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Investigating the Effects of Hypoxia on Macrophages and Their Interactions with Cardiomyocytes

THESIS

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by

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DEDICATION

To all my family
and friends

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ABSTRACT OF THE THESIS

Investigating the Effects of Hypoxia on Macrophages and Their Interactions with Cardiomyocytes

by

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Macrophages are versatile cells that polarize into a continuum spectrum of functional phenotypes depending on the microenvironment physiological and pathological cues. As a major immune regulator, macrophages are of interest especially with the current global COVID-19 pandemic. COVID-19 caused by the SARS-CoV-2 virus negatively impact the health of patients through the respiratory system. However, notable cases of COVID-19 showed cardiac damages triggered by prolonged low oxygenation leading to hyperinflammatory responses within the heart. As cardiac tissues cannot regenerate once damaged, looking into the role of macrophages present in the heart during inflammation in response to hypoxia can provide insightful knowledge of the underlying mechanisms taken place. Before exploring macrophages and cardiomyocytes interactions upon in vitro hypoxic stimulation, an optimal culturing medium is required to ensure healthy growth and proliferation of both cell types in a controlled setting. Interestingly, the combination of M199, a standard cardiomyocytes culturing media, and M-CSF, a potent hematopoietic cytokine known for

differentiating monocytes into macrophages, leads to normal culture of macrophages. To assess the effect of direct and indirect hypoxia on macrophages functions, macrophages cultured in M199 with M-CSF media or neonatal rat ventricular myocytes (NRVMs) media treated with varying degrees and durations of hypoxia were stimulated. Cytokine secretion levels were used as indicators of inflammatory and healing activation. Short term hypoxia is found to modulate the release of TNF-α leading to increased inflammatory responses. In contrast, long term hypoxia regulates the polarization of macrophages to be more M2 or pro-healing-like. These findings function as the framework for developing an experimental system aimed at understanding the interactions between macrophages and cardiomyocytes.

CHAPTER 1
INTRODUCTION

Coronavirus disease 2019 (COVID-19) causes detrimental health effects for millions of individuals worldwide. Their presence continues still today, and the mechanism COVID-19 uses to impose damages to tissues and organs remains unclear. Most COVID-19 patients experienced respiratory tract symptoms, and a great deal of knowledge has been gathered and investigated these past two years. However, a notable portion of these patients showed cardiac symptoms, and little is known about COVID-19 effects on chronic damage to the cardiovascular system[7]. Current knowledge of the effects of COVID-19 on the respiratory system indicate that cardiac trauma can be immune system and/or hypoxia mediated. As a result, this paper focuses on macrophages (a major immune regulator), their interactions with cardiomyocytes, and the effects of hypoxia on macrophages' physiology and functions.

Hypoxia

Life is only possible with the availability of oxygen. Hypoxia is a state in which there is not enough oxygen to maintain body's homeostasis as there are inadequate oxygen supply for tissues to perform their biological needs. Silent hypoxia associated with COVID-19 has been shown to be a key contributor of the "cytokine storm" where large quantity of inflammatory mediators such as macrophages are recruited. Such excessive inflammation can lead to tissue and organ damage, thus causing silent hypoxia to be a significant factor that results in increased mortality rate of COVID-19 patients[8].

Macrophages Biology

Macrophages are immune cells that play a vital role in maintaining the body's homeostasis. Their roles in the detection, destruction, and phagocytosis of body's foreign invaders makes them a key contributor of the innate immune system[1]. Additionally, macrophages' role as an antigen presenting cell helps initiate the adaptive immune response by activating T and B cells to instigate inflammation. Post inflammatory activation, macrophages also play a role in the regulatory and repair processes of the body to promote homeostasis[2].

Macrophages originate from blood monocytes which disperse into various tissues upon infection. They are versatile cells and their ability to polarize in response to various physiological and pathological cues makes them a cell of interest for immunology. Upon specific microenvironmental stimuli and cues, macrophages have the potential to polarize into a continual spectrum of phenotypic pathway. Representing the two extreme ends, non-activated macrophages (M0) can polarize into classically activated (M1) or alternatively activated (M2) functional phenotypes[2]. Classically activated macrophages (M1) follow an IFN-γ–dependent Th1-type response in which soluble cytokines, interferon gamma (IFN-γ) and lipopolysaccharides (LPS), induce the production of interleukin-12 (IL-12) and interleukin-23 (IL-23) that downstream regulates Th-1 proinflammatory activation[3,4]. IFN-γ, specifically, is key to the secretion of

proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) which undergoes various signaling pathways to regulate cell apoptosis.

Inflammation is an important process to fight infection. However, excessive inflammatory activation is detrimental to the body, hence needing a counteractive mechanism to neutralize that. The alternative activated pathway (M2) is the anti-inflammatory mechanism which follows a Th-2 response to promote healing and tissue remodeling upon exposure to interleukin-4 (IL-4) and interleukin-13 (IL-13). The release of anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF- β) resulting from M2 polarization is crucial to the healing and regenerative processes[3]. IL-10 is responsible for preventing excessive tissue damages due to inflammation allowing the body times to heal[5]. In cooperation, TGF- β heals and regenerates via angiogenesis, immunoregulation, and apoptosis control[6].

CHAPTER 2
EFFECTS OF CULTURE MEDIA ON MACROPHAGES FUNCTION

2.1 Background

The overall purpose of the study is to eventually be able to co-culture cardiomyocytes and macrophages and investigate their responses. To do so, an initial experiment testing the effects of different culture media on these two cell types is pivotal as an optimal culture media for both primary cell types is required. Neonatal cardiomyocytes used for this study, are cultured in M199 media that have supplemental nutrients necessary to promote continuous growth of heart and fibroblast cells. Bone marrow derived macrophages (BMDMs), on the other hand, uses D10 media containing both supplemental ingredients for culturing and a potent hematopoietic cytokine known as macrophage colony- stimulating factor (M-CSF) for differentiating monocytes into macrophages. Current culturing of neonatal cardiomyocytes uses 30% fetal bovine serum (FBS) M199 media during initial seeding and 10% FBS M199 media during feeding 2-3 days after. In contrast, BMDMs uses the same D10 media for the entirety of culturing. These procedural differences in addition to the different media used can have significant effects on macrophages and cardiomyocytes if either cell types are cultured in the other's media.

Neonatal cardiomyocytes cultured in D10 media results in cells not growing and surviving which eliminates D10 as a potential co-culturing medium[9]. In contrast, BMDMs cultured in M199 media show increased inflammatory response compared to when cultured in D10[10]. This result suggests that co-culturing macrophages and cardiomyocytes in M199 media is more promising compared to D10. However, increased inflammatory activation of macrophages in M199 media prior to any treatments is not a positive sign hence motivating this paper to assess a third type of media that combines M199 with M-CSF. M199 with M-CSF media theoretically should satisfy the culturing needs of neonatal cardiomyocytes while the addition of M-CSF, a key component of D10 media necessary for differentiation of macrophages, can potentially rescue macrophages from increased inflammatory response as seen when they are cultured in pure M199. To test this theory, an experiment testing all 3 types of media (D10, M199, M199 + M-CSF) is performed. The secretion of proinflammatory cytokine, TNF-α, and anti-inflammatory cytokine, IL-10, are evaluated.

Table 1. Pro-inflammatory (M1) and pro-healing (M2) cytokine markers

Marker	Phenotype	Marker Type	Function	Pro-inflammatory	Anti-inflammatory
TNF- α	M1	cytokine	proinflammatory, apoptotic, cytotoxic	UP	DOWN
IL-10	M2	cytokine	Anti-inflammatory, inhibit host immune response to pathogens, mitigate tissue damage	DOWN	UP

2.2 Materials and Methods

Rat D10 Media Composition

Rat D10 media contains Gibco high-glucose DMEM (Fisher Scientific) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (ThermoFisher), 2 mM L-glutamine, and 20 ng/mL carrier-free recombinant rat M-CSF (Biolegend). High glucose DMEM are used as a basal medium for supporting mammalian cells growth and required supplementation of 10% Fetal Bovine Serum (FBS). L-glutamine is an amino acid supplement aiding in cell growth rate. Penicillin-Streptomycin helps prevent bacterial contamination in cell culture. The most essential component, rat M-CSF stimulates monocytes to differentiate into macrophages.

M199 Media Composition

M199 media contains Medium 199 (Gibco) supplemented with 10% heat-inactivated FBS, 10mM HEPES (Gibco), 1x MEM non-essential amino acids (Gibco), 20 mM glucose, 2 mM L-glutamine (Gibco), 1.5 μM vitamin B-12 (Sigma-Aldrich), and 100 U/mL penicillin (Sigma-Aldrich). M199 with M-CSF media contains the additional 20 ng/mL carrier-free recombinant rat M-CSF (Biolegend).

Macrophage Cell Isolation & Culture

Femur bones from adult Sprague Dawley female rats (Charles River) were harvested and flushed with rat D10 media for bone marrow extraction. Flushed bone marrow was treated with ACK Lysing Buffer (ThermoFisher) prior to centrifuge and resuspension for lysing of red blood cells leaving white blood cells intact. White blood cells were then seeded on standardized non-treated polystyrene petri dishes in rat D10 media. Feeding is done 3-4 days post harvesting to ensure adequate nutrients for cell growth. With the essence of macrophage colony stimulating factor (M-CSF), seeded white blood cells/monocytes were differentiated into bone marrow derived macrophages (BMDMs) ready for use 6-8 days after harvesting. As BMDMs are sticky cells, seeding and freezing down requires the usage of cell dissociation buffer (ThermoFisher) for lifting. Upon lifting, BMDMs were either seeded in experimental wells or freeze down to be stored in a liquid nitrogen tank at a density of 5 million cells/mL.

Culture Media Experimental Setup

BMDMs were seeded on a 24-wells experimental plate at 100,000 cells/0.5 mL. Rat D10, M199, and M199 with M-CSF media were used as the medium for seeding of BMDMs. For each media condition, BMDMs were stimulated for M1 and M2 phenotypes. To achieve M1 polarization, a combination of 10 ng/mL of E. coli-derived LPS (Sigma-Aldrich) and 10 ng/mL of E. coli-derived carrier-free recombinant rat IFN-γ (Biolegend) were used as stimulants 4 hours after seeding of BMDMs. In contrast, 20 ng/mL of 293E derived carrier-free recombinant rat IL-4 (Biolegend) and 20 ng/mL of E. coli derived carrier-free recombinant rat IL-13 (Biolegend) were used to stimulate BMDMs into one branch of M2 phenotype (M2). The addition of 10 ng/mL of E. coli-derived LPS to IL-4 and IL-13 cytokines creates another branch of M2 phenotype (M2*) used for downstream enzyme-linked immunosorbent assay (ELISA) quantification of IL-10 expressions. Stimulated

BMDMs for M1 and M2 phenotypes were left to incubate at 37°C, 5% CO_2 overnight (~18 hours) before supernatant extraction for ELISA.

Enzyme-linked Immunosorbent Assay Quantification of Cytokine Secretion

Collected supernatants of BMDMs treated in rat D10, M199, and M199 with M-CSF conditioned media were assessed by enzyme-linked immunosorbent assay kits (Biolegend) per manufacturer's protocol for TNF-α and IL-10 secretion levels. Unstimulated cells (M0) were used as controls. Optical density of secreted TNF-α and IL-10 concentrations were determined using a Benchmark microplate reader (Bio Rad) with standard curves for its respective cytokine as baseline reference for quantification.

Statistical Analysis

Data from at least three biological rat replicates were represented as mean ± SEM compared to controls. Statistical significance was determined by an unpaired t-test assuming equal variance using GraphPad Prism software. Data were shown as statistically significant if $p < 0.05$.

2.3 Results

BMDMs cultured in standardized macrophage D10 media, standardized cardiomyocyte M199 media, and M199 with M-CSF media were stimulated for M1, M2, and M2* polarization. Cytokine secretions for proinflammatory marker TNF- α, and anti-inflammatory marker IL-10 were assessed via enzyme-linked immunoassay (ELISA).

Five biological replicates were used for this media experiments and the average TNF- α production for M1 polarization was 76447.22 pg/mL for D10, 63196.43 pg/mL for M199, and 61618.67 pg/mL for M199 with M-CSF. As TNF- α secretion responds to inflammatory cytokines such as LPS and IFN-γ, the stimulated M2* conditions containing 10 ng/mL of LPS also show notable production level of TNF- α. The average TNF- α production for M2* condition was 76841.03 pg/mL for D10, 34733.9 pg/mL for M199, and 66977.74 pg/mL for M199 with M-CSF [Figure 1a]. Each biological replicate data sets were normalized to its respective D10 M1 control. When comparing these normalized secretion levels of pro-inflammatory TNF- α marker, D10 and M199 with M-CSF M1 stimulated media follows a similar trend with values of 1 and 0.96, respectively. In contrast, M199 M1 stimulated media has relative TNF- α secretion value of 1.908. Although there are no statistical differences between D10 and M199 with M-CSF compared to M199 media, the normalized standard deviation for M1 conditions were 0, 1.65, and 0.23 for D10, M199, and M199 with M-CSF, respectively. The same trend holds for D10, and M199 with M-CSF M2* stimulated media given the normalized TNF- α production level of 1.11 and 1.07, respectively. M199 M2* stimulated media has normalized TNF- α production value of 0.89. Again, no statistical differences were seen between D10, and M199 with M-CSF compared to M199 M2* stimulated media. However, the normalized standard deviation between D10 and M199 with M-CSF were closer to each other given the values of 0.31 and 0.56, respectively. In contrast, M199 normalized standard deviation was 0.71, which is much greater than the control D10 M2* condition [Figure 1b]. This showed that macrophages stimulated toward pro-inflammatory activation behaved similarly when cultured in the standard D10 and M199 with M-CSF media.

Anti-inflammatory cytokine secretion level of IL-10 in response to IL-4 and IL-13 stimulation were quantified. Looking specifically at the IL-10 production level of M2* conditions for D10, M199, and M199 with M-CSF, their absolute average values were 2651.3 pg/mL, 2003.65 pg/mL, and 3039.07 pg/mL, respectively [Figure 1c]. Normalization of these absolute values with respect to D10 M2* control within each biological replicates were performed, and the relative IL-10 production levels were 1 for D10, 2.06 for M199, and 1.47 for M199 with M-CSF. D10 and M199 with M-CSF M2* stimulated media showed a similar trend of IL-10 production level. There are no statistical differences of relative IL-10 production between all three media conditions. However, normalized standard deviation for D10 and M199 with M-CSF M2* stimulated conditions with values of 0 and 0.31, respectively, are closer to each other if compared to M199 normalized standard deviation of 1.83 [Figure 1d]. This indicated that macrophages stimulated toward anti-inflammatory activation behaved similarly when cultured in the standard D10 and M199 with M-CSF media.

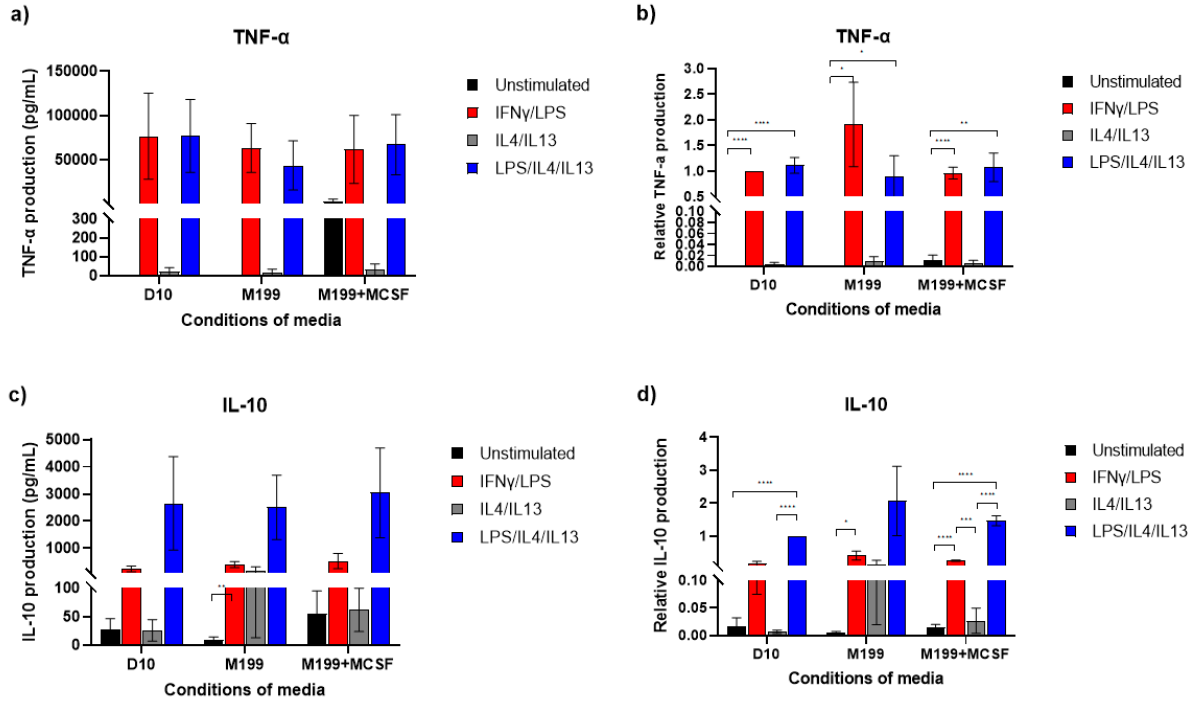

Figure 1. TNF-α and IL-10 cytokine secretion of BMDMs cultured in D10, M199, and M199 with M-CSF culture media. *a) TNF-α production by stimulated BMDMs in D10, M199, and M199 with M-CSF. b) Normalized TNF-α production by stimulated BMDMs in D10, M199, and M199 with M-CSF with respect to D10 M1 control. c) IL-10 production by stimulated BMDMs in D10, M199, and M199 with M-CSF. d) Normalized IL-10 production by stimulated BMDMs in D10, M199, and M199 with M-CSF with respect to D10 M2* control. Each conditioned media was either left unstimulated (first black bar), M1 stimulated with IFN-γ/LPS (second red bar), M2 stimulated with IL-4/IL-13 (third grey bar), or M2* stimulated with LPS/IL-4/IL-13 (fourth blue bar). Data represented 5 biological replicates with error bars denoted Mean ± SEM and *p<0.05 showing statistical significance determined by unpaired t-test.*

2.4 Discussion and Future Directions

The goal of this experiment was to test for the optimal cultured media that can be used for co-culturing of macrophages and cardiomyocytes. Standard D10 media used for culturing of macrophages was previously found to inhibit healthy growth and proliferation of cardiomyocytes. In contrast, standard M199 media used for culturing of cardiomyocytes enhances inflammatory activation of macrophages during LPS/IFN-γ stimulation. Both scenarios are non-optimal for co-culturing of macrophages and cardiomyocytes. Hence, a third type of medium combining M199 which contains essential supplements for cardiomyocytes growth and M-CSF, a stimulating factor for BMDMs maturation, are tested for macrophages response upon stimulation. Compared to M199 media, M199 with M-CSF influence macrophages pro-inflammatory and anti-inflammatory responses similarly to that of the standard D10 media. This showed that M-CSF alone is an essential component for culturing of macrophages. A possible explanation as to why M-CSF in M199 media can potentially rescue macrophages behaviors upon stimulation is its ability to maintain macrophages phenotypic homogeneity. Studies have shown M-CSF potential to influence macrophages to shift into a more M2-like phenotype. This coincide with the fact that macrophages are modulated towards a homogeneity M2 subpopulation[16]. Unlike cultured in pure M199, macrophages can experience phenotypic instability that allow for heterogeneity within the BMDMs population. The heterogeneity can influence BMDMs behavior to have fluctuating pro-inflammatory and anti-inflammatory responses when stimulated. This can also explain why there are larger error bars for TNF-α and IL-10 cytokine secretion levels in BMDMs populations cultured in M199 media. Therefore, the potential of M199 with M-CSF media to maintain macrophages behaviors upon stimulation makes it a promising co-culturing medium of macrophages and cardiomyocytes for future studies about their interactions.

An initial testing of neonatal rat ventricular myocytes (NRVMs) cultured in M199 with M-CSF was performed and the data showed that NRVMs can grow and proliferate similarly as when cultured in its standard M199 media. This is promising, but then again, a conclusive experiment looking at the effect of cardiomyocytes in this medium is required moving forward. Additionally, if M-CSF seems to play a significant role in maintaining BMDMs behaviors, it might be interesting to investigate the varying concentrations of M-CSF in M199 culturing media. Would concentration be a significant factor, or the presence of M-CSF alone is sufficient to maintain standard macrophages behaviors upon stimulation? Furthermore, only TNF-α and IL-10 cytokines secretion in the supernatants were studied. To better draw a conclusive understanding, more pro-inflammatory and anti-inflammatory cytokines and gene markers should be looked at. Studying the effect of different secretion factors being influenced by different cultured media in addition to the expression of gene markers will give insights of the intercellular mechanism that taken place and expand the knowledge of the microenvironmental activities.

CHAPTER 3
EFFECT OF HYPOXIA ON MACROPHAGES FUNCTION

3.1 Background

Hypoxia plays a significant role in modulating macrophages function[11-14]. In the case of silent hypoxia being an important contributing factor of increased mortality rate in COVID-19 patients, studying its effect on macrophages behavior upon pro-inflammatory and anti-inflammatory stimulations can provide insightful knowledge of the intercellular mechanisms that explain why silent hypoxia is so detrimental. Silent hypoxia associated with COVID-19 not only causes an overexpression of ACE-2 receptors which is a major medium of COVID-19 infection, it is responsible for the systemic inflammation via the cytokine storm of inflammatory mediators such as macrophages. The activation of nuclear factor κB (NF-κB) transcription factors responsible for inflammatory and immune responses enhances upon experiencing this phenomenon. This results in serious endothelial damages. Furthermore, silent hypoxia can induce a different immune-metabolic response leading to secondary organ damage like in the case of myocardial ischemia[8,15]. Myocardial ischemia refers to the lack of oxygen received by the heart due to decrease in blood flow usually caused by blockage of plaques in the coronary artery (atherosclerosis). The destabilizing of coronary artery plaques in atherosclerosis results in a heart attack that can be fatal and life threatening[15].

NF-κB are not the only transcription factors that are master regulators of cellular responses to hypoxia. Studies shown that hypoxia-inducible factors (HIF) also function as the cell's oxygen sensing mechanism[11]. HIF are heterodimers that ubiquitinated and degraded in normoxic and healthy conditions. However, upon hypoxia, bacterial infection, cytokines, and inflammatory mediators and stress, HIF translocated into the nucleus and dimerized to induce genes transcriptions responsible for cellular adaptation. Although HIF induces a great adaptive response, over-expression of these transcriptional factors can lead to tumor invasion and metastasis due to activation of pro-angiogenic factors such as vascular endothelial growth factors (VEGFs). Interestingly, macrophages experiencing hypoxia have increased VEGFs expression resulting in the modulation of macrophages into a more M2 or anti-inflammatory phenotype. In correlation, macrophages undergoing hypoxia during stimulation, showed decrease in pro-inflammatory cytokines production, suggesting that hypoxia is a significant regulator of the inflammatory microenvironment and thus should be looked at more closely[13].

The goal of this section is to try to understand the sequence of events or correlation between hypoxia and immune response of macrophages prior to co-culturing with cardiomyocytes. It is of interest to investigate whether hypoxia treatment prior, during, or post inflammatory or healing activation plays a significant role in TNF-α and IL-10 secretion. Additionally, whether the severity of hypoxia for various durations produce interesting results that can be tied back to the intercellular mechanisms at play. Physiologically speaking, atmospheric oxygen concentration is normally 21%. Within that 21%, tissues have oxygen concentration spanning between 2.5-9%. Wounds and necrotic tissues associated with severe hypoxia have oxygen concentration lower than 1%[11]. Hence, to assess the quality of macrophages immune response due to these physiological oxygen

availability ranges, BMDMs were placed in the hypoxia chamber either at 1% pO_2 (severe hypoxia) or 3% pO_2 (moderate hypoxia) for 1, 6, or 24 hours. The order in which the BMDMs were placed in the hypoxia chamber upon cytokines stimulation were also being evaluated.

3.2 Materials and Methods

Macrophage Cell Isolation & Culture

Femur bones from adult Sprague Dawley female rats (Charles River) were harvested and flushed with rat D10 media for bone marrows extraction. Flushed bone marrows were treated with ACK Lysing Buffer (ThermoFisher) prior to centrifuge and resuspension for lysing of red blood cells leaving white blood cells intact. White blood cells were then seeded on standardized non-treated polystyrene petri dishes in rat D10 media. Feeding is done 3-4 days post harvesting to ensure adequate nutrients for cell growth. With the essence of macrophage colony stimulating factor (M-CSF), seeded white blood cells/monocytes were differentiated into bone marrow derived macrophages (BMDMs) ready for use 6-8 days after harvesting. As BMDMs are sticky cells, seeding and freezing down requires the usage of cell dissociation buffer (ThermoFisher) for lifting. Upon lifting, BMDMs were either seeded in experimental wells or freeze down to be stored in a liquid nitrogen tank at a density of 5 million cells/mL.

Hypoxia Chamber

The BIO-V hypoxia chamber (Noxygen) acts as a controlled environment that allow for easy manipulation of oxygen concentration and temperature. The chamber is set to 37°C prior to placement of the experimental plate to mimic the cell's optimal temperature for survival and growth. The gas controller attached to the gas cylinders is used to control different amounts of N_2, O_2, and CO_2 going into the hypoxia chamber. The "VoeReady.exe" program is set up via inputs from a written MATLAB code that automatically calculate the amount of time (1, 6, and 24 hours) and percentage of specific gas (1% and 3% O_2) based on a total flow rate of 50 mL/min. Depending on the desired oxygen concentration, the time require to equilibrate the concentration of gases in the chamber varies. Once equilibrated, cells in the chamber experienced a hypoxic environment for the allotted amount of time.

Hypoxia Treatment Experimental Setup #1

BMDMs were seeded on a 12-wells experimental plate at 300,000 cells/mL. M199 with M-CSF media was used as the medium for seeding of BMDMs. BMDMs were stimulated for M1 and M2 phenotypes for 18 hours. To achieve M1 polarization, a combination of 10 ng/mL of E. coli-derived LPS (Sigma-Aldrich) and 10 ng/mL of E. coli-derived carrier-free recombinant rat IFN-γ (Biolegend) were used as stimulants 4 hours after seeding of BMDMs. In contrast, 20 ng/mL of 293E derived carrier-free recombinant rat IL-4 (Biolegend) and 20 ng/mL of E. coli derived carrier-free recombinant rat IL-13 (Biolegend) were used to stimulate BMDMs into one branch of M2 phenotype. The addition of 10 ng/mL of E. coli-derived LPS to IL-4 and IL-13 cytokines create another branch of M2 phenotype used for downstream enzyme-linked immunosorbent assay (ELISA) quantification of IL-10 expressions. Stimulated BMDMs for the control/normoxia conditions were left to incubate at 37°C, 5% CO_2 overnight (~18 hours) while hypoxia, stimulated

conditions were place in the hypoxia chamber (1% and 3% O_2) post stimulation for the allotted duration (1 and 6 hours). Supernatants were collected for ELISA.

Hypoxia Treatment Experimental Setup #2

BMDMs were seeded on a 24-wells experimental plate at 150,000 cells/0.5 mL. M199 with M-CSF media was used as the medium for seeding of BMDMs for 24 hours.

The first set of experiments look at the effect of hypoxia and cytokines co-stimulation. BMDMs for the control/normoxia and hypoxia conditions were stimulated with 10 ng/mL of E. coli-derived LPS (Sigma-Aldrich) and 10 ng/mL of E. coli-derived carrier-free recombinant rat IFN-γ (Biolegend) 24 hours after seeding to induce M1 polarization. BMDMs for the control/normoxia and hypoxia conditions were also stimulated with 20 ng/mL of 293E derived carrier-free recombinant rat IL-4 (Biolegend) and 20 ng/mL of E. coli derived carrier-free recombinant rat IL-13 (Biolegend) to induce a branch of M2 polarization. The addition of 10 ng/mL of E. coli-derived LPS (Sigma-Aldrich) with 20 ng/mL of 293E derived carrier-free recombinant rat IL-4 (Biolegend) and 20 ng/mL of E. coli derived carrier-free recombinant rat IL-13 (Biolegend) induce another branch of M2 polarization used for downstream enzyme-linked immunosorbent assay (ELISA) quantification of IL-10 expressions. Immediately after stimulation, the control/normoxia conditions are placed in the regular incubator at 37℃, 5% CO_2 overnight for 24 hours. The hypoxia conditions are placed in the hypoxia chamber at 1% O_2 for 6 hours then placed back in the regular incubator for another 18 hours before collection of supernatants.

The second set of experiments looks at the effect of hypoxia prior to stimulation. The purpose is to investigate whether hypoxia alone can induce unpolarized macrophages into a polarized state and to what extent. Additionally, how does stimulation post hypoxia effect cytokine secretion level as cells are already in a stress state? BMDMs for the control/pure hypoxia and hypoxia/stimulation conditions were placed in the hypoxia chamber for 24 hours at 1% O_2. Post hypoxia treatment, the hypoxia/stimulation conditions were stimulated with 10 ng/mL of E. coli-derived LPS (Sigma-Aldrich) and 10 ng/mL of E. coli-derived carrier-free recombinant rat IFN-γ (Biolegend) for M1, 20 ng/mL of 293E derived carrier-free recombinant rat IL-4 (Biolegend) and 20 ng/mL of E. coli derived carrier-free recombinant rat IL-13 (Biolegend) for M2, and an additional 10 ng/mL of E. coli-derived LPS (Sigma-Aldrich) to M2 to induce M2* (another M2 phenotype that allows for detection of IL-10 using ELISA). The control/hypoxia conditions were left unstimulated. Both control/hypoxia and hypoxia/stimulation conditions are then placed in the incubator at 37℃, 5% CO_2 for 24 hours before collection of supernatants.

Enzyme-linked Immunosorbent Assay Quantification of Cytokine Secretion

Supernatants of BMDMs cultured in M199 with M-CSF media treated with hypoxia were assessed by enzyme-linked immunosorbent assay kits (Biolegend) per manufacturer's protocol for TNF-α and IL-10 secretion levels. Unstimulated cells (M0) were used as controls. Optical density of secreted TNF-α and IL-10 concentrations were determined using a microplate reader with standard curves for its respective protein as baseline reference for quantification.

Statistical Analysis

Data from two/three biological rat replicates are represented as mean ± SEM compared to controls. Statistical significance was determined by an unpaired t-test assuming equal variance using GraphPad Prism software. Data were shown as statistically significant if $p < 0.05$.

3.3 Results

Macrophages were stimulated and treated with hypoxia at different time periods to test the pro-inflammatory and anti-inflammatory cytokine secretions via ELISA.

Effect of Hypoxia Post Cytokines Stimulation on Macrophages

For the first experimental setup, BMDMs seeded in M199 with M-CSF were stimulated overnight in normoxic conditions and then placed in 1% or 3% pO_2 for 1 or 6 hours. The purpose was to see the role of hypoxia in correlation to cytokine stimulations for induction of macrophages polarization. It is in question of whether hypoxia enhances, inhibits, or has no effect on the performance of macrophages polarization after overnight stimulation. The results show that neither 1% or 3% pO_2 for 1 and 6 hours affect TNF-α production when compared to control [Figure 2]. There were no significant differences in both 1% and 3% M1 conditions induced by LPS and IFN-γ. TNF-α production of BMDMs experiencing 1% pO_2 at control, 1-hour, and 6 hours were within the ranges of 2000-3000 pg/mL for M1 polarization. Similarly, at 3% pO_2, TNF-α production level ranges within 4200-5200 pg/mL. In comparison, the production of anti-inflammatory marker, IL-10, show differences at 6 hours in 1% hypoxia compared to the control and 1 hour for M2* conditions (LPS, IL-4, and IL-13 stimulated). Although, the difference was not statistically significant, IL-10 production of BMDMs experiencing 1% pO_2 at 6 hours has average value of 622.07 pg/mL compared to the control and 1-hour conditions having values of 268.09 and 191.61 pg/mL, respectively. On the other hand, 3% p O_2 shows no effect on IL-10 production for 1 and 6 hours compared to control [Figure 3].

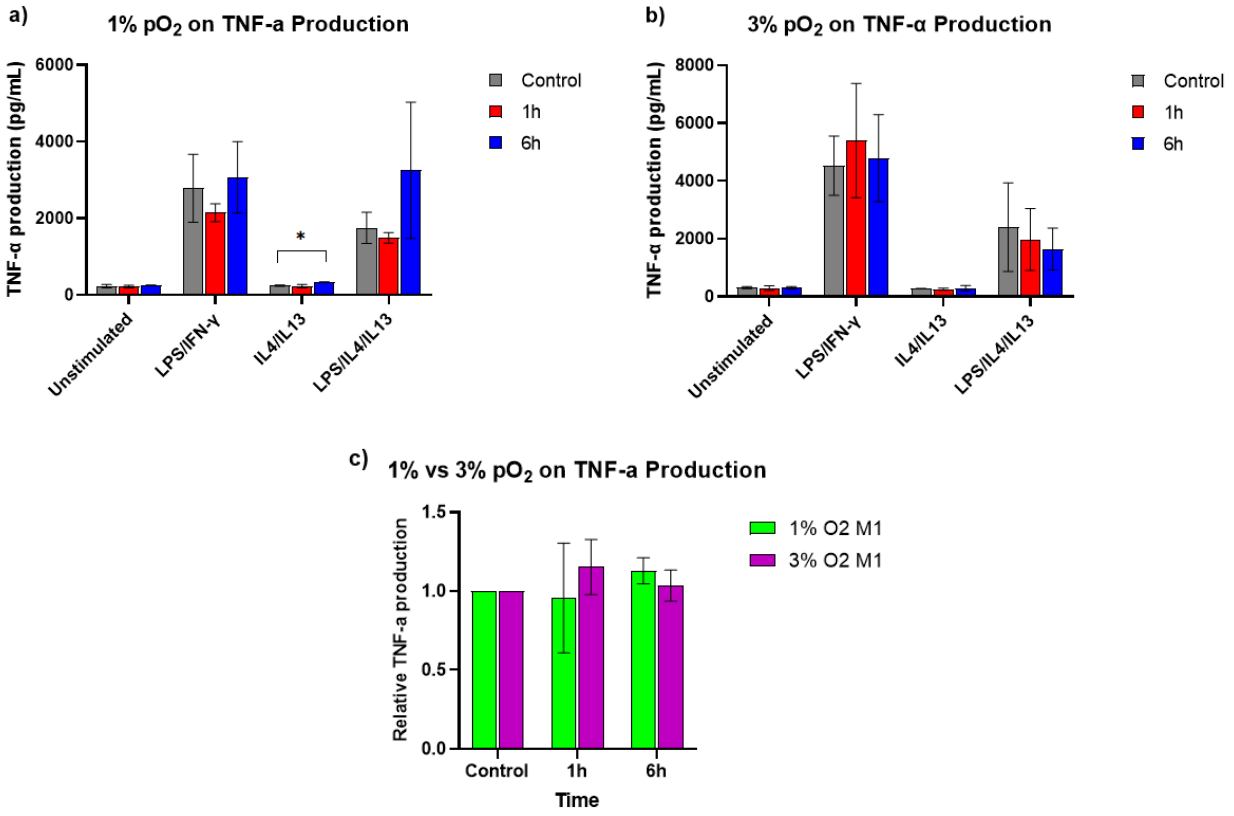

Figure 2. Effect of 1% and 3% pO_2 and cytokine post-stimulation on TNF-α production at 1h and 6h time point. *a) TNF-α production by stimulated BMDMs experiencing 1% pO_2 for 1 or 6 hours compared to control. b) TNF-α production by stimulated BMDMs experiencing 3% pO_2 for 1 or 6 hours compared to control. c) Normalized TNF-α production by LPS/IFN-γ stimulated BMDMs experiencing 1% and 3% pO_2 for 1 or 6 hours compared to control. Data represented 3 biological replicates with error bars denoted Mean ± SEM and *p<0.05 showing statistical significance determined by unpaired t-test.*

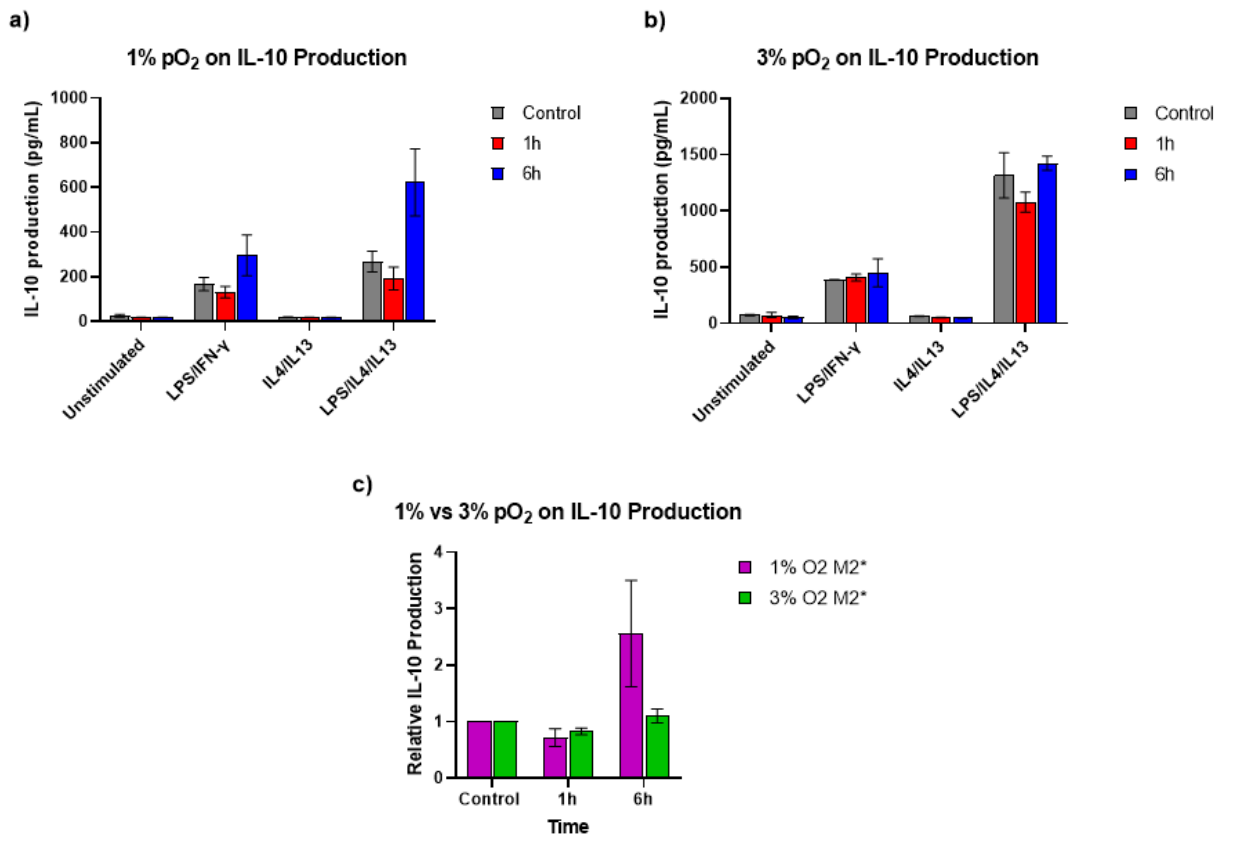

Figure 3. Effect of 1% and 3% pO_2 and cytokine post-stimulation on IL-10 production at 1h and 6h time point. *a) IL-10 production by stimulated BMDMs experiencing 1% pO_2 for 1 or 6 hours compared to control. b) IL-10 production by stimulated BMDMs experiencing 3% pO_2 for 1 or 6 hours compared to control. c) Normalized IL-10 production by LPS/IL4/IL13 stimulated BMDMs experiencing 1% and 3% pO_2 for 1 or 6 hours compared to control. Data represented 3 biological replicates with error bars denoted Mean ± SEM and *p<0.05 showing statistical significance determined by unpaired t-test.*

Effect of Hypoxia and Cytokines Co-stimulation on Macrophages

For the second experimental setup, BMDMs seeded in M199 with M-CSF media were stimulated and immediately placed in the hypoxia chamber at 1% pO_2 for 6 hours. Post 6 hours, BMDMs were placed back in normoxia for another 18 hours of stimulation before collection of supernatants for ELISA analysis. The results showed that LPS/IFN-γ cytokines and 6 hours 1% pO_2 co-stimulation had increased production of TNF-α compared to normoxia control. Although, the difference was not statistically significant, 1% pO_2 for 6 hours conditions had doubled the amount of TNF-α (20336.65 pg/mL) in comparison to normoxia conditions (10035.44 pg/mL). In contrast, LPS/IL-4/IL-13 cytokines and 6 hours 1% pO_2 co-stimulation has little to no effect on IL-10 production. BMDMs in the M2* conditions secreted an average of 676.88 pg/mL of IL-10 in the normoxia control conditions. Whereas in the 1% pO2 conditions, the average IL-10 production value was 478.92 pg/mL [Figure 4].

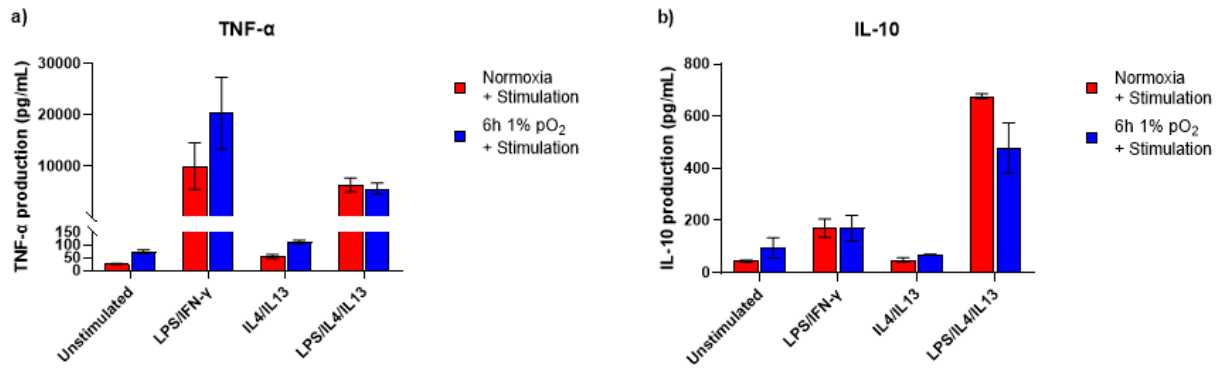

Figure 4: Effect of 1% pO_2 and cytokine co-stimulation on TNF-α and IL-10 production. *a) TNF-α production by co-stimulated BMDMs experiencing 1% pO_2 for 6 hours compared to normoxia control. b) IL-10 production by co-stimulated BMDMs experiencing 1% pO_2 for 6 hours compared to normoxia control. Data represented 2 biological replicates with error bars denoted Mean ± SEM and *p<0.05 showing statistical significance determined by unpaired t-test.*

Effect of Hypoxia Prior to Cytokines Stimulation on Macrophages

For the third experimental setup, BMDMs seeded in M199 with M-CSF media were placed in 1% pO_2 hypoxia chamber for 24 hours prior to cytokines stimulation for another 24 hours. The comparison normoxia controls were taken from the second experimental setup as both experiments were done at the same time using the same cells. The only difference is the duration of which the normoxia controls are in the incubator prior to stimulation for 24 hours less than the third experimental setup. The TNF-α and IL-10 productions for the 24 hours 1% pO_2 and normoxia conditions were plotted and compared for analysis. The results indicated that BMDMs experimenting severe hypoxia conditions (1% pO_2) for 24 hours with additional stimulation afterwards have suppressed pro-inflammatory responses with enhanced anti-inflammatory responses. These conclusions were taken from the production of TNF-α and IL-10 markers for pro-inflammatory and anti-inflammatory activation, respectively. TNF-α production in the normoxia controls have average value of 10035.44 pg/mL compared to the hypoxia conditions with value of 484.33 pg/mL [Figure 5a]. This drastic decrease in pro-inflammatory marker in the LPS/IFN-γ stimulated conditions signify the suppression of inflammation due to severe hypoxia for 24 hours. IL-10 production, on the other hand, has average values of 676.88 pg/mL and 2957.491 pg/mL for the normoxia controls and hypoxia conditions, respectively. The 4-to-5-fold increase of IL-10 production in the severe hypoxic conditions suggests that hypoxia 24 hours prior to LPS/IL-4/IL-13 stimulation enhances healing activation in BMDMs [Figure 5b]. Additionally, it is notable that the level of IL-10 productions tripled the amount of TNF-α [Figure 5c]. This suggests that pre-conditioned macrophages in hypoxia enhance M2 polarization upon stimulation.

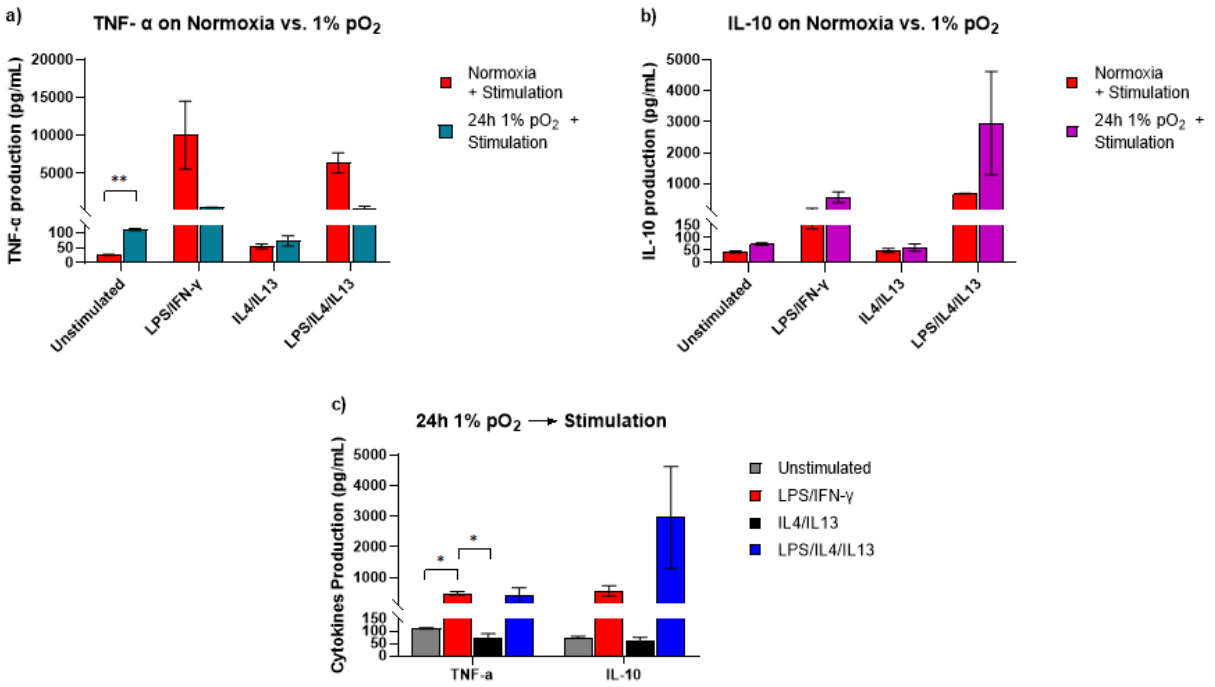

Figure 5. Effect of 1% hypoxia pre-treatment and cytokine stimulation on TNF-α and IL-10 production. *a) TNF-α production by BMDMs experiencing 1% pO_2 for 24 hours prior to stimulation compared to normoxia control. b) IL-10 production by BMDMs experiencing 1% pO_2 for 24 hours prior to stimulation compared to normoxia control. c) TNF-α and IL-10 production by BMDMs experiencing 1% pO_2 for 24 hours prior to stimulation with LPS/IFN-γ for M1 pro-inflammatory phenotype and LPS/IL-4/IL-13 for M2* pro-healing phenotype. Data represented 2 biological replicates with error bars denoted Mean ± SEM and *p<0.05 showing statistical significance determined by unpaired t-test.*

3.4 Discussion and Future Directions

The goal of this section was to investigate the effect of hypoxia on BMDMs upon stimulation. The severity of hypoxic conditions in addition to the duration and time point BMDMs are experiencing hypoxia were evaluated to mimic physiological scenarios and along the way, investigate the most optimal setup for future hypoxia experiments. From the first experimental setup, BMDMs were placed in the hypoxia chamber after overnight stimulation. This resulted in no change in TNF- α production across the tested conditions. An explanation for this phenomenon is that BMDMs producing inflammatory marker, TNF- α, are activated during 4-6 hours of stimulation. Hence, BMDMs would have already exhausted their inflammatory responses upon overnight stimulation. As a result, inducing hypoxia at different concentrations and durations have little to no effects on these cells. In contrast, anti-inflammatory marker, IL-10, are activated at the later stages or 18-24 hours of stimulation. Therefore, increase in IL-10 production at 1% pO_2 for 6 hours post stimulation can be seen compared to the control. This showed that severe cases of hypoxia post stimulation at a longer duration can still influence M2 polarization.

Physiologically speaking, cells are usually influenced by a hypoxic and cytokines mediated microenvironment simultaneously. Thus, the purpose of the second experimental setup was to mimic the hypoxic and cytokines co-stimulation environment in the early stages of inflammation

(6 hours mark). This ensured that inflammatory responses measured by TNF-α correlated the effect of both hypoxia and cytokine co-stimulation and not cytokine stimulation alone like as seen in the first experimental setup. Results showed that LPS/IFN-γ cytokine and 6 hours 1% hypoxia co-stimulation increases TNF-α production compared to normoxia. Inflammatory response due to cytokines stimulation occur within 4-6 hours; hence the addition of hypoxia for 6 hours enhances TNF-a production as cells are in a more stress state. 6 hours after co-stimulation, cells are placed back to normoxia for the rest of the stimulation period. As anti-inflammatory response happens at the later stimulation time point, it is possible that cells are coming back to normal upon the start of anti-inflammatory response. Hence, explaining why data showed little to no changes in IL-10 production after co-stimulation. The notion of placing cells back in normoxia after experiencing hypoxia mimic a possible physiological response of the body to maintain homeostasis after medical intervention. For example, in the case of ischemia due to atherosclerosis in the heart, removal of the plaques allows for the heart to experience normoxia again thus these results can reflect macrophages behavior in such physiological scenarios.

The purpose of the third experimental setup was to assess whether hypoxia alone influences macrophages polarization prior to stimulation. Results indicated that severe hypoxia (1% pO_2) 24 hours before stimulation suppresses pro-inflammatory responses and enhances anti-inflammatory responses. This correlated with what has been found in literatures showing the influence of 24 hours hypoxia toward shifting macrophages into M2 or anti-inflammatory phenotype. To further understand the mechanism why hypoxia enhanced M2 polarization, future works looking at the role of HIF-1α are necessary. Looking at VEGFs production and gene expression can also provide insights to the correlation between hypoxia, M2 polarization, and undesired over-expression of angiogenesis factors. Furthermore, for a more conclusive understanding of the effect of hypoxia on macrophages, it is recommended that a 24 hours 1% hypoxia with cytokine co-stimulation experiment is perform.

CHAPTER 4
EFFECTS OF NEONATAL RAT VENTRICULAR MYOCYTES SUPERNATANTS TREATED WITH HYPOXIA ON MACROPHAGES

4.1 Background

Before the start of co-culturing of macrophages and cardiomyocytes, evaluating the effect of neonatal rat ventricular myocytes (NRVMs) hypoxia-treated supernatants on macrophages can be beneficial to explaining the interaction of macrophages and cardiomyocytes. Literatures showed that excessive TNF-α expression through hypoxia negatively affect cardiomyocytes in acute myocardial infarction[17]. Therefore, investigating the amount of secreted TNF- α in NRVMs media treated with hypoxia in correlation to macrophages' behavior and their secretion level can provide insights to macrophages and cardiomyocytes microenvironmental interactions.

NRVMs in M199 media were treated in 1%, 3% and 6% pO_2 for 0, 0.5, 1, 3, 6, or 24 hours. Post treatment, the supernatants were collected and used as a culturing medium of macrophages. As macrophages were stimulated the same way for all conditions, the factors in questions are the compositions of the NRVMs media that can influence the behavior of macrophages.

4.2 Materials and Methods

Cardiomyocyte Cell Isolation & Culture

Neonatal rat ventricular myocytes (NRVMs) were harvested from ventricles of Sprague Dawley neonatal rats. The ventricular tissues were washed with Hanks' balanced salt solution buffer (HBSS) and treated overnight with 1 mg/mL trypsin solution. M199 culture medium was used to neutralized the trypsin solution followed by 1 mg/mL collagenase type II wash to dissolve HBSS. Cardiomyocytes were then isolated and seeded at 1 million cells/well in a 12-well tissue culture plate. After seeding, cells were incubated in M199 30% FBS media at 37°C with 5% CO2 for 24 hours. Then wells were washed and replaced with fresh M199 10% FBS media for removal of dead cells. After 48 hours, fresh M199 10% FBS media was used for feeding to ensure cardiomyocyte health and limited fibroblast proliferation.

Macrophage Cell Isolation & Culture

Femur bones from adult Sprague Dawley female rats (Charles River) were harvested and flushed with rat D10 media for bone marrows extraction. Flushed bone marrows were treated with ACK Lysing Buffer (ThermoFisher) prior to centrifuge and resuspension for lysing of red blood cells leaving white blood cells intact. White blood cells were then seeded on standardized non-treated polystyrene petri dishes in rat D10 media. Feeding is done 3-4 days post harvesting to ensure adequate nutrients for cell growth. With the essence of macrophage colony stimulating factor (M-CSF), seeded white blood cells/monocytes were differentiated into bone marrow derived macrophages (BMDMs) ready for use 6-8 days after harvesting. As BMDMs are sticky cells, seeding and freezing down requires the usage of cell dissociation buffer (ThermoFisher) for lifting.

Upon lifting, BMDMs were either seeded in experimental wells or freeze down to be stored in a liquid nitrogen tank at a density of 5 million cells/mL.

Enzyme-linked Immunosorbent Assay Quantification of Cytokine Secretion

Supernatants of BMDMs cultured in different NRVM hypoxia treated conditioned media were assessed by enzyme-linked immunosorbent assay kits (Biolegend) per manufacturer's protocol for TNF-α and IL-10 secretion levels. Unstimulated cells (M0) were used as controls. Optical density of secreted TNF-α and IL-10 concentrations were determined using a microplate reader with standard curves for its respective protein as baseline reference for quantification.

Statistical Analysis

Data from one/two biological rat replicates are represented compared to controls. Statistical significance was determined by an unpaired t-test assuming equal variance using GraphPad Prism software if allowed. Data were shown as statistically significant if $p < 0.05$.

4.3 Results

BMDMs were originally seeded in D10 media before changing to its respective NRVMs conditioned media. Each condition experienced overnight stimulation to achieve pro-inflammatory or anti-inflammatory phenotype. Supernatants were collected for ELISA analysis post stimulation. Results showed a plateau of TNF-α production at 1% pO_2 during 6-24 hours' time point. Notably, for 3%, and 6%, there was an increasing trend of TNF- α production from control to 6 hours which then drop at 24 hours. The drop at the 24 hours mark appeared to be more subtle at 3% pO_2 compared to 6% pO_2 [Figure 6]. In contrast, the effect of NRVMs conditioned media on IL-10 production were non-conclusive [Figure 7]. There seemed to be no patterns which might suggest that hypoxia treatment of media prior to culturing BMDMs has little to no effect on M2 polarization.

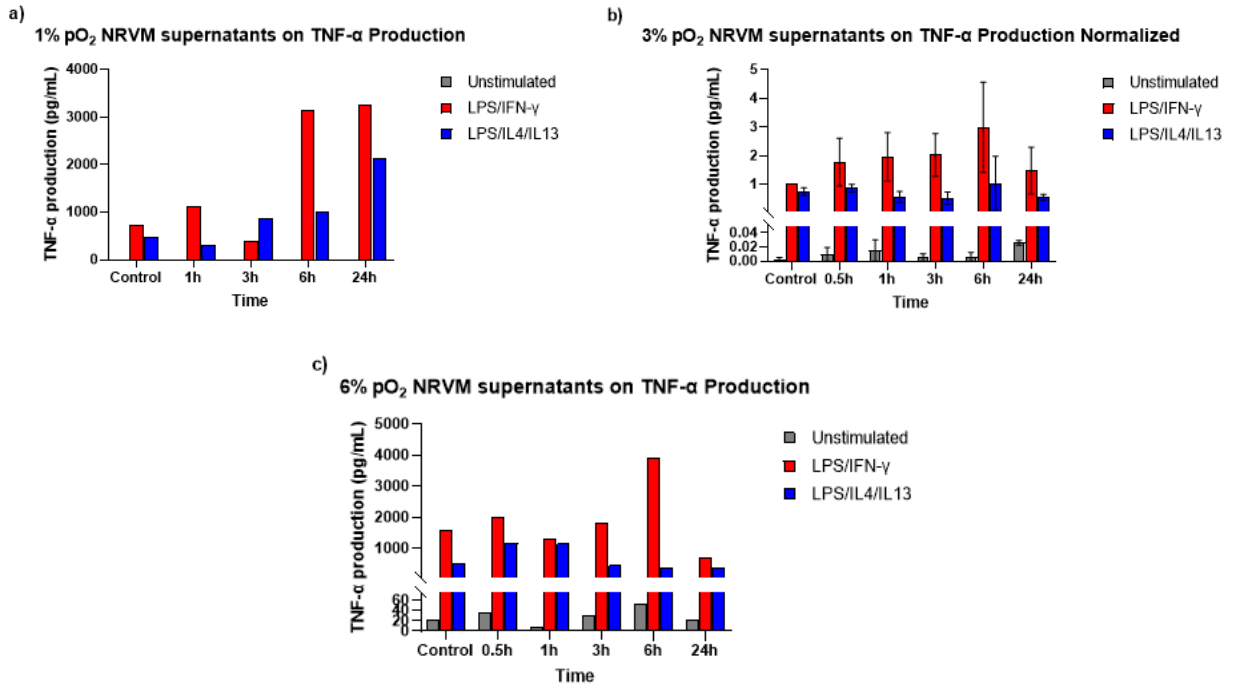

Figure 6. Effects of media conditioned by NRVMs under hypoxia on TNF-α production of BMDMs. *a) TNF-α production by stimulated BMDMs cultured in NRVMs media conditioned at 1% pO_2 for 1, 3, 6, 24 hours compared to control. b) Normalized TNF-α production by stimulated BMDMs cultured in NRVMs media conditioned at 3% pO_2 for 0.5, 1, 3, 6, 24 hours compared to control. c) TNF-α production by stimulated BMDMs cultured in NRVMs media conditioned at 6% pO_2 for 0.5, 1, 3, 6, 24 hours compared to control. Data represented 1 or 2 biological replicates with error bars denoted Mean ± SEM and *p<0.05 showing statistical significance determined by unpaired t-test (statistical testing valid for 3% conditions since experiment was done on 2 biological replicates).*

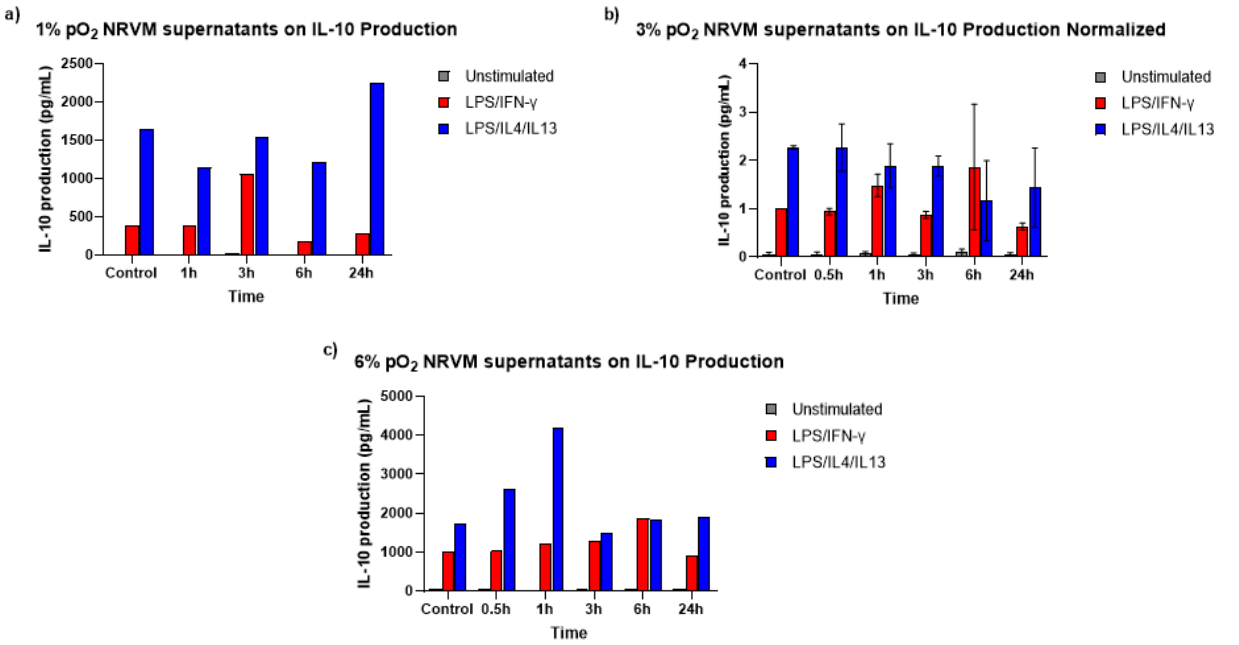

Figure 7. Effects of media conditioned by NRVMs under hypoxia on IL-10 production of BMDMs. *a) IL-10 production by stimulated BMDMs cultured in NRVMs media conditioned at 1% pO_2 for 1, 3, 6, 24 hours compared to*

*control. b) Normalized IL-10 production by stimulated BMDMs cultured in NRVMs media conditioned at 3% pO_2 for 0.5, 1, 3, 6, 24 hours compared to control. c) IL-10 production by stimulated BMDMs cultured in NRVMs media conditioned at 6% pO_2 for 0.5, 1, 3, 6, 24 hours compared to control. Control refers to NRVMs not experiencing any hypoxia. Data represented 1 or 2 biological replicates with error bars denoted Mean ± SEM and *p<0.05 showing statistical significance determined by unpaired t-test (statistical testing valid for 3% conditions since experiment was done on 2 biological replicates).*

4.4 Discussion and Future Directions

The goal of this experiment was to see the effect of NRVMs conditioned media on macrophages. What was expected from NRVMs experiencing hypoxic conditions at different severity and durations was cell deaths. However, a cell variability test for NRVMs after hypoxia treatment is required for validation. Based on the TNF-α results, the fluctuating increase between 0 to 6 hours suggested that there was "something" in the NRVMs media at varying increased concentration. That "something" could be cell debris. Remaining cell debris that has not been filtered out will activate macrophages inflammatory response. Thus, the longer NRVMs are in hypoxia, there will be increasing cell deaths which explains the fluctuating increase of TNF-α. However, the drop at 24 hours was unexpected and interesting. It is a possibility that NRVMs go through some adaptive response at 24 hours which decrease the amount of cell debris presents. It is also a possibility that NRVMs adaptability decreases in correlation to decreasing pO_2 concentrations. Other than cell debris, molecules secreted by NRVMs are potential modulators of TNF-α or any other pro-inflammatory cytokines. Thus, investigating the type of molecules and their effects on macrophages will be interesting as the findings can provide insights to the microenvironmental regulators of macrophages produced by NRVMs during hypoxia. For future directions and improvements, more replicates will be required for validation of trends. Additionally, cell viability check should be done on both NRVMs and BMDMs after hypoxia treatment to ensure, validate, and explain results. A quick way to test whether that "something" was cell debris is by filtering NRVMs conditioned media and re-run the experiment to compare and verify results.

CHAPTER 5
CONCLUSION

Macrophages are an important immune regulator that helps promote the body's homeostasis. Due to its significant role as an immune mediator targeted by silent hypoxia in COVID-19 patients, investigating macrophages' behaviors upon hypoxia treatment can increase our understanding of this disease that killed millions. With macrophages as the main cell interest, this paper was also interested in looking at its interaction with cardiomyocytes. To prep for such study, the initial portion of this paper looked at the effects of different cultured mediums that allow for healthy growth of both macrophages and cardiomyocytes. The combination of M199 media used for standard culturing of cardiomyocyte and M-CSF, an irreplaceable component of D10 (standard macrophages culturing medium) were found to perform similarly to the standard medium for macrophages. Further studies looking at cardiomyocytes' behavior when cultured in M199 with M-CSF media are needed to validate the usage of the medium for co-culturing studies.

Without a doubt, hypoxia plays a significant role in modulating the microenvironment and the cells that operates within it. Macrophages are one of the many cells whose responses are hypoxia dependent. Therefore, the second and third portion of this paper looked at the effect of hypoxia on macrophages polarization. Rather than chemically inducing hypoxia to the cells, a hypoxia chamber was used to modulate a closed hypoxic environment with 1%, 3%, and 6% pO_2 at different durations. Stimulated pro-inflammatory and anti-inflammatory macrophages treated with hypoxia or hypoxia treated supernatants at variable timepoints suggested that short term severe hypoxia amplify pro-inflammatory responses while long term severe hypoxia amplify pro-healing responses. This work serves as preliminary data for future studies on macrophages and cardiomyocytes interactions upon co-culturing. Results provide interesting leads and directions for explaining the mechanism behind macrophages' behavior during hypoxia. These understandings can be used as references to current physiological scenarios like those experienced by COVID-19 patients.

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APPENDIX

Rat Bone Marrow Derived Macrophages Harvest Protocol

Purpose: Harvest and derive rat primary macrophages

Materials

1. Nine petri dishes
2. Two 15 mL conical tubes
3. One 50 mL conical tube
4. Four 5 mL syringe
5. Four 20 G x ½ needle
6. One scissor
7. One forceps
8. One bucket for ice
9. Pasteur pipettes
10. Serological pipettes
11. Micropipette tips

Reagents

1. Rat D10 media
2. ACK Lysis Buffer
3. PBS
4. 70% Ethanol

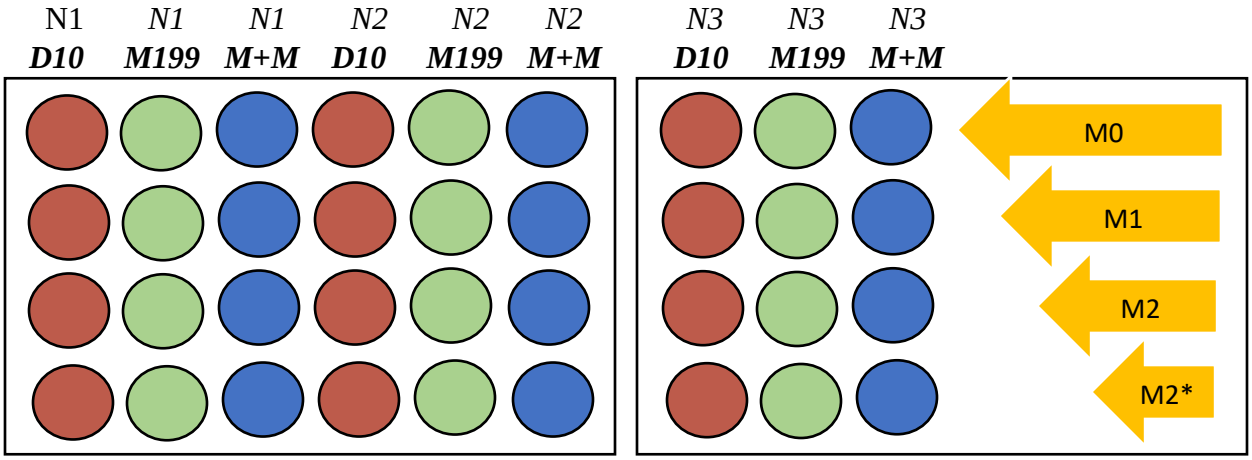
Methods

1. Place rat D10 media in water bath before heading to animal house.
2. Transfer ~25-30 mL of PBS in 50 mL conical tube than submerge in ice.
3. Place ice bucket in fume hood inside the animal house.
4. Get rat info card and a clear plastic bag for later placement of dissected rat.
5. Spray down scissor, forceps, and napkins with ethanol and place inside hood.
6. Spray down rat with ethanol spray before dissection.
7. Carefully dissect out both femur bones with hip bone intact and excess muscles and skin removed.
8. Place dissected femur bones in prepared PBS ice bath.
9. Clean up and place dissected rat in prepared clear plastic bag with rat info card and place in designated freezer.
10. Ethanol spray/clean scissor and forceps and place back in harvest box.
11. Use bleach to spray down hood and head back to core lab.
12. Take rat D10 media out of water bath.
1. Spray media, PBS with femurs, scissor, forceps, petri dishes, needles/syringe, serological pipettes, Pasteur pipettes, and 15 mL conical tubes into hood.
13. Use 10 mL serological pipette to extract 8 mL of rat D10 media into 8 petri dishes (each dish should contain 10 mL of solution: 8 mL of rat D10 + 2 mL of cell mixture).

14. Use 25 mL serological pipette to transfer 25 mL of rat D10 media into an extra petri dish and extract 5 mL using each syringe.
15. Grab a femur bone out of the PBS solution and place in the extra petri dish containing leftover media.
16. Cut/scrap/clean femur bone by removing excess muscles.
17. Cut top and bottom of bone to expose bone marrow.
18. Use rat D10 media in syringe to flush 5 mL of media into each side of bone ends which get collected into the 15 mL conical tube (move up and down to extract maximum bone marrow).
19. Repeat with the second femur bone and flush into another 15 mL conical tube.
20. Centrifuge 15 mL tubes at 300 G for 5 minutes.
21. Aspirate excess PBS from 50 mL conical tube.
22. Spray and clean hood while waiting.
23. Spray in ACK Lysis Buffer into hood.
24. Take out finished centrifuge samples and aspirate out media leaving cells pellet intact.
25. Add 1 mL of ACK Lysis Buffer to sample and break it up to lyse red blood cells.
26. Pipette up and down and let it sit for ~2 minutes.
27. Add 5 mL of rat D10 media to neutralize reaction into each 15 mL conical tubes.
28. Centrifuge samples at 300 G for 5 minutes.
29. Aspirate media leaving only white blood cells (monocytes) intact.
30. Add 8 mL of rat D10 media and mix using micropipette to break up cells.
31. Transfer 2 mL of cells into each petri dish (total 8 petri dishes from 2 femur bones).
32. Gently shake to mix and spread cells out.
33. Check cells under microscope.
34. Place in incubator 37℃, 5% CO_2 for 3-4 days until feeding.
35. Clean up hood.
36. 3-4 days after harvesting, check to see if cells are growing and sticking onto plates.
37. Add 3 mL of fresh rat D10 media into each petri dishes and place back in incubator at 37℃, 5% CO_2
38. Freeze down cells at Day 5 of harvest if not using for experiments.
39. If need cells for experiment, use matured and differentiated cells at Day 6-8 of harvest.

Rat Bone Marrow Derived Macrophages Cultured Media Experiment Protocol

Purpose: Compares the effect of M0, M1, and M2 polarization using rat D10, M199, and M199 with M-CSF (M+M) media on 3 biological rat replicates (N1, N2, N3).



Materials

1. Two 24-wells plates
2. Twelve 15 mL conical tubes
3. Three microcentrifuge (Eppendorf) tubes
4. Two cell countess slides
5. Micropipette tips
6. Serological pipettes
7. Pasteur pipettes
8. Three cell scrapers

Reagents

1. Rat D10 media
2. M199 10% FBS media
3. Stock LPS cytokines
4. Stock IFN- γ cytokines
5. Stock IL-4 cytokines
6. Stock IL-13 cytokines

Methods

Lifting of fresh and/or frozen rat BMDMs

1. Use Day 6-8 freshly harvested BMDMs and/or thaw frozen cells 2 days prior to usage (using 3 biological replicates)
2. Check harvest plates for cells confluency (make sure 90% confluency) before lifting.
3. Aspirate culture media from harvest plates using Pasteur pipette.
4. Add 5 mL of cell dissociation buffer into each harvest plates for lifting of cells.

5. Incubate at 37℃, 5% CO_2 for ~15 minutes.
6. Tap cells to aid in lifting.
7. Check if majority of cells lift under the microscope.
8. Gently scrape cells to ensure lifting with a cell scraper.
9. Transfer lifted cell solution to a 15 mL conical tube using 1 mL micropipette tips.
10. Centrifuge at 300 G for 5 minutes.
11. Aspirate out cell dissociation buffer leaving cell pellet intact.
12. Resuspend and break up cells pellet in 1 mL using micropipette tips.
13. Add additional 5 mL of rat D10 media and mix well.

Seeding of rat BMDMs into experimental wells

14. Transfer 10 μL of each cell solutions into its respective microcentrifuge tube.
15. Add 10 μL of trypan blue into each microcentrifuge tube to create a 2-fold dilution mixture for cell counting.
16. Mix and transfer 10 μL into cell countess slide to count cells.
17. Use microscope to count cells in all 4 squares and multiply by 2 (dilution factor) and 10^4 to get the cell density.
    Example:
    100 cells (cells counted in 4 squares) * 2 (dilution factor) * 10^4 = 2 x 10^6 cells/mL
18. Multiply cell density by resuspension volume (6 mL) to get total cell count
19. Separate cell suspension into three 15 mL conical of 2 mL each (should have 9 tubes total, 3 for each biological replicates).
20. Centrifuge at 300 G for 5 minutes.
21. Aspirate and resuspend in desired media volume (Rat D10, M199, M199 with M-CSF) making sure that each well have 100,000 cells/ 0.5 mL (24-wells plate).
22. Transfer 0.5 mL of cell suspensions into its respective wells.
23. Incubate for ~4 hrs. at 37℃, 5% CO_2 to ensure sticking before stimulation.

Stimulation of M1, M2, and M2 conditions*

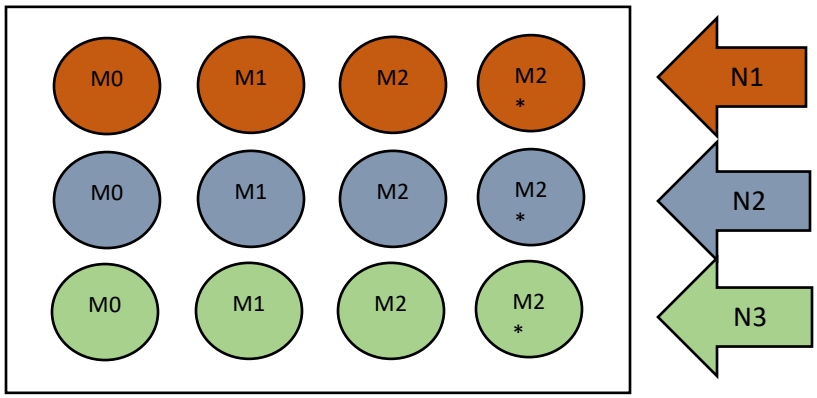
24. Prior to stimulation, follow stimulation table to make stimulating solutions for all conditions.
25. Transfer both experimental plates from incubator into hood after 4 hrs. is up.
26. Remove 125 μL of old media from each well and add 125 μL of stimulating solutions to its corresponding wells.
27. Stimulate overnight (18 hrs.).
28. Collect supernatant and store in -20℃ until performing ELISA test per manufacturer's protocol.

Table 2. Stimulation worksheet

M1		Total volume	1	mL	stock (ng/mL)	original (ng/mL)
	10ng/mL	LPS	10	ul	1.0E+03	1.0E+06
	10ng/mL	IFNg	1	ul	1.0E+04	2.0E+05
		Media	0.989	mL		
M2		Total volume	1	mL	stock (ng/mL)	original (ng/mL)
	20ng/mL	IL-4	1	ul	2.0E+04	2.0E+05
	20ng/mL	IL-13	1	ul	2.0E+04	2.0E+05
		Media	0.998	mL		
M2*		Total volume	1	mL	Stock ng/ml)	original (ng/mL)
	20ng/mL	IL-4	1	ul	2.0E+04	2.0E+05
	20ng/mL	IL-13	1	ul	2.0E+04	2.0E+05
	10ng/ml	LPS	10	ul	1.0E+03	1.0E+06
		Media	0.988	mL		

Rat Bone Marrow Derived Macrophages Hypoxia Protocol #1

Purpose: Treating cultured BMDMs in various degree of hypoxic conditions for 3 biological rat replicates and investigate their response.



Materials

1. 12-wells plates
2. Petri dishes
3. 15 mL conical tubes
4. Cell scrapers
5. Serological pipettes
6. Pasteur pipettes
7. Micropipette tips
8. Cell countess slides
9. Microcentrifuge (Eppendorf) tubes

Reagents

1. Cell Dissociation Buffer
2. Rat D10 media
3. M199 10% FBS with M-CSF media
4. Stock LPS cytokines
5. Stock IFN- γ cytokines
6. Stock IL-4 cytokines
7. Stock IL-13 cytokines

Equipment

1. Hypoxia chamber

Methods

1. Use Day 6-8 freshly harvested BMDMs and/or thaw frozen cells in rat D10 media 2 days prior to usage (using 3 biological replicates).
2. Lift, count, and seed 300,000 cells/mL in M199 10% FBS with M-CSF media into 12 wells plates.

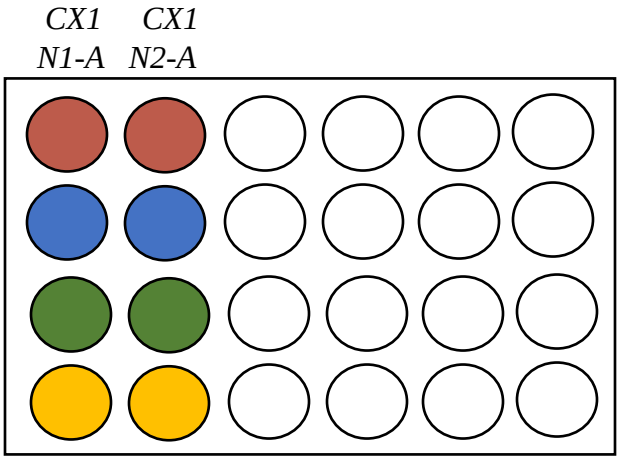
3. Incubate for ~4 hrs. at 37℃, 5% CO_2 to ensure sticking before stimulation.
4. Make stimulating solution for M1, M2, and M2* conditions using the stimulation table.
5. Stimulate each corresponding sample and left to incubate overnight (18 hrs.).
6. Start up in order of hypoxia chamber, computer with "VoeReady.exe" system, gas flow controller, and gas tanks.
7. Setup the "VoeReady.exe" system and input script-maker codes for desired percentage and duration of hypoxic condition.
8. Ensure hypoxia chamber is at 37℃ prior to placement of experimental plate into the chamber
9. Collect supernatants after hypoxia treatment and store at -20℃ until performing ELISA per manufacturer's protocol.

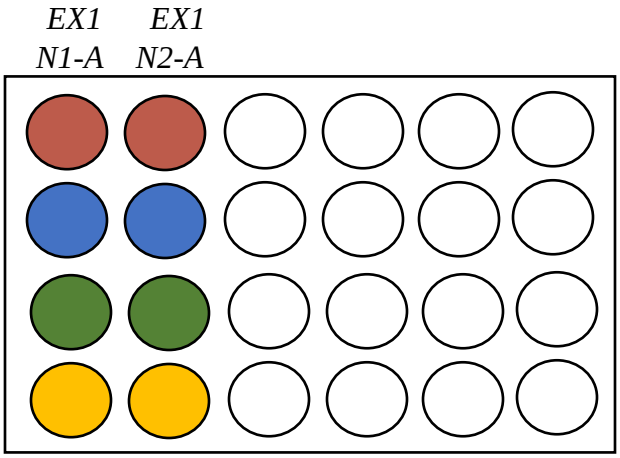
Table 3. Script-maker codes for the hypoxia chamber

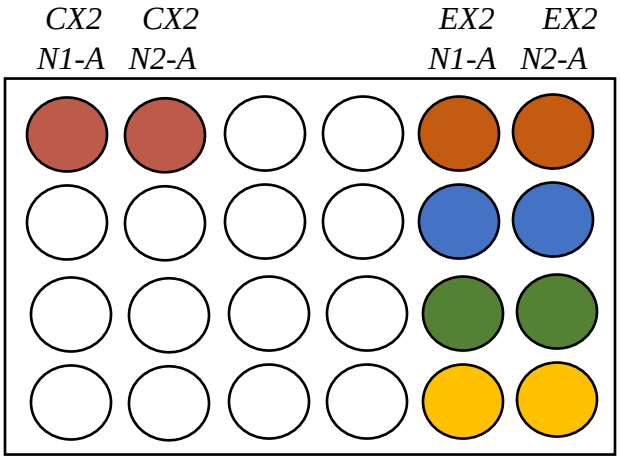
Hypoxia Condition	**Script for N_2**	**Script for CO_2**	**Script for O_2**
1% pO_2 for 1 hr.	wait 1 step 95% wait 547 step 94% wait 3600 step 0% wait 10	wait 1 step 8.3333% wait 547 step 8.3333% wait 3600 step 0% wait 10	wait 1 step 0% wait 547 step 1.6667% wait 3600 step 0% wait 10
1% pO_2 for 6 hrs.	wait 1 step 95% wait 547 step 94% wait 21600 step 0% wait 10	wait 1 step 8.3333% wait 547 step 8.3333% wait 21600 step 0% wait 10	wait 1 step 0% wait 547 step 1.6667% wait 21600 step 0% wait 10
1% pO_2 for 24 hrs.	wait 1 step 95% wait 547 step 94% wait 86400 step 0% wait 10	wait 1 step 8.3333% wait 547 step 8.3333% wait 86400 step 0% wait 10	wait 1 step 0% wait 547 step 1.6667% wait 86400 step 0% wait 10
3% pO_2 for 1 hr.	wait 1 step 95% wait 349 step 92% wait 3600 step 0% wait 10	wait 1 step 8.3333% wait 349 step 8.3333% wait 3600 step 0% wait 10	wait 1 step 0% wait 349 step 5% wait 3600 step 0% wait 10
3% pO_2 for 6 hrs.	wait 1 step 95% wait 349 step 92% wait 21600 step 0% wait 10	wait 1 step 8.3333% wait 349 step 8.3333% wait 21600 step 0% wait 10	wait 1 step 0% wait 349 step 5% wait 21600 step 0% wait 10

Rat Bone Marrow Derived Macrophages Hypoxia Protocol #2

Purpose: Treating cultured BMDMs in 1% hypoxic condition during different stage of stimulation and investigate their responses.







Setup

A. Stimulate and place in hypoxia chamber immediately

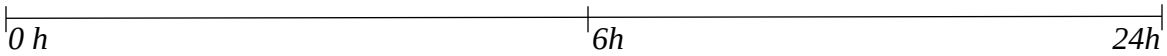
a. Reasoning: Initial hypoxia protocol #1 have cells that's already been stimulated overnight prior to placement in hypoxia chamber. This results in insignificant change in TNF-α secretions between different hypoxic conditions. A possible explanation for this result is that cells are activated toward inflammation during the first 4-6 hours of stimulation. Cells have already exhausted their inflammatory responses hence different hypoxic conditions have little to no effect on these cells. Therefore, to see the effect of hypoxia, cells can be stimulated and placed in the hypoxia chamber simultaneously.

b. 2 biological replicates are used for each test conditions (denoted as N1 and N2). For test set up A, there will be a control group (CX1) which have cells that are stimulated and placed in the incubator for the entirety of experiment (24 hours). There will also be an experimental group (EX1) which have cells that are

stimulated and immediately placed in hypoxia chamber for 6 hours. After 6 hours, cells are placed in the regular incubator for the rest of the experiment (24 hours total). Supernatants and lysates for qPCR will be collected the next day for analysis.

B. *Place in hypoxia chambers for 24 hours then stimulate*
    a. *Reasoning: Test the effect of hypoxia alone compared to hypoxia with stimulation. Want to see if hypoxia alone can induce unpolarized macrophages into a polarized state and to what degree. How does stimulating post hypoxia effect cytokine secretion level as cells are already in a stress state?*
    b. *2 biological replicates are used. For test set up B, there will be a control group (CX2) and experimental group (EX2) which have cells placed in hypoxia chamber for 24 hours. After 24 hours, only the EX2 group are stimulated. After stimulation, both groups are placed in the regular incubator for another 24 hours. The reason for incubating overnight after hypoxia treatment is to allow the expression of M2 markers which happens at the later time point post stimulation.*

C. *EX1 Timeline*

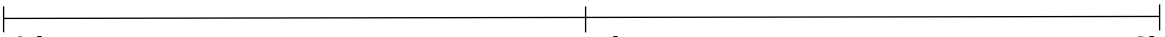


Stimulate CX1, EX1 then place EX1 in 1% hypoxia chamber, place CX1 in incubator

Place EX1 in incubator

Collect supernatant

EX2 Timeline



Place CX2, EX2 in 1% hypoxia chamber

Stimulate EX2, then place CX2, EX2 in incubator

Collect supernatant

Materials

1. 24-wells plates
2. Petri dishes
3. 15 mL conical tubes
4. Cell scrapers
5. Serological pipettes
6. Pasteur pipettes
7. Micropipette tips
8. Cell countess slides
9. Microcentrifuge (Eppendorf) tubes

Reagents

1. Cell Dissociation Buffer

2. Rat D10 media
3. M199 10% FBS with M-CSF media
4. Stock LPS cytokines
5. Stock IFN- γ cytokines
6. Stock IL-4 cytokines
7. Stock IL-13 cytokines

Equipment

1. Hypoxia chamber

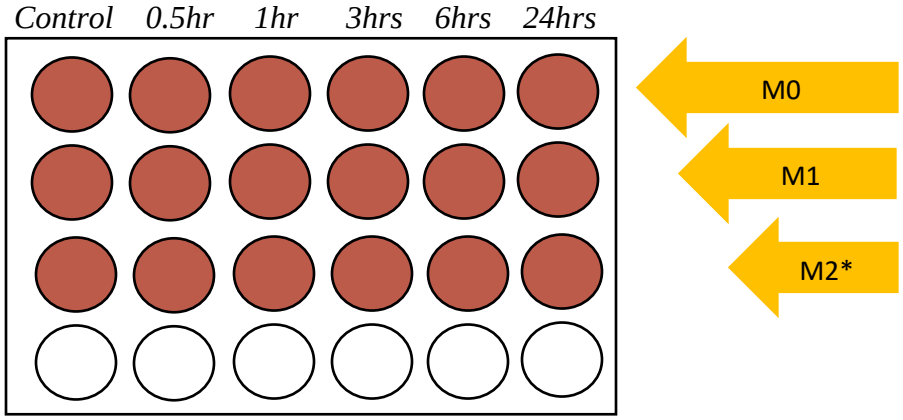
Methods

1. Use Day 6-8 freshly harvested BMDMs and/or thaw frozen cells in rat D10 media 2 days prior to usage (using 2 biological replicates).
2. Lift, count, and seed 150,000 cells/mL in M199 10% FBS with M-CSF media into 24 wells plates.
3. Incubate overnight at 37℃, 5% CO_2 to ensure sticking.
4. Make stimulating solution for M1, M2, and M2* conditions using the stimulation table.
5. Start up in order of hypoxia chamber, computer with "VoeReady.exe" system, gas flow controller, and gas tanks.
6. Setup the "VoeReady.exe" system and input script-maker codes for desired percentage and duration of hypoxic condition (1% hypoxia for 6 hours).
7. Stimulate CX1, EX1 conditions then place CX1 condition in incubator.
8. Ensure hypoxia chamber is at 37℃ then immediately place experimental plate with EX1, condition into the chamber for 6 hours.
9. After 6 hours, set "VoeReady.exe" system to 1% hypoxia for 24 hours and place CX2 and EX2 conditions in the chamber.
10. Place EX1 condition in incubator for another 18 hours to make 24 hours of total stimulation.
11. After 24 hours of placing CX2 and EX2 in hypoxia chamber, stimulate only EX2 with M1, M2, and M2* cytokines.
12. Place both CX2 and EX2 in incubator for another 24 hours.
13. Collect supernatants the next day and store at -20℃ until performing ELISA per manufacturer's protocol.

Rat Bone Marrow Derived Macrophages in NRVM Conditioned Media Protocol

Purpose: Culture BMDMs in neonatal rat ventricular myocytes (NRVMs) conditioned media that has been treated in various hypoxic environments and investigate BMDMs' responses.

Example: 1% hypoxia treated NRVMs conditioned media culturing N1 rat BMDMs



Materials

1. 24-wells plates
2. Petri dishes
3. 15 mL conical tubes
4. Cell scrapers
5. Serological pipettes
6. Pasteur pipettes
7. Micropipette tips
8. Cell countess slides
9. Microcentrifuge (Eppendorf) tubes

Reagents

1. Cell Dissociation Buffer
2. Rat D10 media
3. NRVMs conditioned media treated with hypoxia
4. Stock LPS cytokines
5. Stock IFN- γ cytokines
6. Stock IL-4 cytokines
7. Stock IL-13 cytokines

Methods

1. Use Day 6-8 freshly harvested BMDMs and/or thaw frozen cells in rat D10 media 2 days prior to usage.
2. Lift, count, and seed 150,000 cells/mL in rat D10 media.
3. Incubate for ~2 hrs. at 37℃, 5% CO_2 to ensure sticking before replacing with its corresponding NRVMs conditioned media treated with hypoxia.

4. Incubate for 2 hrs. at 37℃, 5% CO_2 to ensure further sticking before stimulation.
5. Make stimulating solution for M1, M2, and M2* conditions using the stimulation table.
6. Stimulate each corresponding sample and left to incubate overnight (18 hrs.).
7. Collect supernatants and store at -20℃ until performing ELISA per manufacturer's protocol.