Effects of increased mitochondrial DNA mutation on the macrophage response to *Listeria monocytogenes*

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Abstract

Mitochondria are important for cellular function, and as cells divide, their mitochondria also divide by replicating their DNA. The integrity of mitochondria DNA (mtDNA) replication, carried out by Polymerase G (PolG), is critical for the maintenance of mitochondria and their functions. In this study, mice carrying a mutant PolG, $PolG^{D257A}$, were used to determine the effect of increased mtDNA mutations on the macrophage population and polarization in response to bacterial and cytokine challenge. It was hypothesized that increased mtDNA mutations will inhibit pathogen clearance by macrophages. To test this hypothesis, the $PolG^{D257A}$ mice were used, along with *Listeria monocytogenes* (LM) as a model of bacterial infection. Three days post LM infection, the bacterial load and the macrophage population was determined in the spleen and liver of $PolG^{D257A}$ and WT mice. No statistical difference was observed in the bacterial load in the liver or spleen, or in the macrophage population in the spleen of the $PolG^{D257A}$ and WT mice. However, the $PolG^{D257A/D257A}$ mice were associated with a higher percentage of macrophages in the liver during LM infection. Polarization of peritoneal macrophages into classically activated (M1) and alternatively activated (M2) macrophages was also studied in vitro. In a single experiment, increased mtDNA mutations in $PolG^{D257A}$ mice seemed to elicit increased M1 and decreased M2 macrophage polarization. Replication of the experiment is warranted to confirm these results. These experimental findings could lead to a better understanding of the role of the mitochondria and macrophages in infectious disease.

Introduction

1.1 *Listeria monocytogenes*

Listeria monocytogenes (LM) is a facultative intracellular bacterium which is the leading cause of a foodborne infection known as listeriosis. Listeriosis is a relatively rare disease with 0.1 to 10 cases per 1 million people per year, depending on the countries and regions of the world (WHO, 2018). Though rare, listeriosis is associated with a high mortality rate in certain groups of people, therefore making the infection a public health concern. The disease primarily affects pregnant women, the elderly (>65), and the immunocompromised (CDC, 2020). Upon infection through ingesting contaminated food, LM moves through the bloodstream and infects the liver, spleen, lymphoid system, and the brain (Zhang et al., 2017). The macrophage internalizes the bacteria into a phagosome (Abuaita et al., 2018). Proteases in the phagosome of infected cells break down LM proteins which are then recognized by innate immune cells like macrophages (Eitel et al., 2011). Internalization of LM induces the cytosolic surveillance system resulting in the expression of Tumor Necrosis Factor alpha (TNF-α) and Interleukin 12 (IL-12). These cytokines stimulate T cells to produce Interferon gamma (IFN-γ) which drives the clearance of bacteria (Zenewicz et al., 2007). The virulence of LM is enhanced by Listeriolysin O, a cytolysin that mediates the escape of LM from the phagosome to avoid humoral defenses such as antibodies, thus,

enabling cytosolic LM replication and spread through actin polymerization and actin-based motility (Nguyen et al., 2019, Cheng et al., 2018).

1.2 Macrophage polarization

Macrophages play a unique role in phagocytosis and clearance of pathogens. Macrophages produce and secrete anti-microbial peptides, generate protons, and secrete anti-microbial effectors such as ROS and nitric oxide (Ramond et al., 2019). Macrophages are known to exhibit heterogeneity in that they can polarize into two main functional types, M1 and M2 macrophages. During infection, macrophages can differentiate into M1 macrophages under the influence of bacterial Lipopolysaccharide (LPS) and increase tissue inflammation and phagocytic capacity to support pathogen clearance. M1 macrophages can induce an expedited ATP production through glycolysis to provide cell energy for the macrophage during inflammation (Yarbro et al., 2020). On the other hand, M2 activation is directly induced by signals from IL-4, IL-13, and IL-33. M2 macrophages are responsible for inflammation resolution, angiogenesis, and tissue regeneration (Yao et al., 2019). M2 macrophages have lower microbicidal activity, which can present a favorable niche for bacterial replication leading to chronic infections. M1 polarization is dependent on glycolysis and mitochondrial fission whereas M2 polarization is reliant on oxidative phosphorylation, fatty acid oxidation and mitochondrial fusion (Ramond et al., 2019). All the aforementioned cell processes are dependent on mitochondria and contribute to the eradication of bacteria by macrophages.

1.3 Mitochondria, Polymerase G, and mitochondrial DNA replication

The mitochondrion is a double membrane organelle that generates energy through the electron transport chain system. Products from the electron transport chain also fuel an array of signaling pathways that help macrophage activation and function. The mitochondrion genome is approximately 16,500 nucleotide base pairs, which form the circular mitochondrial DNA (mtDNA) and encode 13 proteins (Gahl, 2019). The replication of the mtDNA is essential for cell proliferation and mitochondrial biogenesis (Kujoth et al., 2005). One protein that is important in this process is DNA Polymerase gamma (PolG). PolG replicates mtDNA and eliminates mismatched nucleotides in the mitochondrial genome. Mutations in PolG disrupt the integrity of the mtDNA, thereby making the gene a disease locus, responsible for over 180 human disease mutations for the PolG gene including Alpers' syndrome (Zhang et al., 2011). The PolGD257A mutant mouse model harbors a mutation in the 257th amino acid, a change from Aspartic Acid to Alanine, in the exonuclease domain II of PolG, which results in the polymerase lacking proofreading function in all cell types. The ablation of the PolG proofreading capabilities in these mice results in accelerated aging-associated features such as hair loss, graying and kyphosis (Kujoth et al., 2005). Sequencing revealed that the frequency of mtDNA mutations in the $PolG^{D257A}$ mice was ~3 to 8 times higher than that of the Wildtype mice in various cell tissues including the liver of 5-6 months old mice (Kujoth et al., 2005). This $PolG^{D257A}$ mouse model is used herein to determine the effect of increased mtDNA mutations and the resulting mitochondrial dysfunction on LM infection, induced macrophage recruitment and polarization. It is hypothesized that both LM infection and macrophage polarization will be subdued owing to the increased mtDNA mutations.

Materials and Methods

2.1 Mice

The mice used in this experiment are the wildtype (WT) $PolG^{+/+}$ mice, the heterozygous $PolG^{D257A/+}$ mice and the homozygous $PolG^{D257A/D257}$ mice. The mice were between 4 to 6 months of age. All mice used in this study were bred in the East Campus Research Facility at Cornell University. All studies were performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH guide for Care and Use of Laboratory Animals,

federal and state regulations, and was approved by the Cornell University Institutional Animal Care and Use Committee (IACUC, protocol #2013-0014).

2.2 LM culture and infection

A colony of LM was used to inoculate 3 mL of Brain Heart Infusion (BHI) LM selective media for culture at 37 °C. WT and $PolG^{D257A/D257A}$ mice were infected with 1 x 10^6 CFU of LM via peritoneum injection. Three days post infection, the mice were euthanized, and their spleens were collected. The livers were also harvested after perfusion with 10 mL of Phosphate Buffered Saline (PBS) through the hepatic portal vein. The spleens and livers were homogenized in 4 mL and 3 mL of 1% Triton-X100, respectively. The homogenates were distributed into 1:10 serial dilutions to 10^5 dilutions. 0.1 mL of each dilution was plated onto BHI agar plates and incubated at 37 °C. The LM CFU/organ were counted after 24 to 48 hours of culture.

2.3 In vitro macrophage polarization

The peritoneal fluid from WT, $PolG^{D257A/+}$ and $PolG^{D257A/D257A}$ mice were collected and cultured at 1 x10^6 cells/well as described (Alatery et al. 2008). The cells were cultured in RPMI media with 5 ng/mL macrophage colony stimulating factor (M-CSF), 10% Fetal Bovine Serum (FBS), 1% glutamine and 1% penicillin/streptomycin. The cells were incubated in a humidified incubator with 5% CO2 at 37°C. Three days later, the cells were rinsed twice with 1X PBS and the macrophage stimulating media was replaced with a fresh batch. On Day 7, some of the wells were stimulated towards M1 polarization with 100 ng/mL LPS, and some were stimulated towards M2 with 20 ng/mL IL-4. A third set of the wells were cultured in RPMI media without M-SCF for M0 polarization — 10% FBS, 1% glutamine and 1% penicillin/streptomycin. There were three cell culture replicates for each cell type, cultured at 1 x10^6 cells/well in three different wells. After 48 hours, the cells were detached with 0.25% trypsin in EDTA and washed with 2 mL per well with 10% FBS in PBS (Zhao et al., 2017). The cells were stained for flow cytometric analyses as described in 2.5.

2.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total mRNA was collected from peritoneum macrophages in Trizol following in vitro polarization as described (Wellcome, 2007). The cells were separated into an aqueous phase with 200 µL of chloroform per ml of Trizol and the resulting RNA was precipitated in 500 µL 2-propanol. The RNA pellet was washed with 1 mL of 75% ethanol (prepared using RNase-free water), air dried for 10 minutes in the hood and later dissolved in 30 µL of RNase-free water on ice. The concentration of RNA was measured with Quawell Q3000 UV Spectrophotometer. The 260/280 value for each sample was close to 2. The RNA was diluted (1:10) with RNase free water in preparation for Reverse Transcription to cDNA. Each sample received a mixture of 5x iScript Reaction Mix, iScript Reverse Transcriptase and nuclease free water following instructions on the iScript cDNA synthesis kit from Bio-Rad Laboratories, Inc. The resulting cDNA underwent PCR reaction using the following forward and reverse primers respectively; 5'-AAACGGCTACCACATCCAAG-3' and 5'-CCTCCAATGGATCCTCGTTA-3' for the control - 18S rRNA (155 bp) and 5'-GGGCATACCTTTATCCTGAG-3' and 5'-CCACTGAAGTCATCCATGTC-3' for M2 Ym-1 (304 bp) (Mulder et al. 2014). All primers were purchased from Integrated DNA Technologies (Coraville, Iowa). The Denville Scientific Inc DNA marker 100bp ladder (cb3602) was used in the Gel Electrophoresis.

2.5 Flow cytometric analyses

All macrophage populations and phenotypes were determined with the Thermo Fisher Attune NxT Flow cytometer and analyzed using FlowJo. Macrophages were stained directly with fluoro-chrome-labeled antibodies against the following markers: eBioscience AF700 Anti-mouse MHC Class II (I-A/I-E) (clone M5/114.15.2), BioLegend PE/Dazzle Anti-mouse/human CD11b (clone M1/70), eBioscience PE anti-mouse F4/80 (clone BM8),

BioLegend APC/Cyanine7 Anti-mouse CD86 (clone GL-1), BioLegend FITC anti-mouse CD206 (MMR) antibody (clone C068C2), eBioscience Fixable Viability Dye eFlour 506 (clone v506) and eBioscience FC block Anti-Mo CD16/CD32 Purified (clone 93). Macrophages in the spleen and the liver were identified with F4/80+MHCII+CD11b+ markers using flow cytometric analyses.

2.6 Statistics

All data representations shown in this study were illustrated with GraphPad Prism version 9.0. Statistical significance between groups ($P<0.05$, $n>3$) were determined using Student's t-test.

Results

3.1 Increased mtDNA mutations leads to reduced liver weights in LM infected mice

To understand the effect of $PolG^{D257A}$ mutation on the response to LM infection, the weights of mice, livers, and their spleens of WT and $PolG^{D257A/D257A}$ mice were compared 3 days after LM infection. As shown in Figure 1, the WT and $PolG^{D257A/D257A}$ mice had similar weights post infection except the livers, where the $PolG^{D257A/D257A}$ livers weighed less than WT livers ($p<0.05$). There was no difference in weights of the mice and spleens.

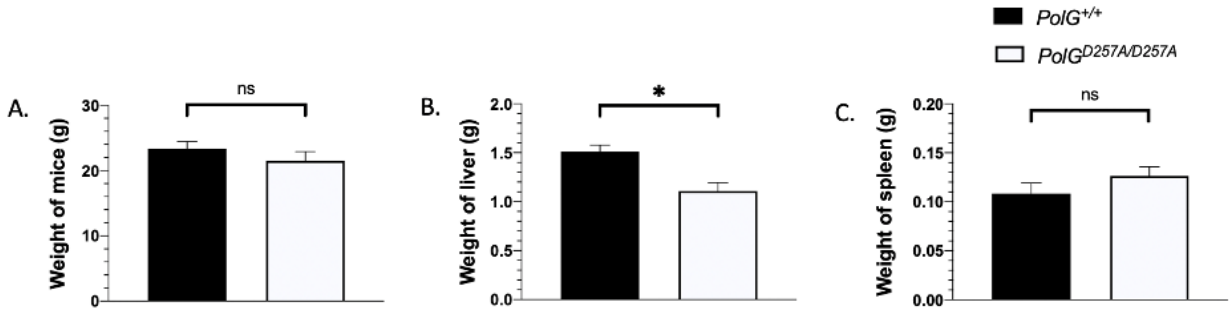

Figure 1: Reduced weights of the liver of PolGD257A/D257A 3 days post LM infection. WT (n=6) and PolGD257A/D257A (n=8) mice were infected with LM and sacrificed after 3 days for analysis of spleen and liver. (A) Weight of the WT and PolGD257A/D257A mice. (B) Weight of liver of WT and PolGD257A/D257A mice ($p<0.05$). (C) Weight of spleen of WT and PolGD257A/D257A mice.

3.2 Increased mtDNA mutations does not affect LM CFUs in the spleen and liver of infected mice

To determine the effects of the $PolG^{D257A}$ mutation on LM colonization 3 days after infection, spleen and liver homogenates were plated on BHI agar plates and the LM colonies were counted. LM CFU was calculated per gram of organ and the results are displayed in Figure 2. Although the difference was not statistically significant, $PolG^{D257A/D257A}$ mice had higher bacterial load in the spleen than the WT mice. However, the reverse was observed in the liver, although again the difference was not statistically significant.

3.3 Increased mtDNA mutations are associated with a higher percentage of macrophages in the liver during LM infection

Three days post LM infection, splenic and liver cells were isolated and stained for macrophages. Viable cells that were positive for F4/80, MHCII and CD11b were gated as macrophages using flow cytometric analyses (Figure 3). The number of macrophages were determined as a product of the percentage of F4/80+MHCII+CD11b+ cells and the total cell counts for each group. There was no statistically significant difference in the percent and number of splenic macrophages (Figure 4). By contrast, the livers of $PolG^{D257A/D257A}$ mice had a higher percent of macrophages when compared to the WT mice ($p<0.05$) (Figure 5). This suggests that increased mtDNA

This suggests that increased mtDNA mutation may be linked to high macrophage recruitment in the liver during LM infection.

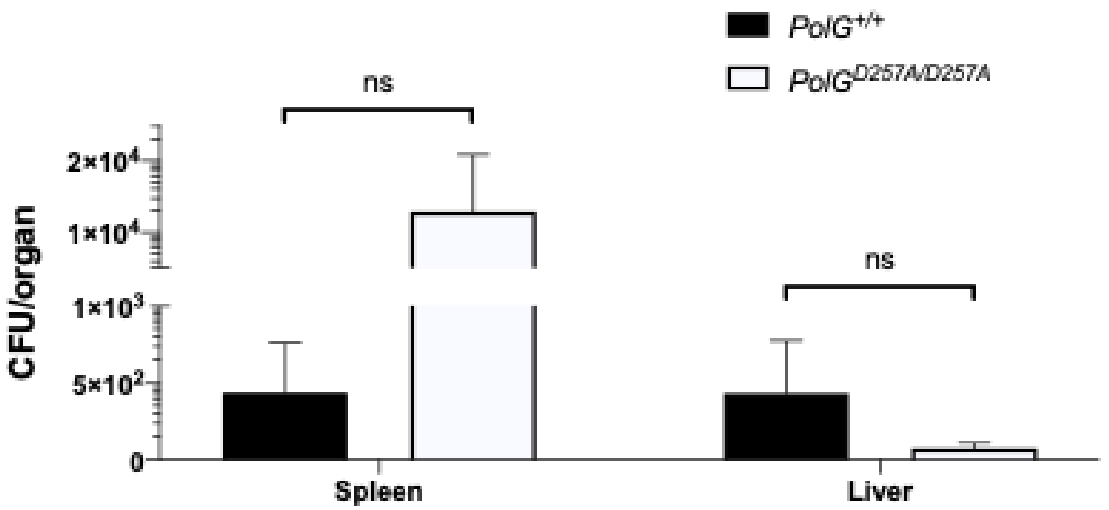

Figure 2: No statistically significant difference in LM CFU/organ in the spleen and liver of WT and PolGD257A/D257A mice 3 days post infection. WT and PolGD257A/D257A mice shown in Figure 1 were analyzed for LM CFUs in the spleen and liver. LM colonies were counted from tissue homogenates 24 to 48 hours after plating. There was no statistical significance in the difference in bacterial load between the WT and PolG$^{D257A/D257A}$ mice. WT (n=6) and PolG$^{D257A/D257A}$ (n=8).

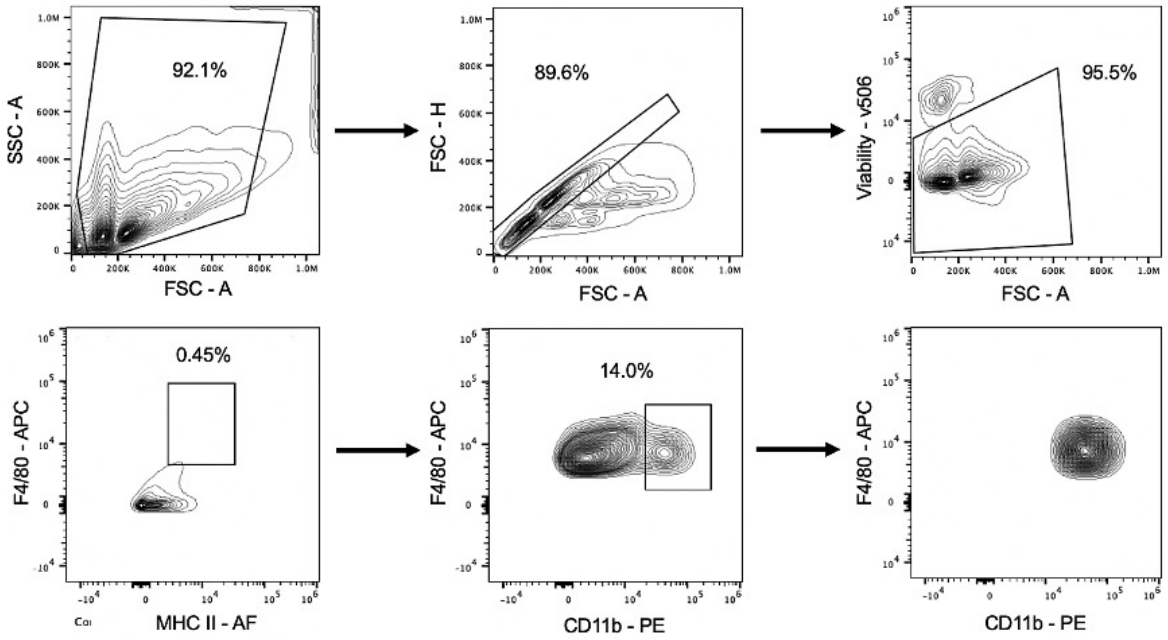

Figure 3: Macrophage Gating Strategy. Macrophages were gated from isolated cells from the spleen and liver of mice infected with LM shown in Figure 1. Viable macrophage populations were detected with F4/80+MHCII+CD11b+ markers using flow cytometric analyses.

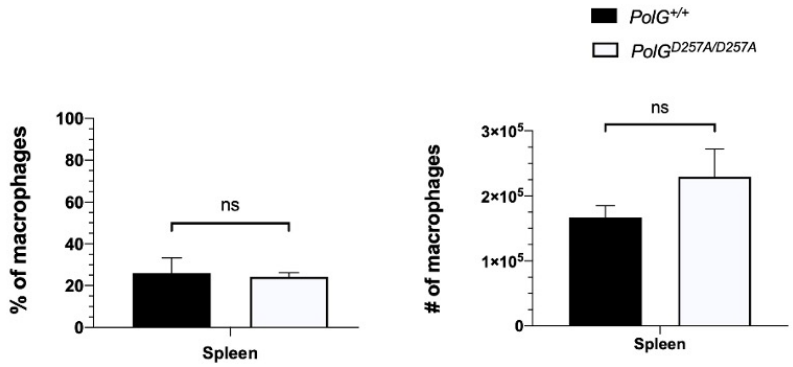

Figure 4: No difference in percent and number of macrophages present in the spleen of WT and PolG$^{D257A/D257A}$ 3 days post LM infection. Macrophages were gated from isolated cells from the spleens of mice infected with LM as shown in Figure 3. Viable macrophage populations were detected with F4/80+MHCII+CD11b+ markers using flow cytometric analyses. There was no significant difference in the percent or number of macrophages between the WT and PolG$^{D257A/D257A}$ mice in the spleen. WT (n=6) and PolG$^{D257A/D257A}$ (n=8).

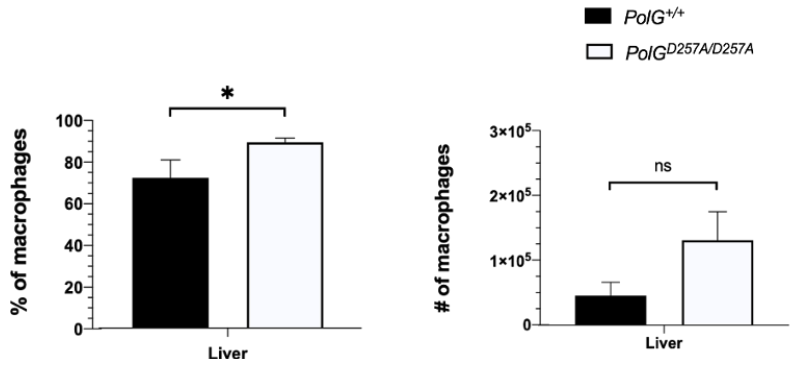

Figure 5: Statistically significant increase in percent, but not number of macrophages in the liver of PolGD257A/D257A mice 3 days post LM infection. WT and PolGD257A/D257A mice shown in Figure 1 were analyzed for liver macrophages. Increased mtDNA mutations were associated with a higher percentage of macrophage in the liver. WT (n=6) and PolG$^{D257A/D257A}$ (n=8) ($p<0.05$).

3.4 Increased M1 and decreased M2 macrophage polarization with increased mtDNA mutations

To investigate the effect of mitochondrial dysfunction due to increased mtDNA mutations on macrophage polarization, classically activated M1 and alternatively activated M2 macrophages from the peritoneum of the WT, heterozygous PolG$^{D257A/+}$ and PolG$^{D257A/D257A}$ mice were evaluated. The peritoneal cavity is a common and easy source of primary macrophages compared to the spleen and liver (Zhao et al., 2017), and so peritoneal macrophages were used for polarization experiments. Cells collected from the peritoneal cavity of the WT, PolG$^{D257A/+}$ and PolG$^{D257A/D257A}$ mice were stimulated with 5 ng/mL M-CSF media to differentiate monocytes into macrophages. After 7 days, the macrophages were treated with no cytokine (M0), 100 ng/mL LPS for M1 polarization and 20 ng/mL IL-4 for M2 polarization. 48 hours after polarization, the macrophages were stained with antibodies for flow cytometric analyses. Viable macrophages were gated as double positive for F4/80 and CD11b markers using the strategy shown in Figure 6. There were 90 to 100% F4/80+CD11b+ cells in both the polarized and non-polarized cell types (Figure 7). This is indicative of the presence of macrophages in all genotypes and testing conditions. Further, the LPS-induced M1 macrophages were detected as F4/80+CD11b+CD86+ cells (Figure 8). CD86 is an antigen surface marker that is highly expressed under inflammatory conditions and is considered an M1 macrophage marker (Zhao et al., 2017). Macrophage M1 wells had a higher percentage of CD86 markers in comparison to the non-polarized M0 macrophages and IL-4-induced M2 macrophages (Figure 8). Statistical significance was not computed for

these results because the data was obtained from the pooling of 2 to 3 mice in one experiment. However, the trend shows a gradual increase in percentage of M1 macrophages as the mtDNA mutations increase (Figure 8). The macrophage mannose receptor, CD206, was used as an M2 gene surface marker (Rőszer, 2015). All macrophages showed elevated percent and number of F4/80+CD11b+CD206+ cells (Figure 9), although more experiments need to be performed to confirm these results. RT-PCR was performed to examine functional M2 markers such as the Ym-1 gene, which is also upregulated by IL-4 in macrophages (Rőszer, 2015). The gel electrophoresis display demonstrates that the Ym-1 gene, unlike the control, was expressed at 304 bp in the IL-4-induced M2 macrophages alone, and not the M0 or M1 macrophages (Figure 10). Also, the brightness of the bands faded with increased PolGD257A mutations. The PolG$^{D257A/D257A}$mice showed no band at all (Figure 10).

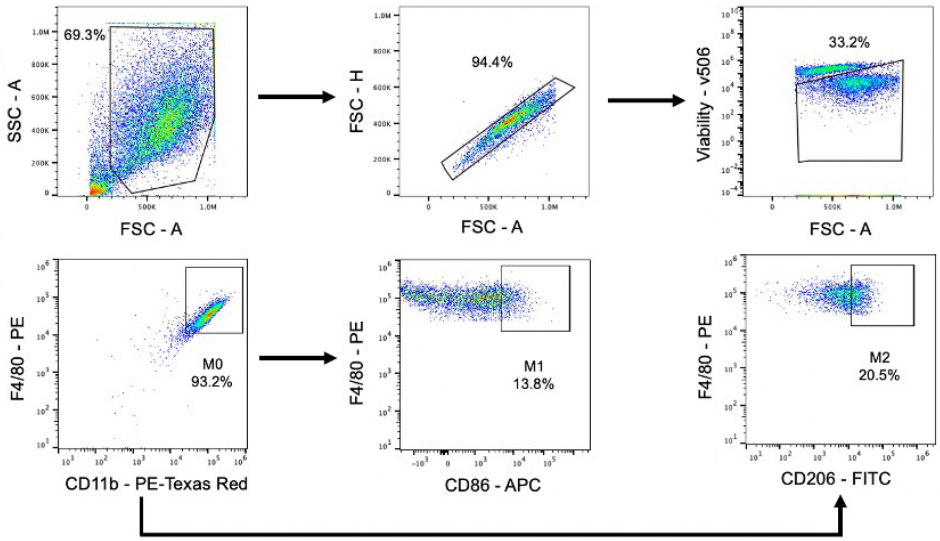

Figure 6: Gating Strategy for Macrophage Polarization. 48 hours after polarization to M0, M1 and M2 peritoneal macrophages, cells were gated using flow cytometric analyses as shown above. Viable macrophage populations were detected as F4/80+CD11b+. LPS-induced M1 macrophages were detected with F4/80+CD11b+CD86+ markers and IL-4 induced M2 macrophages were detected as F4/80+CD11b+CD206+.

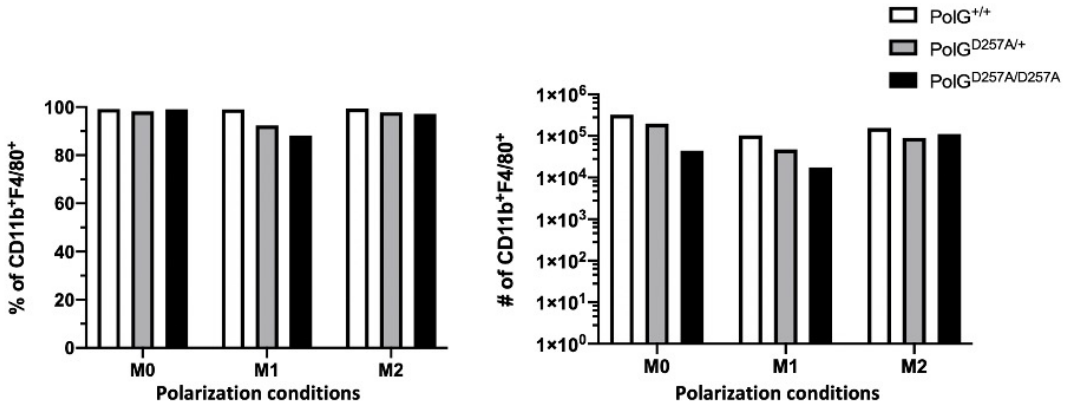

Figure 7: Percent and number of F4/80+CD11b+ macrophages present in the M0, M1 and M2 peritoneal macrophage cultures of WT, PolGD257A/+ and PolGD257A/D257A mice. Peritoneal cells from WT (n=3), PolG$^{D257A/+}$ (n=2), and PolG$^{D257A/+}$ (n=2) were pooled and cultured under M0, M1 and M2 conditions as described in the Methods section. Cells were 90 to 100% F4/80+CD11b+ for macrophage markers after polarization. The number of F4/80+CD11b+ macrophage cells varied from 1x10^4 to 1x10^6.

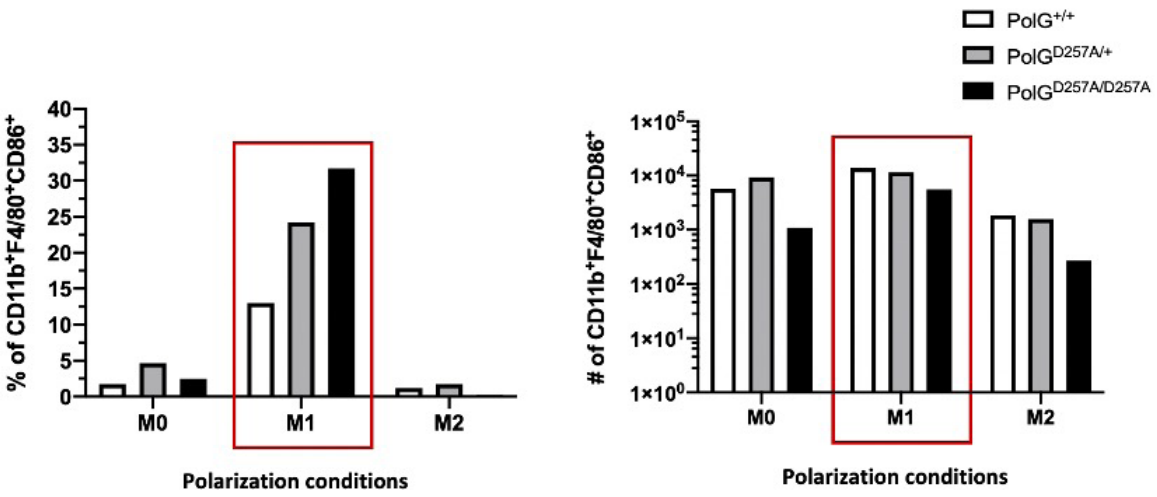

Figure 8: Percent and number of F4/80+CD11b+ CD86+ macrophages present in the M0, M1 and M2 peritoneal macrophage cultures of WT, PolGD257A/+ and PolGD257A/D257A mice.Peritoneal cells from WT (n=3), PolGD257A/+ (n=2), and PolGD257A/+ (n=2) were pooled and cultured under M0, M1 and M2 conditions as described in the Methods section. The red boxes indicate LPS-induced M1 macrophage cultures.

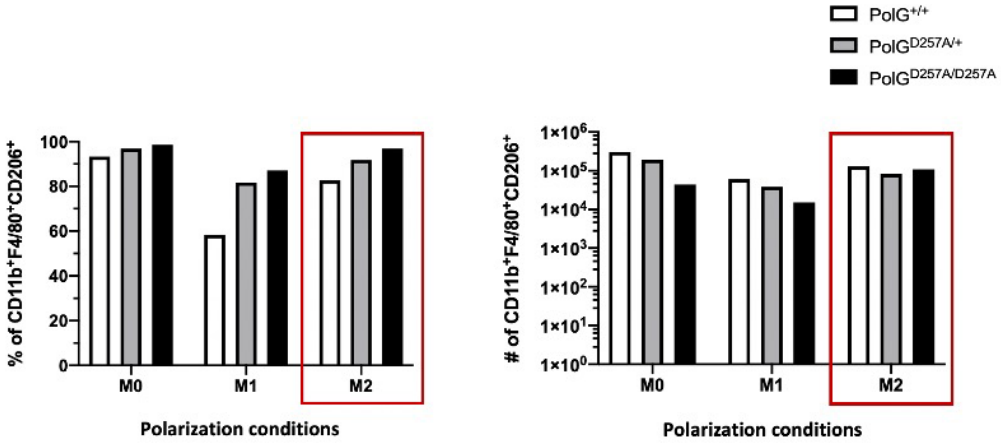

Figure 9: Percent and number of F4/80+CD11b+ CD206+ macrophages present in the M0, M1 and M2 peritoneal macrophage cultures of WT, PolGD257A/+ and PolGD257A/D257A mice. Peritoneal cells from WT (n=3), PolGD257A/+ (n=2), and PolGD257A/+ (n=2) were pooled and cultured under M0, M1 and M2 conditions as described in the Methods section. The red boxes indicate IL4-induced M2 macrophage cultures.

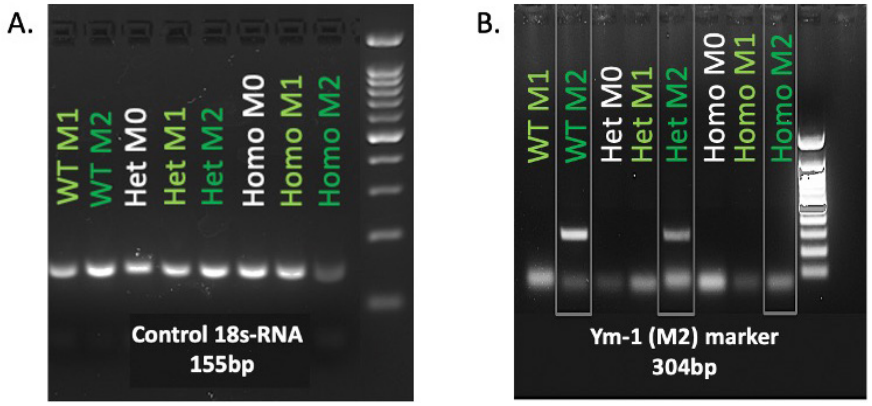

Figure 10: Decreased M2 macrophage polarization in PolGD257A mice. Peritoneal cells from WT (n=3), PolGD257A/+ (n=2), and PolGD257A/+ (n=2) were pooled and cultured under M0, M1 and M2 conditions as described in the Methods section. RT-PCR results of (A) Control 18s-RNA and (B) Ym-1, an M2 associated gene, 48 hours after polarization.

Discussion

Macrophages are among the cells that make up the first line of immune defense and they adopt diverse activation states in response to infectious diseases. While mitochondria have been shown to be important for macrophage function, the effect of increased mtDNA mutations on the macrophage population and polarization in response to bacterial and cytokine challenge is not known. Here, the hypothesis that increased mtDNA mutations and accompanying mitochondrial dysfunction will suppress the ability of macrophages to clear pathogens was tested using LM infection of PolG^{D257A} and WT mice, and polarization of peritoneal macrophages into classically activated (M1) and alternatively activated (M2) macrophages from these mice. The data suggest that increased mtDNA mutations affect the percentage of macrophages in the liver during LM infection and may affect M1 and M2 macrophage polarization in vitro. Replication of the experiment is warranted to confirm these results. The findings from these experiments could lead to a better understanding of the role of the mitochondria and macrophages in infection. Furthermore, as the various phenotypes of macrophages are still being characterized, there remains limited knowledge on how macrophage activation is affected by mitochondrial dysfunction (Anders, 2020). Thus, the identification of macrophage phenotypic differences in mice with increased mtDNA mutations will provide novel insights into the role of macrophages and the mitochondria in pathogen clearance. In addition, the study may improve current drugs and therapeutic interventions for pathologies such as Alzheimer's disease, that are associated with mitochondrial dysfunction (Wang et al., 2020).

In this study, the percent of macrophages was identified as being significantly higher in the livers of LM-infected PolGD257A/D257A mice than LM-infected WT mice. Currently, a direct correlation between macrophage response and mitochondrial dysfunction remains understudied. Increased mtDNA is also associated with accelerated aging, and the PolG$^{D257A/D257A}$ mice have also been shown to exhibit accelerated aging in comparison to the WT mice (Kujoth et al., 2005). Moreover, the process of aging in the liver is associated with degenerative modifications including deficits in mitochondrial function in both macrophages and hepatocytes (Kim et al., 2015). LM induces inflammasome activation that promotes the recruitment and bacterial-killing capacity of hepatic macrophages (Jeong et al., 2020). Several laboratories have demonstrated an age-related increase in hepatic macrophages, as high as threefold greater, in aged mice (Bloomer et al. 2020, Covarrubias et al, 2020, Stahl et al., 2020). Thus, it can be proposed that the increase in macrophages in the liver of PolG$^{D257A/D257A}$ mice conforms to the narrative of the aged mice. Also, the macrophages in the liver PolG$^{D257A/D257A}$ may be actively responding to the LM-induced inflammasome activation leading to the corresponding lower bacterial load in the WT mice, though this was not statistically significant.

In contrast to the liver, both the LM colonization and macrophage population in the spleen showed no differences between the WT and PolG$^{D257A/D257A}$mice. Splenic macrophages from older mice secrete substantially lower amounts of TNF-α and IL-6 cytokines in response to LPS and Toll-like receptor signals (Linehan et al., 2015). Geriatric mice that were gavaged with LM displayed higher LM colonization, higher inflammatory response and produced less IFN-γ in the spleen (Alam et al. 2020). IFN-γ is recognized as a key regulator of macrophage activation that enhances macrophage phagocytosis to fight LM infection (Wu et al., 2014). Thus, low IFN-γ production in aged mice can be linked to reduced phagocytic ability, which could be a consequence of several factors including senescence, defective autophagy, and impairments in mitochondrial and increased ROS production (Yarbro et al., 2020). If replicated, the results from this study would generally agree with these findings in

that the increased macrophages in the LM-infected PolG$^{D257A/D257}$A spleen may have phagocytic defects resulting in the high LM colonization, although not statistically relevant. Replication of the study with more mice and further phagocytosis or ROS production assay may reveal statistical significance in the increase in LM colonies and macrophages observed in the spleen of the PolG$^{D257A/D257A}$ mice.

Additionally, macrophages in the peritoneal cavity of the WT, PolG$^{D257A/+}$ and PolG$^{D257A/D257A}$ mice were examined under polarizing conditions. The success of the in vitro culture of monocytes into macrophages was determined by the higher levels of F4/80+CD11b+ cells in all mouse groups. Large peritoneal macrophages in several mouse strains, including C57Bl/6 used in this study, express high levels of F4/80 and CD11b (Cassado et al., 2015). M1 macrophage activation seemed to increase with and M2 macrophage activation seemed to decrease with homozygous PolG^{D257A} mutations, although the macrophage polarization data needs more replicates. Consistent with these observations, macrophages with reduced mitochondrial content were more likely to be polarized into M1 macrophages and exhibited increased sensitivity to inflammation in response to stimuli (Adesso et al., 2013). Additionally, increased mitochondrial metabolism has been shown as both a requirement and characteristic of M2 macrophage polarization (Van den Bossche et al., 2016 & Tan et al., 2015). In the context of LM infection, LM has been shown to induce M1 macrophage polarization as well as promote repolarization of M2 macrophages into M1 macrophages (Thiriot et al., 2020). Given that mitochondrial dysfunction enhances M1 and impairs M2 polarization, the PolG$^{D257A/D257A}$ mice may activate M1 macrophages in response to LM infection.

A major limitation of this study was the lack of experimental replication and the small sample size used. Thus, repetition of the study with more mice, uninfected controls, and possibly time-course experiments to confirm the results is warranted. Additionally, functional M1 and M2 markers, such as iNOS and Arginase, respectively, will need to be quantified using RT-PCR to help formulate definite conclusions about the phenotypic variations observed, and also, to provide a perspective on the controversy surrounding acceptable M1 and M2 macrophage biological markers. Finally, mitochondrial uncoupling protein 2 has been shown to influence macrophage polarization (Angajala et al., 2018). Hence, future works will identify mitochondrial protein expression in the polarized macrophages using western blot technique to further characterize the players involved in each phenotype. In conclusion, this study used mice with mutated PolG, PolG^{D257A}, to investigate the effects of mitochondrial dysfunction on the macrophage recruitment and polarization following LM infection. A higher percentage of macrophages was observed in the liver of the PolG^{D257A} mice than the wildtype mice. In a single experiment, increased mtDNA mutations were associated with increased M1 and decreased M2 peritoneal macrophages. The findings suggest that mitochondrial dysfunction may lead to increased susceptibility to LM infection, although not statically significant, and increased M1 macrophage recruitment to LM infection. Replication of the experiments is warranted to confirm these findings. This work represents the first study of the potential effects of increased mtDNA mutations in the PolG$^{D257A/D257A}$ mice on the macrophage response to LM infection, and the findings contribute to the understanding of inflammation activation and mitochondrial dysfunction in infectious diseases.

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