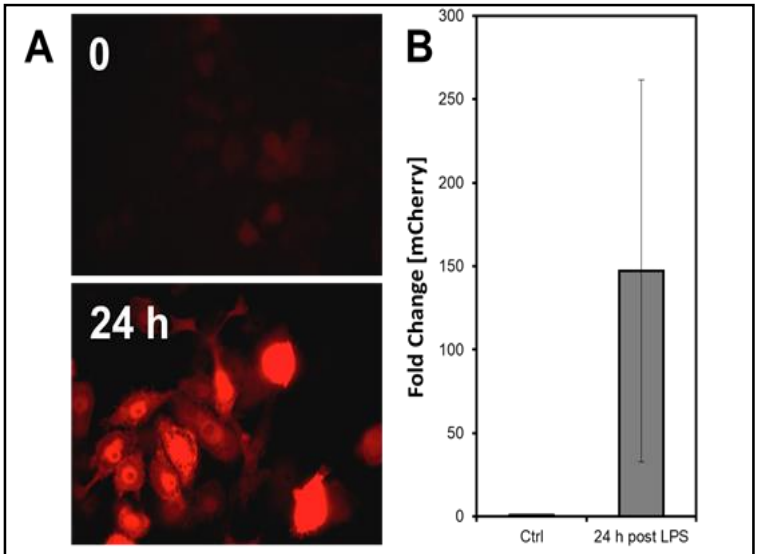

Figure 4. Initial polyclonal line testing by microscopy. Live cell imaging of the *Nos2* reporter cell line before and 24 hours post treatment with 100 ng/mL LPS (A). Quantification of mCherry expression at time 0 (ctrl) and 24 hours post LPS treatment, with fold change calculated. (n > 100 cells) (B)

cells is suggestive of a synergistic response. However, the LPS and co-treatments were not significantly different from each other (Fig. 5 D). These statistical results could be caused from low *n* numbers, resulting in averages that are not statistically different for the LPS treatments and the IFN-γ treatments.

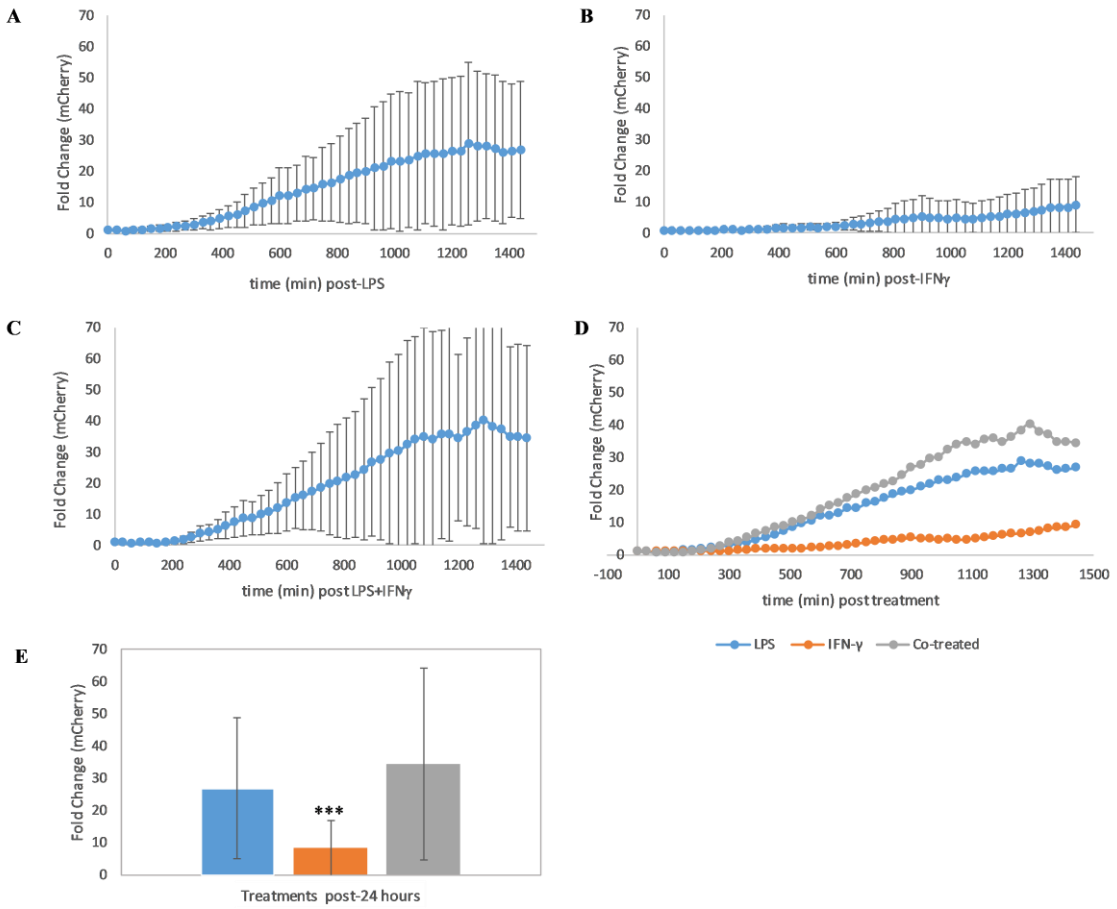

Figure 5 Synergistic response to co-treatment of RAW 264.7 *Nos2* reporter macrophages with LPS and IFN-γ. Reporter macrophages were stimulated with LPS (A), IFN-γ (B) or a co-treatment of both (C), and the fold change of mCherry fluorescence imaged over a 24 hour time course is displayed, as well as a comparison of the three kinetic time courses (D). Comparison of data at 24 hrs post treatment time point (E). 24 hour time point data failed Shapiro-Wilks normality test ($n \geq 15$ cells for each treatment). *** Kruskal-Wallis/Dunn comparison test found IFN-γ induced expression was significantly different than LPS and co-treatment. Error bars are displayed as standard deviations.

Protein lysates from the clonal lines created by serial dilution were tested by western blot analysis after a treatment of 100 ng/mL LPS, 100 U/mL IFN-γ, or a co-treatment of both. Western blot analysis confirmed that the clones tested and used were expressing iNOS and mCherry at similar levels (Fig 6), showing that the clonal lines expression of both the exogenous and endogenous gene exhibited similar dynamics. There also was a noticeable increase in expression with the co-treatment, suggesting genuine evidence of a synergistic response in the reporter cell line.

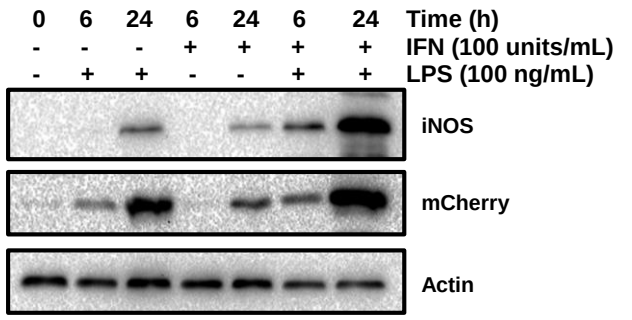

Figure 6 Validation of clonal line via Western blotting. Clonal lines were treated with LPS, IFN-γ, or a co-treatment of both cytokines for validation of properly expressing clonal lines. All clonal lines showed similar expression in the endogenous gene and the exogenous mCherry reporter product as seen in this representative Western Blot.

The hysteresis phenomenon of the NF-κB signaling pathway was tested in *Nos2* reporter cells to confirm that the reporter showed responses similar to those observed in the prior Sung *et. al*. study. Three biological repeats of the hysteresis experiment, switching from low and high doses, were performed with the reporter cell line. Cells were lysed and western blotted to measure mCherry expression (Fig 7 A-C). Subsequent densitometry analysis showed that after being initially stimulated with a high dose of LPS the mCherry expression remained elevated even in those cells that received a low dose or remained untreated for the second treatment (Fig 7 D). This maintenance of expression

after removal of stimuli suggests that *Nos2* expression is also affected by the positive feedback loop found within the NF-κB pathway in macrophages and that this can be observed using this reporter.

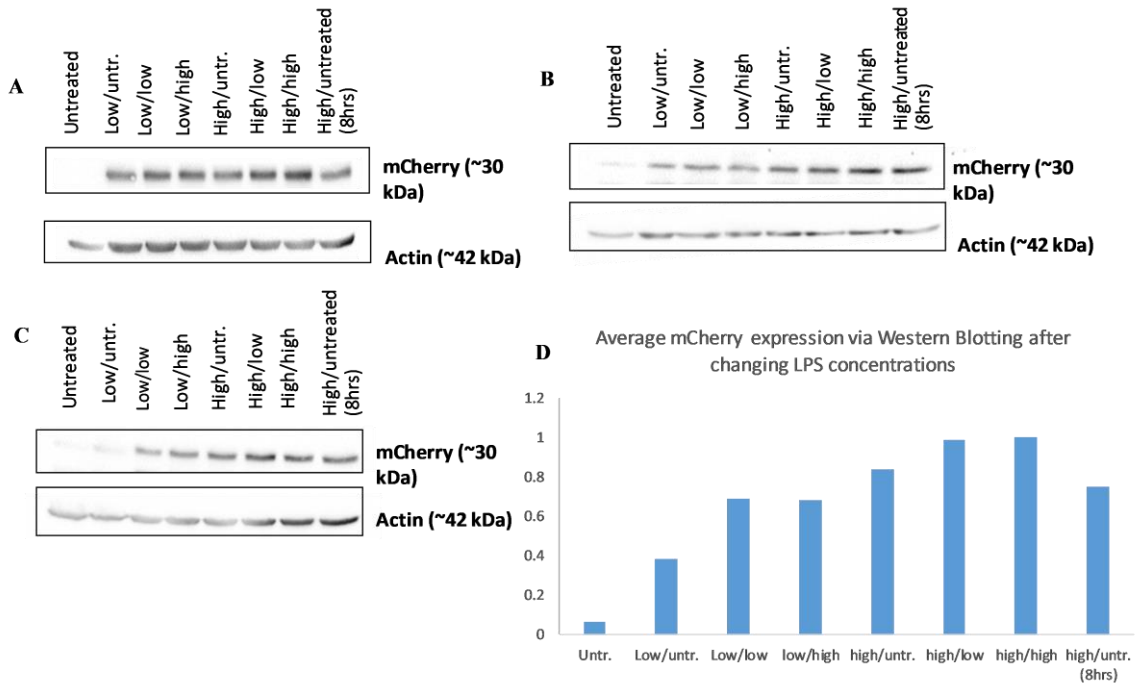

Figure 7 Hysteresis in NF-κB pathway of LPS stimulated RAW 264.7 *Nos2* reporter cells. The *Nos2* reporter cell line exhibited hysteresis in mCherry expression post LPS treatment changes. Three biological repeats of the hysteresis experiment were performed (A-C), and analyzed via densitometry of western blot assay. The mCherry expression seen via western blot analysis for the three repeats was averaged and normalized to actin and then to the high/high treated cells (D) to potentially display the positive feedback effect.

To test if the JAK/STAT1 signaling pathway produced a hysteresis response, and to validate the reporter cells exhibited the expected response to IFN-γ, a dose response curve was performed to determine "low" and "high" doses. A low dose being defined as being able to elicit a measurable increase in reporter levels above the basal level of

expression, but not maximal and a high dose being defined as eliciting the maximal amount of reporter expression possible. After treating the reporter line with different doses of IFN-γ across several orders of magnitude and measuring mCherry levels from these cells by western blotting (Fig 8 A), the reporter response to IFN-γ stimulation proved to be dose-dependent as expected. Based on these data, a low dose of 1 U/mL and a high dose of 100 U/mL was chosen for the hysteresis test. In this experiment, which was performed only once, the expression of mCherry resembled the results from the hysteresis experiments performed with LPS treatments. This experiment should be repeated and an appropriate statistical analysis performed before any firm conclusions are arrived at (Fig 8 D). The similarity in the results may not be due to similarities in feedback loops in the pathways or a hysteresis effect in the IFN-γ pathway in the reporter line, but may be due to the methods used. Prior experiments that were performed by Sung *et al* to test for hysteresis in the NF-κB response to LPS measured the change in expression of mRNA transcripts from NF-κB-responsive genes. Our assay measured changes in mCherry proteins by Western blotting. Although the mCherry protein is destabilized through the inclusion of a PEST sequence, the half-life of the protein is likely different (possibly longer) than *Nos2* mRNAs and therefore western blotting and the use of this reporter may not be ideal for studying hysteresis in NF-κB-regulated gene expression. Other assays, such as qPCR for *Nos2* mRNAs, would be more accurate at measuring hysteretic response in macrophages.

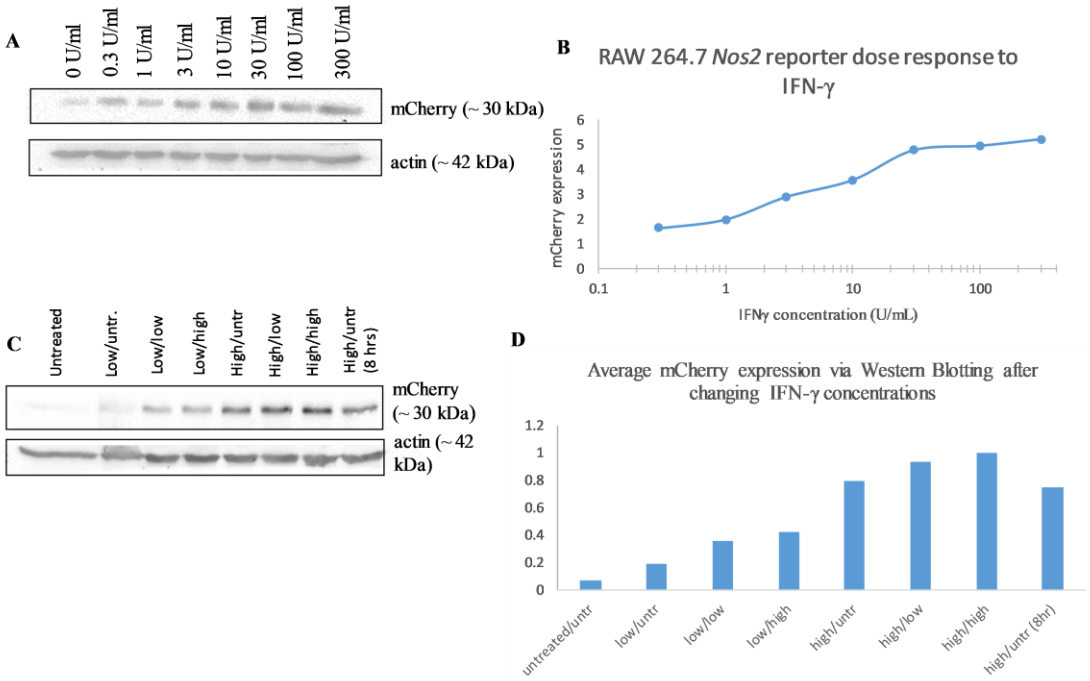

Figure 8 Dose-response and Hysteresis in IFN-γ stimulated RAW 264.7 *Nos2* reporter macrophages. A dosage response test was run using IFN-γ, covering 4 magnitudes of activity in concentration to determine a "high" and "low" dose of IFN-γ. Using Western Blotting (A) the *Nos2* reporter was found to be dose dependent to IFN-γ. Densitometry (B) showed a dose response-like curve, and 1 U/mL and 100 U/mL were chosen as the "low" and "high" dose, respectively. Concentrations of 1 U/mL showed confident stimulation in a lower magnitude, and 100 U/mL showed adequate stimulation of the *Nos2* gene before plateauing in mCherry expression. The western blot analysis suggested the absence of the hysteresis response in the JAK/STAT1 pathway, with untreated and low dose treated cells showing lower expression than high dose treated cells (C, D).

Once the reporter had been successfully validated, clonal lines were used to determine the ability of polysaccharide compounds extracted from American ginseng to stimulate *Nos2* expression in macrophages as a marker of M1 activation. This was performed by treating the reporter cells with a panel of purified polysaccharide extracts,

designated AGC1 and AGC4. The mCherry fluorescence was measured by confocal microscope at T=0 h and at 24 hours post-treatment. Both AGC extracts stimulated mCherry expression in the reporter cell line (Fig 9 A). The fluorescence was found to be less than that of cells treated with 1 μg/mL LPS, which was used as a positive control alongside the AGC compounds. The fold change in mCherry fluorescence was quantified and compared among all treatments using a Kruskal-Wallis/Dunn statistical analysis. The expression stimulated by AGC compounds were significantly different from all other treatments (vehicle and LPS), but the two AGC compounds were not significantly different from each other ($p < 0.0001$, $n > 153$ cells for each treatment; Fig 9 B).

In order to determine whether the dynamics of reporter gene expression differed between AGC1 and LPS-treated cells, a live cell imaging experiment was conducted. Quantification of mCherry fluorescence in the time course showed *Nos2* reporter kinetics from AGC1 treatment to differ slightly from LPS. In AGC1 treated cells, mCherry fluorescence peaked around 12 hours and then began declining. In contrast, mCherry fluorescence peaked at around 16 hours and stayed constant in LPS stimulated cells (Fig 9).

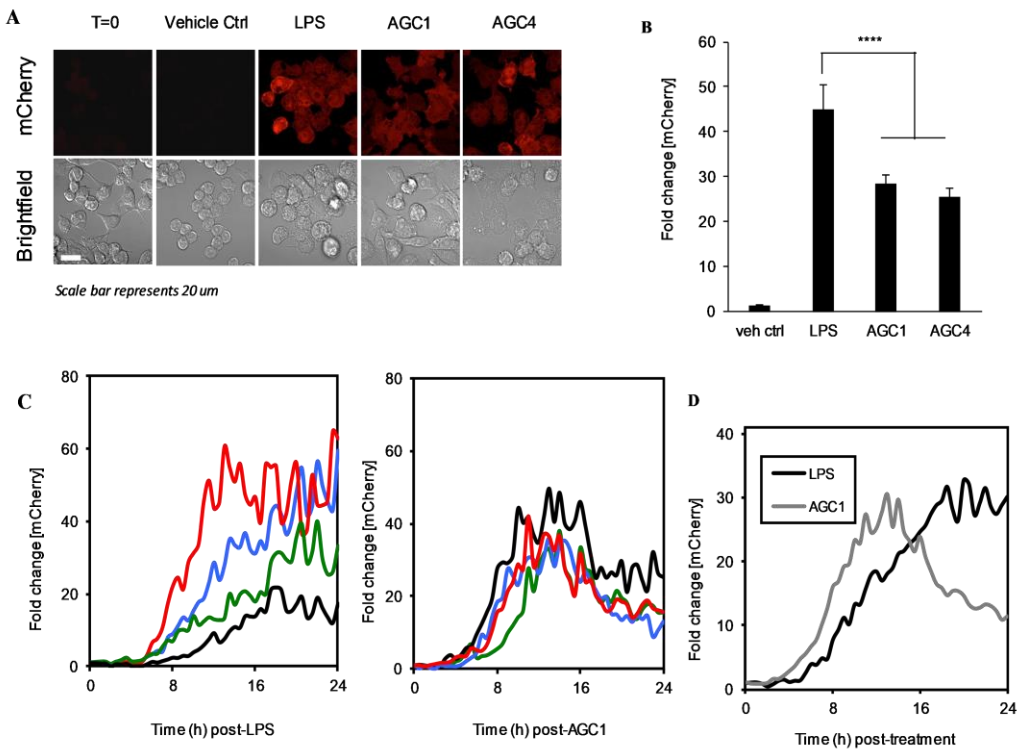

Figure 9 *Nos2* reporter response to compounds extracted from American ginseng. The *Nos2* reporter cell lines were treated with compounds extracted from American ginseng (25 μg/mL), along with an LPS (1 μg/mL) control and a PBS control. The treated cell lines were imaged at T = 0 and at 24 hours post treatment (A). The fold change in mCherry flourescence was measured at the 24 hours post treatment (B) and compared to each treatment. Kruskal-Wallis/Dunn comparison statistical analysis on the fold change showed that all treatments were statistically different from one another except for the AGC compounds ($p < 0.0001$, $n > 150$ cells for each treatment). AGC1 was chosen for 24-hour time course imaging in comparison to LPS, and the kinetics of *Nos2* expression was measured over 24 hours for each treatment. A representative 4 cells from each treatment is shown (C), along with an average of all cells analyzed for each treatment, compared (D). It is shown that AGC1 has slightly different kinetic activity than LPS, peaking around 12 hours and then decreasing again, where LPS peaks around 16-20 hours and remains constant throughout 24 hours.

After these experiments were complete, an LAL assay run in the Farone lab tested positive for endotoxin contamination of 0.07 ng/μg in the AGC-1 samples, approximately equivalent to 1.75 ng/mL of LPS in the compounds when at a concentration of 25 ug/mL, the concentration of compound used for the majority of our tests. Typically, endotoxin is

found within bacterial cells. It was unknown when the contamination occurred during AGC-1 purification, as there was no detectable contamination at the start of the experimentation process, or whether this result represented cross-reactivity between the endotoxin assay and the polysaccharide itself.

To further investigate the effects of the endotoxin by comparing signaling pathway kinetics from LPS stimulation and AGC stimulation, a RAW264.7 NF-κB reporter cell line existing within the Nelson lab that expresses an EGFP-tagged version of the NF-κB transcription factor, p65, was utilized. Exposure of these cells to LPS promotes the translocation of p65 from the cytoplasm to the nucleus (and subsequent return to the cytoplasm). The dynamics of the response differ when cells are exposed to different concentrations of LPS or even different inducers of NF-κB signaling. It was hypothesized that if endotoxin contamination of AGC-1 was solely responsible for the observed expression of the *Nos2* reporter, the dynamics of the NF-κB response would be identical after AGC-1 treatment when compared to the response elicited by LPS at concentrations equivalent to the assumed level of endotoxin contamination.

The compound's activation of the NF-κB reporter was compared to a 1 ng/mL LPS control, to help consider if activation was truly produced by the polysaccharide or the endotoxin contamination detected. After 6-hour time course imaging, the live cell images of the NF-κB reporter were quantified for p65 translocation from the cytoplasm to the nucleus. A ratio of nuclear to cytoplasmic translocation was calculated for both 25 μg/mL AGC1 treatment and 1 ng/mL LPS treatment, and were plotted together for comparison (Fig. 10). The dynamics of the NF-κB response appeared highly similar

between conditions. The nuclear to cytoplasmic ratio peaked at ~25 minutes after both treatments, declining rapidly with near-identical kinetics. Furthermore, the magnitude of the response was similar with a peak nuclear to cytoplasmic ratio of ~3.5 and ~3 ($n > 15$ cells for each treatment) observed in LPS and AGC-1 treated cells, respectively. A Shapiro-Wilks test showed that the data set for the peak time points of each treatment was not normally distributed, so a Mann Whitney U test was performed and concluded that the two treatments were not significantly different ($p = 0.389$), resulting in the conclusion that M1 activation seen from AGC1 could potentially be from low-level endotoxin contamination.

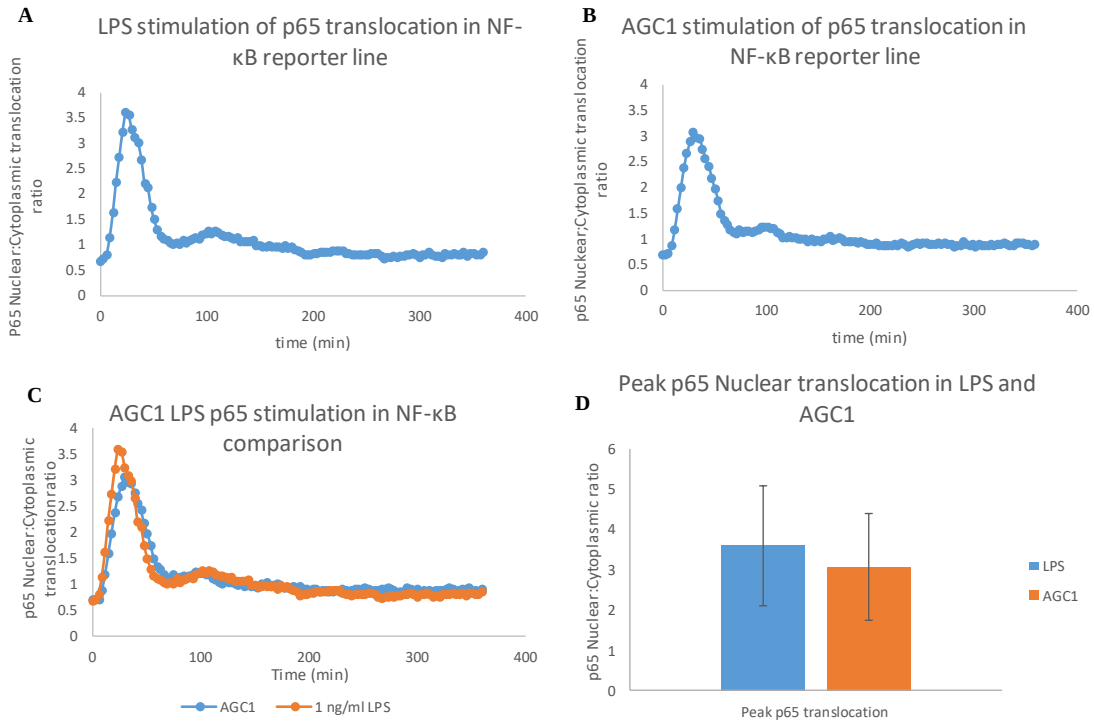

Figure 10 NF-κB reporter cell line response to LPS and AGC1 stimulation. An existing NF-κB reporter cell line was treated with 1 ng/mL LPS or 25 µg/mL AGC1 and imaged every 3 minutes for 6 hours to determine how AGC1 worked kinetically with NF-κB signaling in comparison to 1 ng/mL LPS, comparable to the level of endotoxin found within 25 µg/mL AGC1. The eGFP fluorescence of p65 was measured within the cytoplasm and the nucleus, and a ratio of nuclear : cytoplasmic translocation of p65 was created for each image timepoint. LPS showed a peak of p65 translocation around 25 minutes post treatment, rapidly declined, and showed a small amount of translocation again around 100 minutes post treatment (A). AGC1 also peaked around 25 minutes post treatment and showed a very slight translocation again around 100 minutes post treatment (B). An overlay of the two treatment's translocation ratio was created to visualize the two treatments for determination if AGC1 activity was caused from endotoxin contamination (C). The two compounds work extremely similarly when activating the NF-κB signaling pathway. The peak translocation ratios were compared for each treatment (D). Treatments failed Shapiro-Wilks normality test. ($p = 0.021$ (null is that data is normally distributed)). The Mann Whitney U comparison test showed no significant difference between the two at peak translocation ($p = 0.389$).

Discussion

Classical macrophage activation is crucial to a proper inflammatory and anti-microbial response in everyday life (1). However, too much activation can cause chronic inflammation, and not enough activation can cause increased risk of some diseases and can contribute to immunocompromised diseases like macrophage activation syndrome (6,7). A reporter to monitor M1 activation can be a very useful tool to test potential therapeutic compounds for their abilities to stimulate activation. However, it must be validated before being put into use as a research tool.

Initial testing using fluorescence microscopy and western blot analysis of the *Nos2* reporter cell line confirmed that the mixed polyclonal reporter line and the clonal lines faithfully recapitulate the response of the endogenous gene to inducers of M1 polarization and can therefore likely be used as a reliable marker of macrophage M1 activation. More specifically, the western blotting experiments showed that the reporter cell line is potentially capable of exhibiting a synergistic response to co-treated with LPS and IFN-γ, similar to the endogenous gene. Similar results were achieved in corresponding live cell imaging experiments. However, possibly due to large cell-to-cell variation and small sample numbers, the difference in response between LPS treated and LPS and IFN-γ co-treated samples was not significant. This was a simple validation experiment of the reporter gene to determine if it was capable of recapitulating the responses of the endogenous gene to known activating stimuli. Due to the length of the imaging times (typically 24 hours), the lengthy analysis process, and the time constraint around the project, each experiment was performed once causing low *n* numbers and

potentially causing large standard deviations. While this can provide a preliminary view, the experiments should be repeated to ensure accuracy.

Clonal reporter lines were selected from 96 well plates that only had single cells after dilution, and were puromyicin selected to ensure that populations remained single cell clones. This was followed by western blot-based assays to validate the clonal lines, testing that changes in mCherry expression mirrored the changes in expression of the endogenous iNOS proteins. This avoided the need for lengthy time course imaging experiments on each and every clonal line while still enabling us to identify the most suitable clones for the subsequent experiments.

The NF-κB hysteresis response was validated through three biological repeats in clonal cell lines. Hysteresis was potentially observed through western blot results. Process and signaling pathways that exhibit hysteresis typically incorporate positive feedback, where once the pathway is stimulated above a specific threshold, the pathway remains active after the stimuli is removed. Hysteresis has been demonstrated in the NF-κB response to LPS in macrophages using qRT-PCR (13). While qRT-PCR was unavailable for *Nos2* reporter validation, western blot analysis of the mCherry protein gave results that suggested that this response extended to NF-κB-regulated expression of *Nos2*.

At the beginning of this project, we were unaware of positive feedbacks within the macrophage JAK/STAT1 signaling pathway and therefore did not expect to observe hysteresis in *Nos2* expression in IFN-γ-stimulated cells. Testing this hypothesis required that a dose response curve for the reporter be produced at different IFN-γ doses. Due to

time constraints, only one repeat of the dose response curve was performed. The western blot showed that 1 U/mL elicited a minimal but measurable response, and that 100 U/mL elicited a maximal response, identical to that achieved by higher IFN-γ doses (i.e. 300 U/mL). It would be preferred to have more repeats, but for the purposes of simple validation of the reporter, 1 U/mL and 100 U/mL were selected as 1 U/mL appears to elicit a response greater than the basal response of untreated RAW 267.4 cells in the western blot, and 100 U/mL appears to elicit a maximal response. The hysteresis experiment, by changing the concentration on the reporter cell line after 16 hours, and western blotting 4 hours after the concentration change showed that those cells initially treated with higher doses of IFN-γ still had higher expression than those initially treated with lower doses, similar to the results seen when the same experiments were run with LPS treatments. Recent studies have begun to show a possibility of autoregulation and positive feedback mechanisms existing within the STAT1 signaling pathway, which would explain the similarity of the hysteresis responses in IFN-γ and LPS treatments (14). The mechanisms of a potential feedback in the JAK/STAT1 pathway are complex and have not yet been completely verified, but would be an explanation for the results seen in hysteretic tests run with the reporter cell line. The results could also potentially be seen because the *Nos2* reporter works on the transcriptional level, requiring time before being translated into proteins that can be detected by western blotting. The stability of the mCherry reporter may not be exactly identical to the *Nos2* transcripts, and may therefore not be an ideal method for studying hysteresis at the level of gene

transcription. A more accurate test for both JAK/STAT and NF-κB hysteresis responses would be a qRT-PCR or other assays quantifying mRNA.

After various validation experiments, the reporter can be used with confidence as a tool to measure *Nos2* transactivation and possibly as a marker of macrophage M1 polarization. Potential therapeutic compounds that are thought to alter NO production by macrophages, like those from American ginseng, can now be tested using the reporter. Polysaccharides were purified from sterile plant tissue culture material. The polysaccharide elicited an M1 response lower than that of 1 μg/mL of LPS on the *Nos2* reporter line and was shown to be significantly different than the LPS response.

The obvious next course of action would be to test AGC1 against an NF-κB reporter, as ginseng polysaccharides were believed to activate the NF-κB pathway, to determine how it compared to LPS activation (9). Before starting the NF-κB reporter tests, an LAL assay was conducted in the Farone Lab to ensure the compounds were clean of any contaminating endotoxin. It was found that there was endotoxin contamination in the polysaccharide compound. Endotoxin typically is found within the cell walls of gram negative bacteria, similar to LPS. The amount of contamination detected by the assay was found to be around 0.07 ng/μg, equivalent to about 1.75 ng/mL of LPS in 25 μg/mL of polysaccharide, the concentration of the compound used for the majority of our assays. It has been shown as little as 1 ng/mL of LPS is sufficient to elicit an NF-κB response in murine macrophages (14). Therefore, the NF-κB tests were done with 1 ng/mL of LPS as a control, as it was close to the 1.75 ng/mL potential endotoxin contamination and was still known to produce a response. If 1 ng/mL produced a

response similar to the AGC compound, it could be assumed that 1.75 ng/mL could also elicit a highly similar response. The NF-κB reporter data showed that 1 ng/mL of LPS and 25 μg/mL of AGC1 produced responses that were not statistically different from each other at the peak of p65 translocation to the nucleus.

After review, it is believed that interference due to cross-activity within the polysaccharide structures could result in a false positive endotoxin contamination result. There are several control experiments to be done to determine the source of endotoxin contamination, if any, and for proper determination of endotoxin concentration within the sample. Samples will be tested pre and post purification dialysis to determine if endotoxin activity is within the sample originally, or after dialysis. PBS will be passed through the endotoxin removal column to determine if the column could be contaminating samples with endotoxins. An LPS sample will be passed through the endotoxin removal column to determine if the column is removing endotoxins effectively. The AGC samples will also be treated with NaOH, which is able to neutralize endotoxins, and will be able to determine if the results seen are actually endotoxins or cross-activity within the polysaccharide. All controls will be tested with ELISA, which can detect and quantify lipopolysaccharide within samples. If it is found that the positive endotoxin result is actually cross activity, testing with NF-κB and *Nos2* reporters will continue in order to increase sample size and get an accurate representation of data and standard deviations for publication.

In conclusion, a *Nos2* reporter for live cell macrophage activation was created and validated through a series experiments. These experiments tested the function of the

reporter to properly display and mCherry fluorescent reporter synonymously with the transcription of the iNOS protein as *Nos2* is transactivated. Two signaling pathways are incorporated in M1 polarization, the NF-κB and the JAK/STAT1 pathway, activated by stimuli binding to TLRs on the cell membrane. The function of both pathways were tested as part of the validation process. The reporter has been successfully validated, and can now be used as a tool to test a number of potential immunomodulatory compounds. More control tests are required to confidently confirm that polysaccharides extracted from American ginseng genuinely create an immune response, but the reporter data suggests that these compounds induce a response lower than that of LPS stimulation.

This reporter cell line for M1 activation can serve multiple uses as a research tool. As shown, it can be used through live cell microscopy to measure the effect of potentially therapeutic substances on macrophage activation. It could also be used to observe how pathogens are able to activate or potentially manipulate macrophage activation for survival within the body. When used with live cell microscopy, the reporter can show the kinetics of macrophage activation stimulated by various compounds or pathogens. In the future, a dual reporter could be made by combining the *Nos2* reporter with a reporter for M2 activation, allowing for a complete view of the effects of various pathogens on macrophages within the body.

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Appendices

Appendix I- Definition of Terms

Arg-1 - An enzyme encoded for by the *Arg1* gene, that converts the amino acid L-arginine to urea and ornithine. It is a marker of M2 activation.

CD14 – A part of the pattern recognition receptor family, a co-receptor for the detection of bacterial LPS.

Cytokine – A peptide secreted by different types of cells that can influence other immune cells.

Fluorescent protein – Proteins that absorb light and re-emit it at a longer wavelength. These are frequently used for visualization of the expression and localization of specific proteins within a cell when used as a tag. They can also be used as reporters to measure promoter/gene transactivation

Hysteresis – A phenomenon in which a response to a given dose is based on the history of past stimulation.

Interferon-γ (IFN-γ) - An endogenous cytokine produced by other immune cells, like T cells and natural killer cells, that triggers macrophage M1 activation.

Inducible Nitric Oxide Synthase (iNOS) – An enzyme encoded for by the *Nos2* gene, that utilizes the amino acid L-arginine to produce nitric oxide. It is a marker of M1 activation.

Immunomodulatory - capable of altering the function of immune cells

Lipopolysaccharide (LPS) - A product of gram-negative bacteria that stimulates an immune response, composed of a lipid and a polysaccharide.

Macrophage - A type of phagocytic, innate immune cell.

mCherry – A fluorescent protein that emits red light when excited with green light.

Nitric Oxide (NO) - A reactive nitrogen species that reacts with oxygen in an oxygen rich environment, resulting in antimicrobial activity. It can also act as a signaling molecule in other contexts.

Pathogen - Any invading microorganism that can colonize and damage a (human) host.

PEST – A short amino acid sequence that targets proteins for degradation. When attached to a fluorescent protein, the sequence acts as a destabilization motif giving the fluorescent protein a shorter half-life.

Phagocytose – A type of endocytosis used by phagocytic immune cells to ingest pathogens and cell debris.

Plasmid - Circular DNA used by bacteria to exchange genetic material. It is also commonly employed by molecular biologists to introduce exogenous genes in to bacterial, yeast, and mammalian cells.

Positive Feedback Loop – When the output of the system amplifies the system's response.

Toll-like receptor 4 (TLR4)– A transmembrane receptor protein, part of the pattern recognition receptor family, which binds LPS and activates the NF-κB signaling pathway.