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Functional Comparison of RAW 264.7 Cells and Murine Bone Marrow-Derived Macrophages (BMDM)

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Abstract:

RAW 264.7 cells are an immortalized line of mouse cells that are commonly used in immunological research. They will be used to compare against murine bone marrow-derived macrophages (BMDM) to see if the two lines of cells are experimentally comparable in basic inflammatory testing. A standard method of measuring cell activity is through the analysis of nitric oxide production, which can be accomplished using a Griess reaction. Cell counts will be standardized to account for differing growth rates between the two cell lines. A similar output of nitric oxide concentration as an indicator of cellular activity between the RAW 264.7 cells and the BMDM will lead to the decreased need of animal testing and cellular extraction for inflammation research.

Keywords: RAW 264.7 cells, bone marrow-derived macrophages (BMDM), Griess reaction, nitric oxide, macrophage, animal testing.

Introduction.

Macrophage Cell Type.

Macrophages are a large cell type that is commonly associated with immunological and inflammatory processes. These cells can be activated to prepare them for their physiological duty through interaction with the cytokine IFN-γ, an immunological stimulus, or by lipopolysaccharide (LPS), a bacterial endotoxin. Stimulation by either of these two compounds encourages macrophages to produce nitric oxide, a major component in inflammation. Macrophages can deal with inflammatory threats either through engulfing large invaders in a process called phagocytosis or by degrading particles using their secreted nitric oxide (Mosser 2003). Because of their physiological role, macrophages exhibit a characteristic spreading pattern after activation, protruding large arms from their cell bodies out towards their attacking threats (Figure 1).

RAW 264.7 Cell Line.

Immortalized cell lines have been used as a tool in microbiological research for decades as a means to avoid repeated animal testing. One very common line used across many different forms of microbiological testing is the RAW 264.7 cell line. These are macrophage-like cells that were derived from murine cells modified by Abelson leukemia virus (Raschke et al. 1978). They have been widely accepted as a model for macrophage research due to their comparable experimental performance. Their type falls under the M1 macrophage subgroup, classifying them as pro-inflammatory cells that can be stimulated by the administration of lipopolysaccharide (LPS), and they are semi-adherent in their growth pattern (Taciak et al. 2018). The use of this

Figure 1. *Activated macrophage spreading pattern. BMDM in M-CSF, day 5. Image captured at 20X magnification using an Olympus B202 inverted microscope and OPELCO (OPtical ELements COrportation) software.*

cell line for the past 40 years has made the immunological study of macrophages accessible and more ethical than the constant use of animal-extracted cells.

LPS Activation.

Macrophage activation using LPS is a common way to study the activity of cultured cells. Macrophages react strongly to the introduction of LPS, which makes up a large portion of the outer membrane of Gram-negative staining bacteria. LPS are glycolipids, or carbohydrate-based lipid molecules, that sit in the membrane of these bacteria and activate the inflammatory response in the receiving macrophages (Fenton and Golenbock 1998). One LPS molecule is composed of a core polysaccharide attached to one O side chain or antigen and one Lipid A, which is the portion embedded in the cellular membrane and consisting of a dimer bonded to several fatty acid chains, as shown in Figure 2. The Lipid A region is the damaging toxic component, with the O antigen varying between bacterial species. LPS is a known endotoxin, stimulating fever and shock responses in host cells by triggering a massive release of inflammatory cytokine compounds. LPS induces the production of nitric oxide by the macrophage enzyme, inducible nitric oxide synthase (iNOS). This nitric oxide (NO) compound is produced by respiring macrophages to kill bacterial threats (Perecko et al. 2014). Production of NO is a natural biological process that occurs in response to toxins or antagonists to create an inflammatory reaction. The compound contains an uneven number of electrons, making nitric oxide very unstable and eager to react with other reactive species; this identifies it as an oxidizing free radical that is very capable of breaking down other compounds, such as in its interaction with LPS (Bloodsworth et al. 2000). In larger organisms, NO aids in smooth muscle relaxation through the induction of cyclase enzyme production (Sipes 2021). However, the nitric oxide produced does not stay in the cell for long. Its half-life is usually less than a second, after

Figure 2. *LPS structure. (Botolph 2009)*

which it is quickly oxidized to nitrite and nitrate by oxygen (Sun et al. 2003). The concentration of this nitrite end product can be easily measured to quantify the amount of cellular activity. Increasing the amount of LPS added should increase the amount of cellular activity, and therefore the amount of nitric oxide produced.

LPS and Cell Proliferation.

While the addition of LPS activates nitric oxide synthesis in macrophage cells, it can also have inhibitory effects in relation to cell proliferation. Xaus et al. observed a progressive decline in cell proliferation after the addition of LPS, starting around 12-15 hours after exposure (1999). LPS may also induce apoptosis, or programmed cell death, after its introduction. Aside from decreasing typical cell counts, halting proliferation should not significantly affect the measurements for cellular activity, as the pathway of LPS in the cell cycle operates independently from nitric oxide production (Vadiveloo et al. 2001).

Griess Reaction.

Measuring the nitrite concentration in the suspended cellular solution can be achieved by performing a Griess reaction. This procedure utilizes a colorimetric change in solution that can be measured for absorbance, and therefore concentration, using a spectrophotometer. The reaction is achieved by treating the cellular suspension with sulfanilamide in an acidic solution. This creates an intermediate salt that is then coupled with N-naphthyl-ethylenediamine (NED). The azo compound that is produced creates a purple color change that is measurable at 540 nm and allows even very low concentrations of nitrite to be detected (Sun et al. 2003). Sometimes, the concentration of nitrates is more prevalent than that of nitrites. Since these molecules can act as intermediates to each other, nitrate can be reduced using a chemical reductant to produce the

quantifiable nitrite for the Griess reaction. The reaction is always run against a nitrite standard curve, made of a range of known nitrite concentrations. This allows the samples tested to be plotted against the line of best fit, which gives a reliable estimate of dissolved nitrites and concurrent cellular activity.

Problems with Cell Count Methods.

Griess reactions can be used to show cellular activity, but to determine relative cellular activity, they must be compared against the cellular density in each sample. The semi-adherent growth of RAW 264.7 cells cause them to stick together and grow as aggregates in solution, especially when cultured in low-adherence plates. These plates allow for the natural, three-dimensional growth of cells, which is appropriate for their formation in culture when testing (Yasuda et al. 2009). However, cells growing in aggregate are much harder to quantify, especially without the use of expensive, specialized cell counting technology and software.

The attempts by Soares et al. to standardize procedures for both counting these cells and managing their aggregate size has led to several methods that they believe are useful in creating better conditions (2014). Their techniques include multiple wash steps, physical disturbance using centrifugation or pipetting, and transferring to larger, conical flasks for resuspension before counting. These steps were taken to modify automated processes formatted for large-scale applications in a smaller, manual method for ease of use. Their result produced a feasible reproduction of automated methods, but they admitted a lack of fine details and specific procedural parameters for a replicable result (Soares et al. 2014). Although standard cell culture plates are commonly used for microbiological testing, the semi-adherent nature of the RAW 264.7 cells onto these plates causes a problem when extracting the cells after growth.

EDTA.

Ethylenediaminetetra-acetic acid (EDTA) is a cation chelating agent, which binds molecules between metal ions. It is commonly used to inhibit biofilm formation in experimental and medical practices. EDTA is believed to inhibit filamentation of the target microorganism, preventing adhesion at high levels (Ramage et al. 2007). Independent work by both Ramage et al. and Dunne and Burd found a decrease in cell adhesion in cell cultures treated with EDTA (2007, 1992). This is an important addition when working with RAW 264.7 cells in normal cell culture plates, as this cell line is semi-adherent. EDTA can offer a solution to get accurate cell counts that avoids the clumping problem associated with low adherence cell culture plates.

Bone Marrow-Derived Macrophages (BMDM).

Bone marrow-derived macrophages (BMDM) are a cell type commonly extracted from mice for microbiological testing. Unlike other cell lines used in research, BMDM are differentiated from bone marrow stem cells once cultured *in vitro*, meaning that any underlying health conditions of the donor mouse will not affect the performance of the BMDM cells being used (Marim et al. 2010). Additionally, a large number of these macrophages can be cultured from just one mouse, reducing the need for large numbers of animals for testing. Techniques described by Marim et al. and briefly by Trouplin et al. also mention the cryopreservation of these cells using liquid nitrogen, allowing them to be viably stored for longer periods (2010, 2013). The gene expression of these cells can be altered for more specific testing purposes (Trouplin et al. 2013). This highly adaptable cell line is commonly used for testing involving drug or genetic screening methods, host-pathogen interactions, and studies on macrophage function.

Not much information is available in regards to comparing the performance *in vitro* of RAW 264.7 cells and BMDM. One immediate problem is the difference in growth rate between the two forms of macrophages, which would have to be accounted and controlled for when administering the activation by LPS. While the two cell lines are quite similar, only one study has directly compared them experimentally. Perecko et al. examined the difference between RAW 264.7 and BMDM responses to chloroquine and hydroxychloroquine effects on nitric oxide production (2014). They found that there was no difference in NO production after introduction of their reactive agent, but general cellular activity by LPS was only used as a control group. There was no direct comparison between their normal cell functioning of the two cell lines after activation by LPS. However, their work provides a strong background for the experimental set-up necessary for creating conditions to compare the two cell lines, including differing culturing periods and media constituents.

Monocyte-Colony Stimulating Factor (M-CSF).

BMDM can be activated by culturing them in media containing macrophage-colony stimulating factor (M-CSF). M-CSF is a carbohydrate-bound protein, or glycoprotein, that belongs to a family of cytokine growth factors (Rosenfeld et al. 1992, Campbell et al. 2000). These function by encouraging cell differentiation and proliferation into their mature state. They also work to prolong survival of bone-degrading cell (osteoclast) precursors (Kitaura et al. 2005). In humans, M-CSF plays a role in autoimmune diseases causing inflammation, such as arthritis, and is secreted by tissue cells in joints (Campbell et al. 2000). In *in vitro* studies, M-CSF can be added to media to activate mature macrophages prolong their viable window for usage.

Macrophages operate by contributing to the inflammatory response after activation by foreign invasion or intoxication. Nitric oxide is a natural product of cellular interaction with the

bacterial endotoxin LPS, working to metabolize the toxin to reduce its inflammatory effects. This response is consistent across macrophage types, making it a reliable factor to use to compare cellular inflammation activity. A similar output in nitric oxide concentration, measured using the Griess reaction, would indicate comparable inflammatory responses between different cell lines. Differences in cell line growth rates can easily be accounted for by adjusting the nitric oxide concentration as a factor of cell numbers; this standardizes the NO output per cell as opposed to the concentration of the general cellular solution, which may contain different cell population densities according to the speed with which they proliferate. Cell counting by hemocytometer allowed for the calculation of a sample's cell density to normalize the NO production. A comparable response after this adjustment would support similar cellular response to LPS stimulation per capita between the two cell lines.

Materials and Methods.

RAW 264.7 Cell Culturing.

RAW 264.7 cells (ATCC, TIB-71, Arlington, VA) were incubated at 37˚C and at 5% CO_2. A new cell passage was made every four days into a new offset flask using DMEM (Lonza, Walkersville, MD) complete culture media for an acclimation period of four passages before use for experiments ["complete" indicating that the media is supplemented with 10% fetal bovine serum and other additions]. This is done to keep the cells in fresh media and in their most responsive stage of their growth before testing, keeping them viable for several weeks. For each new pass, the supernatant media was carefully disposed, then fresh DMEM was administered before scraping with a rubber scraper (Cell Treat scientific products) to detach the DMEM cells

from the flask. A sample of the cells were combined with Trypan blue and cell density and cell viability were measured with a hemocytometer. Cells were allowed to acclimate 24 hours before respective LPS treatments and dispensing 500 µL of cell solution in polystyrene 24-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ).

Cell Counting.

To obtain a more accurate cell count and detach the RAW 264.7 cells from the cell culture plate, EDTA was used. A vacuum filtration system was used with an autoclaved Pasteur pipette tip to remove all used media from the wells. 500 µL of EDTA (Sigma, St. Louis, MO) in 1X Phosphate Buffered Saline (PBS, Sigma, St. Louis, MO) was administered for 5 minutes, after which a pipetman was used to triturate and physically remove the cells from the bottom of the well. The well bottom was examined visually to ensure the absence of any cloudy areas indicating the presence of adhered cells. The triturated solution was transferred to a microtube to be dyed with Trypan blue (Sigma, St. Louis, MO) and counted with a hemacytometer (Figure 3). The following equation was used to estimate cell density from the hemacytometer counts:

Equation 1. *Calculation of cell density.*

$$(average\ number\ of\ cells\ in\ large\ \ squares\ of\ hemocytometer\ ) \times \frac{10^4}{mL} \times \frac{1}{dilution\ factor} = \frac{cells}{mL}$$

Mice Handling.

Ten *Mus musculus* female Swiss mice were obtained for use in testing from Hilltop Lab Animals, Inc. The mice were received at six weeks of age and weighing approximately 20 grams. They were kept in cages with adequate food and water that was checked daily, along with stimulation equipment and corn-based bedding. The cages were replaced and cleaned weekly.

Figure 3. *Hemocytometer grid. Counts are taken from the 16 boxes in each of the four corners to calculate cell density. (Alcibiades 2006)*

The mice were kept at standard lab conditions as described by the NCBI to best suit their natural physiological conditions (NCBI 2011).

Bone Marrow Derived Macrophage Collection and Culturing.

Mice were sacrificed with the use of a CO_2 chamber. Before dissection, the mice were weighted with an electronic scale and sprayed liberally with 70% ethanol. Autoclaved surgical instruments were used to begin to remove the skin and tissue from around the hind leg joint and dislocate it at the hip. The tissue was removed from the femur and tibia and the disarticulated bones were placed in a petri dish of 70% ethanol. The bone was washed in 1X PBS (Sigma, St. Louis, MO) before each end was grasped firmly with tweezers and the epiphyses cut off with scissors. They were held as close as possible to each epiphysis to avoid shattering the bone. A 26-gauge needle was used to slowly push 5 mL of cold DMEM through the marrow cavity to collect through a 100 μm cell strainer into a 50 mL conical tube. The bone marrow suspension was transferred to a 100 mm bacterial petri dish and allowed to incubate at 37˚C and at 5% CO_2 for 2-4 hours to allow mature dendritic cells and stem cells to adhere to the dish, leaving the immature BMDM of interest free in the supernatant.

The supernatant was transferred to two 15 mL conical tubes and centrifuged at 2000rpm for 5 minutes in a Sorvall RT 7 at 4˚C. The resultant supernatant was discarded, and the cell pellet briefly resuspended in 1 mL cold H_2O to perform an osmotic lysis. This created a highly hypotonic solution for the unwanted red blood cells, which would lyse more quickly than the macrophages. To avoid the macrophages also lysing, 10 mL of DMEM was added and the solution centrifuged again at 2000 rpm for 5 minutes at 4˚C. The supernatant was discarded again, and the pellet resuspended in 10 mL of Bone Marrow Maintenance Media (BMMM), which is composed of 5 μL of stock 50 μg monocyte-colony stimulating factor (M-CSF,

PeproTech, East Windsor, NJ) and 10 mL DMEM. A sample was taken for counting by hemocytometer and 5x10^6 cells were added to new bacteriological 100 mm polystyrene petri dishes. These were left to incubate at 37˚C and at 5% CO_2 for 7 days (Figure 4).

Three days after incubation, 5 mL of fresh pre-warmed BMMM was added to each petri dish. On day 5 all supernatant was aspirated and discarded and replaced with 10 mL of fresh BMMM. On day 7, the BMDM are ready to be transferred to flasks for testing.

Cold 5mM EDTA in 1X PBS was added to each petri dish and incubated on ice for 20 minutes. A rubber cell scraper was used to remove adherent cells and the resultant cell suspension was transferred to a conical tube, ready to be counted by hemocytometer to calculate cell number and adjust cell density for experimental use.

LPS Activation.

A serial dilution was made using *E. coli* LPS stock solution (O55:B5, Sigma, St. Louis, MO, Lot#117K4131) at 5 mg/mL. Concentrations for LPS treatments were 0 ng/mL, 1 ng/mL, 10 ng/mL, and 100 ng/mL. 500 µL of DMEM complete was used for the 0 ng/mL LPS dose. Cells were plated in 24-well polystyrene culture plates at a concentration of 4x10^5 cells per well and incubated overnight (12-16 hours). Cells were washed twice with 500 µL of DMEM complete and the used media was discarded using vacuum filtration between washes. 500 µL of treatment was administered to each well and allowed to stimulate for 24 hours.

Griess Reaction.

At 24 hours after LPS introduction, a 50 µL sample of the cell cultures were subjected to the Griess reaction to determine concentration of nitrites in solution. A standard curve was created in a 96-well microtiter plate by setting up a range of known sodium nitrite (Sigma, St.

Figure 4. *BMDM cultured in M-CSF at days 3, 5, and 7. Image captured at 20X magnification using an Olympus B202 inverted microscope and OPELCO (OPtical ELements COrportation) software.*

Louis, MO) concentrations to run the samples against. A stock nitrite solution of 250 μM was used to prepare the standard curve. The plate was organized as described in Table 1.

The 24-well cell culture plate was centrifuged at 1000 rpm for 2 minutes to pellet the cells, and the supernatant was collected for testing. A 50 μL sample from each well was run against the nitrite standard curve.

The Griess reagents are composed of an equal part mixed of 1% sulfanilamide in 2.5% phosphoric acid with 0.1% of naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid. A multichannel pipettor was used to deliver 100 μL of the reagents to each testing well. The plate was read using the μQuant Microplate Reader at 540 nm and a standard curve was created to determine sampled nitrite concentrations.

Following nitrite determination, a 150 μL sample of each treatment well was collected and frozen at -80˚C for later cytokine analysis.

Adherent Cell Removal from Tissue Culture Plate.

Each plate well was washed with 500 μL of PBS before removing the supernatant and wash solution with vacuum filtration. 500 μL of EDTA was added and incubated on ice for 5 minutes. A P200 pipetman was used to triturate and use manual force to help dislodge the cells adhering to the bottom of the well. The solution was then transferred into a microtube, and cell density was calculated using a hemocytometer. The wells were treated in pairs to keep EDTA incubation times consistent.

Table 1. *Conditions used to generate nitrite standard curve.*

Statistical Analysis.

A one-way ANOVA (SPSS 28) was performed to determine if there were significant differences between cell lines groups with significance set at $p < 0.05$. Post hoc analysis by Dunnett's *t* test was performed to determine which groups differed significantly from untreated controls. A two-sample t test assuming equal variances (Excel) was performed to determine if responses between the two groups differed significantly at the same treatment level, with significance set to $p < 0.05$.

Results.

A direct comparison between the RAW 264.7 cell line and BMDM will allow for a better understanding of how similar the inflammatory responses are under equivalent conditions. A Griess reaction was used to quantitate cellular activity in response to inflammation across two cell lines to determine their experimental comparability. Differences in cell proliferation rates complicated the prospect of direct comparison. Rather than attempting to alter the growth conditions and normalize their proliferation, cellular activity was standardized across total cell counts. The similarity between the RAW 264.7 and BMDM dose responses was closest in trial 2, and the RAW 264.7 nitric oxide production response increased with each trial.

After the addition of LPS to the RAW 264.7 cells, there was a significant decrease in cell proliferation. This was noted even from the lowest concentration. Cell counts were reduced by about 50%, results supported by the findings of Xaus et al. in their studies compared against M-CSF proliferation (1999). The counts decreased with an inverse relationship to LPS concentration, yielding fewer cells at the highest dose responses. This relationship was not found

in the cell counts of BMDM, which actually experienced a slight increase in cell numbers with LPS activation (Table 2).

The stunted cell proliferation can clearly be seen within the well plate after LPS administration. Microscopy pictures taken from trial 2 provide a visual for the effect that LPS has over cellular growth patterns (Figure 5). Photos taken of the wells with 0 ng/mL of LPS show the dense proliferation of the RAW 264.7 cells compared to the quite sparse growth in the BMDM well. The pictures also show the effects of LPS administration on RAW 264.7 cell proliferation, showing the decrease in cell numbers concurrent with the dose response along with the absence of this decrease in the BMDM.

Trial 1 shows a slightly elevated but variable dose response to LPS by the BMDM cell line compared to the RAW 264.7, shown below in Figure 6. Controlling for nitrite concentration per 10,000 cells kept the results looking quite similar to the original data (Figure 6, B).

In trial 2, the baseline response to LPS activation changed significantly compared to trial 1. The RAW 264.7 cell line far surpassed the BMDM in nitrite production, but again with a high level of variability within each dose response (Figure 7, A). Adjusting for cell counts altered the results to show a trend comparable to the adjusted counts from trial 1 (Figure 7, B).

Trial 3 was conducted several weeks after the initial two experiments, using cells extracted from much older mice. This resulted in a highly increased unadjusted dose response by the RAW 264.7 cells as opposed to the BMDM from aged mice (Figure 8, A). The ability of the BMDM to proliferate as rapidly as in the earlier trials was also negatively impacted, producing far fewer BMDM in the same time period. Adjusting the dose response per 10,000 cells did show results comparable to trials 1 and 2, but with a slightly better production from the RAW 264.7

Table 2. *Effects of 1 ng/mL LPS versus 0 ng/mL LPS dosage on RAW 264.7 and BMDM cell counts.*

cells (Figure 8, B). While there is still no statistically significant difference between the two cell lines in trial 3, the age of the mice did create a new trend in the opposite direction than what was observed in previous trials.

Statistical analysis using the SPSS 28 software on the trials show a positive Analysis of Variance (ANOVA) test, indicating that there was a successful dose response to the LPS. T-tests conducted on each dose response between the RAW 264.7 and BMDM cells calculate that there is no statistical difference between their activity. The statistical analyses are provided in Appendix 1.

Figure 5. *Photographs of BMDM and RAW 264.7 cell lines before and after LPS administration.* ***A:*** *RAW 264.7 cell line with 0 ng/mL LPS.* ***B:*** *RAW 264.7 cell line with 100 ng/mL LPS at 24 hours.* ***C:*** *BMDM cell line with 0 ng/mL LPS.* ***D:*** *BMDM cell line with 100 ng/mL LPS at 24 hours. All images captured at 200X magnification using an Olympus B202 inverted microscope and OPELCO (OPtical ELements COrportation) software.*

A

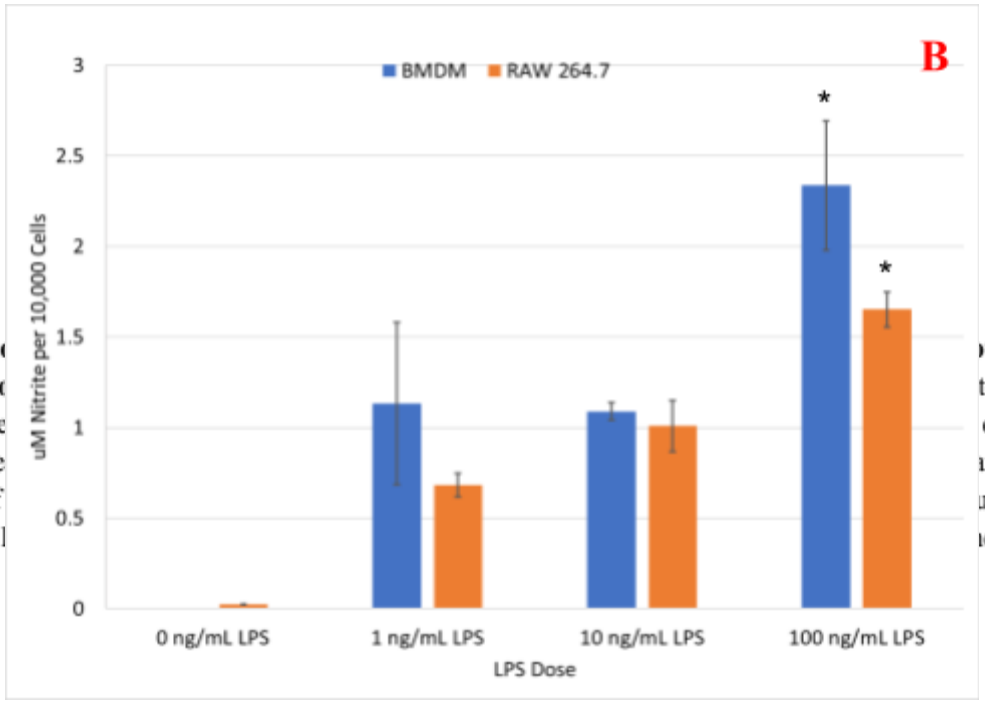
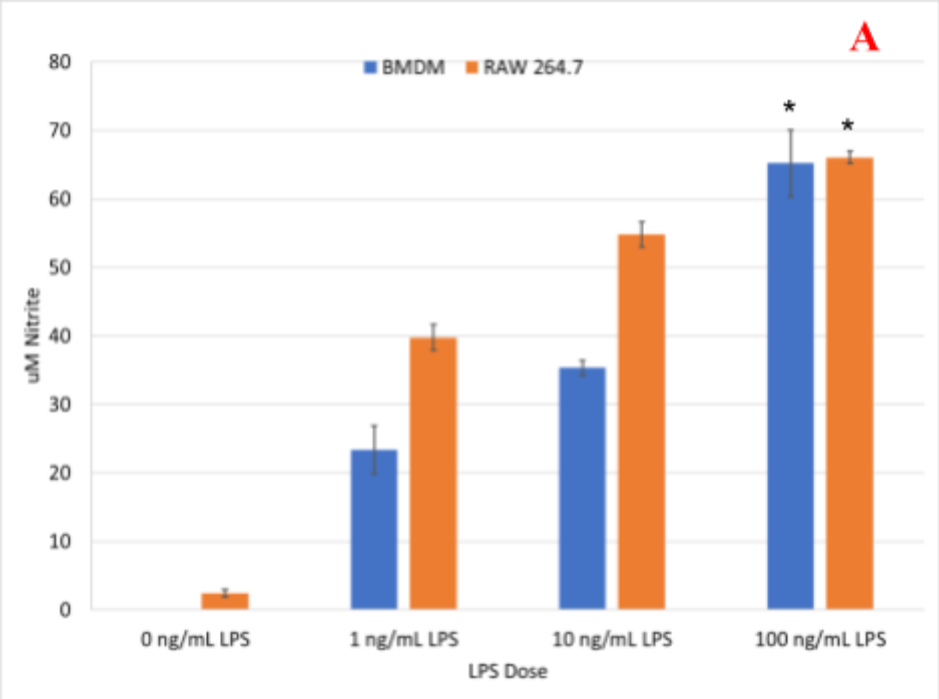
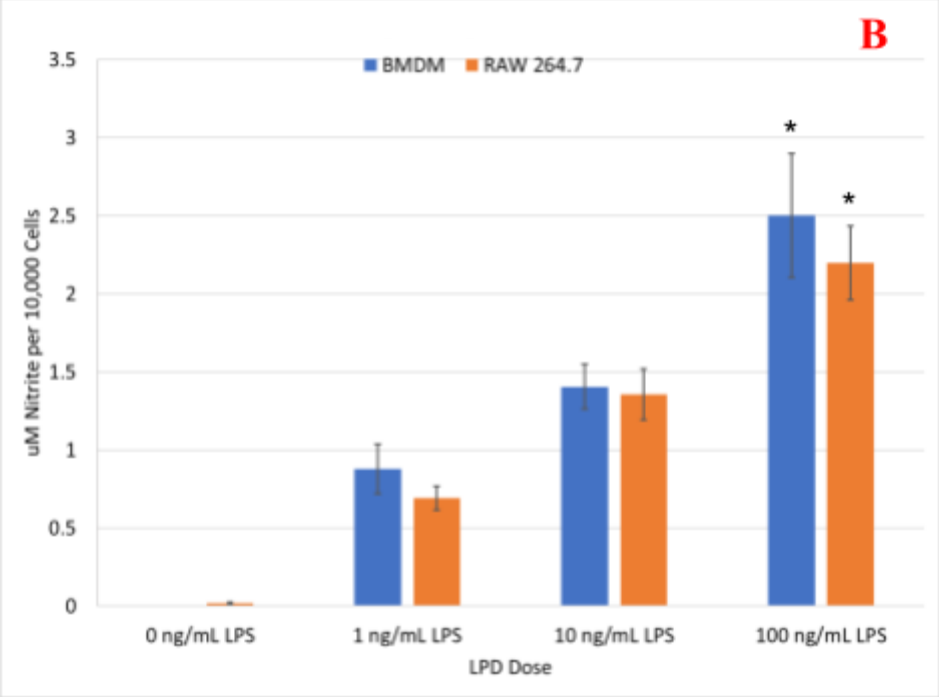


Fig 6. Resp **unts**
manipulati tometry at
550 nm. The of a 24well
plate and tre ndard
deviation of ual
variances. *I er at $p \leq$
0.05.


A
BMDM
RAW 264.7
80
70
60
50
40
30
20
10
0
uM Nitrite
*
*
0 ng/mL LPS
1 ng/mL LPS
10 ng/mL LPS
100 ng/mL LPS
LPS Dose


B
BMDM
RAW 264.7
3.5
3
2.5
2
1.5
1
0.5
0
uM Nitrite per 10,000 Cells
*
*
0 ng/mL LPS
1 ng/mL LPS
10 ng/mL LPS
100 ng/mL LPS
LPD Dose

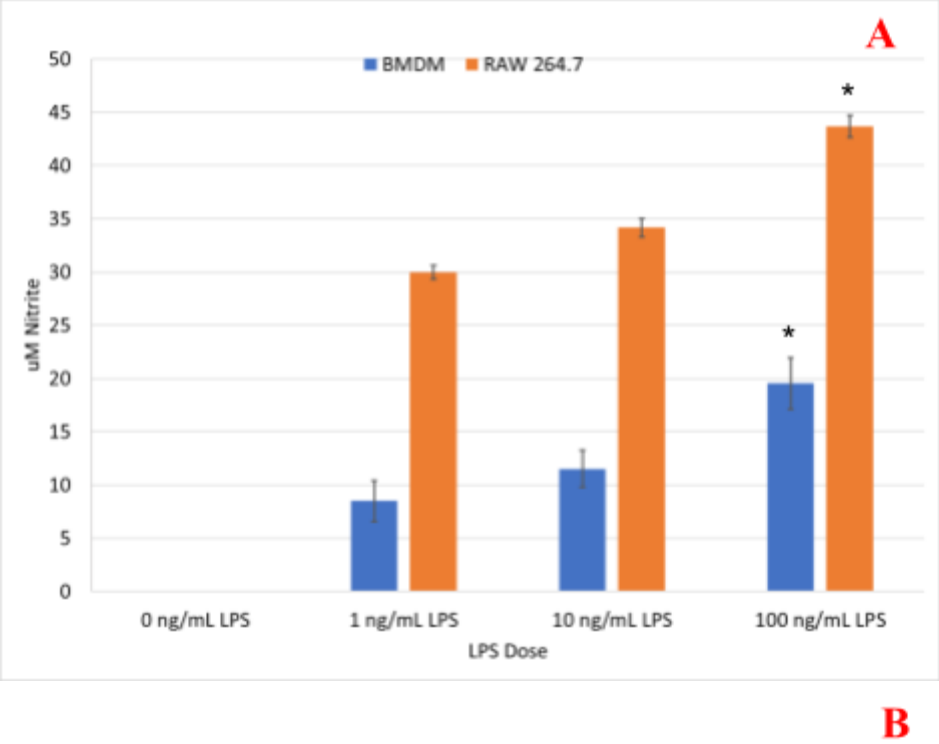


Fig 8. Response of RAW 246.7 macrophages compared to BMDM (A) with concurrent adjusted counts manipulation (B): trial 3. Nitrite in supernatants measured by Griess reaction, followed by spectrophotometry at 550 nm. The RAW 246.7 macrophages were grown in DMEM complete, plated at $4x10^5$ cells in 500uL of a 24well plate and treated with 0 ng/ml, 1 ng/ml, 10 ng/ml, or 100 ng/ml LPS. Each bar represents the mean $\pm$ standard deviation of three equivalent wells. Significance was determined using a two-sample *t* test assuming equal variances. *Indicates all data sets between treatment groups are not significantly different from each other at $p \leq 0.05$.

Discussion.

No studies on a functional research comparison have yet been reported between bone marrow-derived macrophages and their immortalized macrophage-type RAW 264.7 counterparts. Comparing their responses to LPS activation can judge their normal cell functioning when subjected to inflammation. The inflammatory response can be quantitated by measuring nitrite concentrations produced as a means to metabolize the LPS endotoxin. Adjusting the nitrite responses to account for differing cell numbers allows for a functional comparison.

Administering a concentration of LPS even as small as 1 ng/mL to the RAW 264.7 cell line showed a drastic decrease in cell proliferation, as supported by the existing literature. Their numbers were cut almost by half with the first dose response, with a slight but steady decrease continuing with greater LPS concentrations. Though not as drastic as the change seen in the RAW 264.7 cells, the BMDM line actually showed a slight increase in cell counts. Kapetanovic et al. reported that LPS stimulation causes a slight uptick in murine BMDM cell growth, followed by a rapid growth arrest (2012). This study incubated their cell cultures in 60-hour time increments, meaning that it is possible that our studies utilized the BMDM before this cessation in cell growth while still in the middle of their growth increase at our 24-hour mark. this difference in effect on cell proliferation serves as another advocation for the adjustment of cell numbers to create a reliable comparison.

The variable responses observed between the three trials in the unadjusted data highlight the effect that cell number has on nitrite production. RAW 264.7 cell counts were much higher in the later trials, corresponding with higher concentrations of nitrite in solution; unlike in Figure 6, their levels surpassed that of the BMDM output. BMDM cell counts saw a decrease in numbers in each progressive trial, furthering the difference between nitrite production levels in the Griess

reaction. Stout and Suttles reported that macrophages extracted from aging rodents showed functional changes and decreased inflammatory responses in certain tissues (2005). However, they observed no age-associated decline in inflammation of macrophages isolated from murine bone marrow. While their nitrite production remained relatively stable throughout all three trials, the cell proliferation in the aging mice experienced a significant decline. This gives a positive association with the use of RAW 264.7 cells if extended testing is desired, allowing the immortalized line to be cultured for more passages and have more reliable cell numbers without having to consider time restraints on aging animal subjects.

Adjusting the nitric oxide production to consider cell number normalized the output between trials. The standard deviation range remained somewhat high in both adjusted data sets, but the analysis of variance indicated that there is not a significant difference between the two (Figure 6-8). There was an improved decrease in nitrite concentration difference between the two cell lines in trial 2, which could be due to improvement in technique efficiency and familiarity with the methods. This is supported by the increased p-value in trial 2's t-test, shown in Appendix 1, calculating that there is less difference between the adjusted value's two cell lines ($p = 0.86$) as opposed to the unadjusted data ($p = 0.63$).

Further comparable analysis could be performed by examining a Western blotting protocol. Western blotting can select for specific proteins, which in our testing would comprise the inducible nitric oxide synthase (iNOS) which is activated in the presence of LPS to produce nitric oxide. Correlating results with higher nitric oxide levels from the Griess reaction would help to confirm the cells' increased cellular activity in response to LPS activation. The ELISA assay is another plate-based assay that can create quantifiable results in measuring immunological factors called cytokines in cellular solution. ELISA would allow for function

analysis through a different biochemical pathway than nitric oxide production through the use of antibody/antigen labelling.

Measuring nitrite concentrations in solution of activated macrophages provides a simple and direct means to observe cellular functioning. Human models are typically substituted in early research for animal models, but synthetic *in vitro* methods are highly sought after to avoid the need for animal testing and sacrifice. Showing reliably comparative results between an immortalized cell line and cells cultured from live animals is an effective means to argue for this replacement. Considerations must be taken in reference to growth patterns and culturing methods, but these are easily accounted for. Our results show that the RAW 264.7 immortalized cell line responds comparatively after LPS activation to BMDM generated from mice; this supports the conclusion that RAW 264.7 cells may be exchanged for BMDM in research to avoid any unnecessary need for animal testing and sacrifice.

Appendix A: Statistical Analyses.

3/5/2022 Data (Trial 1)

Oneway of RAW 264.7 Dose Response

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3808.462	3	1269.487	1111.368	<.001
Within Groups	9.138	8	1.142		
Total	3817.600	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Nitrite
Dunnett t (2-sided)[a]

		Mean Difference			95% Confidence Interval	
(I) Category	(J) Category	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
1	4	-48.29333*	.87265	<.001	-50.8063	-45.7804
2	4	-15.25667*	.87265	<.001	-17.7696	-12.7437
3	4	-12.96333*	.87265	<.001	-15.4763	-10.4504

*. The mean difference is significant at the 0.05 level.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Oneway of BMDM Dose Response

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9074.716	3	3024.905	93.536	<.001
Within Groups	258.716	8	32.339		
Total	9333.432	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Nitrite
Dunnett t (2-sided)[a]

		Mean Difference			95% Confidence Interval	
(I) Category	(J) Category	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
1	4	-77.70667*	4.64324	<.001	-91.0776	-64.3357

2	4	-37.63333*	4.64324	<.001	-51.0043	-24.2624
3	4	-40.96333*	4.64324	<.001	-54.3343	-27.5924

*. The mean difference is significant at the 0.05 level.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

T-test Unadjusted Data

t-Test: Two-Sample Assuming Equal Variances		
	Variable 1	*Variable 2*
Mean	38.1962963	31.92222222
Variance	993.4325194	423.2149063
Observations	4	4
Pooled Variance	708.3237128	
Hypothesized Mean Difference	0	
df	6	
t Stat	0.333387068	
P(T<=t) one-tail	0.375092154	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.750184308	
t Critical two-tail	2.446911851	

T-test Adjusted Data

t-Test: Two-Sample Assuming Equal Variances		
	Variable 1	*Variable 2*
Mean	1.139239054	0.84159479
Variance	0.911107346	0.459122448
Observations	4	4
Pooled Variance	0.685114897	
Hypothesized Mean Difference	0	
df	6	
t Stat	0.508546663	
P(T<=t) one-tail	0.314611233	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.629222465	
t Critical two-tail	2.446911851	

3/17/2022 Data (Trial 2)

Oneway of RAW 264.7 Dose Response

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6922.752	3	2307.584	1156.529	<.001
Within Groups	15.962	8	1.995		
Total	6938.714	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Nitrite
Dunnett t (2-sided)[a]

		Mean Difference			95% Confidence Interval	
(I) Category	(J) Category	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
1	4	37.31333*	1.15333	<.001	33.9921	40.6345
2	4	52.39333*	1.15333	<.001	49.0721	55.7145
3	4	63.62000*	1.15333	<.001	60.2988	66.9412

*. The mean difference is significant at the 0.05 level.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Oneway of BMDM Dose Response

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6775.081	3	2258.360	242.980	<.001
Within Groups	74.355	8	9.294		
Total	6849.437	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Nitrite
Dunnett t (2-sided)[a]

		Mean Difference			95% Confidence Interval	
(I) Category	(J) Category	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
1	4	-66.01333*	2.48923	<.001	-73.1815	-58.8452
2	4	-41.81333*	2.48923	<.001	-48.9815	-34.6452
3	4	-29.85667*	2.48923	<.001	-37.0248	-22.6885

*. The mean difference is significant at the 0.05 level.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

T-test Unadjusted Data

t-Test: Two-Sample Assuming Equal Variances		
	Variable 1	*Variable 2*
Mean	30.96376812	40.75362319
Variance	735.2792131	769.2256529
Observations	4	4
Pooled Variance	752.252433	
Hypothesized Mean Difference	0	
df	6	
t Stat	-0.504788509	
P(T<=t) one-tail	0.315853422	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.631706844	
t Critical two-tail	2.446911851	

T-test Adjusted Data

t-Test: Two-Sample Assuming Equal Variances		
	Variable 1	*Variable 2*
Mean	1.196013514	1.067067024
Variance	1.093537904	0.865545658
Observations	4	4
Pooled Variance	0.979541781	
Hypothesized Mean Difference	0	
df	6	
t Stat	0.184252351	
P(T<=t) one-tail	0.429942569	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.859885137	
t Critical two-tail	2.446911851	

4/22/2022 (Trial 3)

Oneway of RAW 264.7 Dose Response

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3337.506	3	1112.502	1945.047	<.001
Within Groups	4.576	8	.572		
Total	3342.081	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Nitrite
Dunnett t (2-sided)[a]

		Mean Difference			95% Confidence Interval	
(I) Category	(J) Category	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
1	4	-44.50333*	.61750	<.001	-46.2815	-42.7251
2	4	-13.70000*	.61750	<.001	-15.4782	-11.9218
3	4	-9.50333*	.61750	<.001	-11.2815	-7.7251

*. The mean difference is significant at the 0.05 level.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Oneway of BMDM Dose Response

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	681.104	3	227.035	71.833	<.001
Within Groups	25.285	8	3.161		
Total	706.389	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Nitrite
Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
1	4	-21.04667*	1.45157	<.001	-25.2267	-16.8666
2	4	-11.04333*	1.45157	<.001	-15.2234	-6.8633
3	4	-8.02000*	1.45157	.001	-12.2000	-3.8400

*. The mean difference is significant at the 0.05 level.
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

T-test Unadjusted Data

t-Test: Two-Sample Assuming Equal Variances		
	Variable 1	*Variable 2*
Mean	9.893518519	26.9614198
Variance	65.24001359	355.938967
Observations	4	4
Pooled Variance	210.5894903	
Hypothesized Mean Difference	0	
df	6	
t Stat	-1.663323687	
P(T<=t) one-tail	0.073653378	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	0.147306756	
t Critical two-tail	2.446911851	

T-test Adjusted Data

t-Test: Two-Sample Assuming Equal Variances		
	Variable 1	*Variable 2*
Mean	0.41756826	0.571769856
Variance	0.129610637	0.213660588
Observations	4	4
Pooled Variance	0.171635613	
Hypothesized Mean Difference	0	
df	6	
t Stat	-0.52638095	
P(T<=t) one-tail	0.3087528	
t Critical one-tail	1.943180281	

P(T<=t) two-tail	0.6175056	
t Critical two-tail	2.44691185 1	

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