Measuring the Immunomodulatory Effects of beta-Adrenergic Receptor Drugs on THP-1-Derived M1 and M2 Macrophage-Like Cells.

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Abstract

Measuring the Immunomodulatory Effects of beta-Adrenergic Receptor Drugs on THP-1-Derived M1 and M2 Macrophage-Like Cells.

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Macrophages play a crucial role in coordinating the immune response and regulating inflammatory balance. They exist as two main phenotypes: M1 macrophages, which are pro-inflammatory and secrete cytokines like TNF to amplify the immune response, and M2, which are anti-inflammatory and promote tissue healing by secreting cytokines like IL-10. The balance between these phenotypes is essential for effective infection control, and disruptions can lead to autoimmune diseases, severe infections, and other inflammatory disorders. Understanding the modulation of these phenotypes is key for developing effective therapies.

This project investigates the interactions between the sympathetic nervous system (SNS) and the immune system by treating a monocyte-like cell line with β-adrenergic receptor (β-AR) agonist and antagonist drugs. While previous studies showed that β2-AR drugs have anti-inflammatory properties in macrophages (Kast, 2000; Szelenyi et al., 2000), research on their effects on M1/M2 macrophage phenotypes and receptor selectivity is limited. This project aims to address these gaps to better understand the interplay between the SNS and immune system.

β1- and β2-AR expression was confirmed in both macrophage phenotypes, with *ADRB1* and *ADRB2* genes upregulated in M1-like cells (with IFNγ) and downregulated in M2-like cells. Isoproterenol, a non-selective β-AR agonist, decreased TNF in M1-like cells and increased IL-10 in M2-like cells. These effects were reversed by the non-selective β-AR antagonist bupranolol but not by the β2-selective antagonist ICI 118,551. Terbutaline, a β2-selective agonist, showed similar trends and was reversed by both antagonists. This research demonstrates that SNS activity influences immune responses, promoting an anti-inflammatory profile through β-AR activation. These findings suggest potential therapeutic strategies using β-AR drugs for inflammatory and autoimmune conditions.

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List of Abbreviations

ANOVA = Analysis of Variance
APCs = Antigen Presenting Cells
AR = Adrenergic Receptor
cAMP = cyclic AMP
CD = Cluster of Differentiation
ddH_2O = double distilled water
DMSO = Dimethyl-Sulfoxide
EDTA = Ethylene-Diamine-Tetra-Acetic acid
ELISA = Enzyme-Linked Immunosorbent Assay
FBS = Fetal Bovine Serum
GPCR = G Protein-Coupled Receptor
IL = Interleukin
IL-10R = Interleukin-10 Receptor
IFNγ = Interferon-γ
IFNγR = Interferon-γ Receptor
LPS = Lipopolysaccharide
MHC II = Major Histocompatibility Complex II
MLCK = Myosin Light Chain Kinase
mRNA = Messenger RNA
NF-κB = Nuclear Factor-κB
PAMP = Pathogen-Associated Molecular Pattern
PBS = Phosphate Buffered Saline
PKA = Protein Kinase A
PMA = Phorbol 12-Myristate 13-Acetate
PPIA = Peptidylprolyl Isomerase A
PRRs = Pattern Recognition Receptors
RPMI = Roswell Park Memorial Institute
RT-qPCR = Quantitative Real-Time Polymerase Chain Reaction
SD = Standard Deviation
SNS = Sympathetic Nervous System
Th = T Helper
THP-1 = Tohoku Hospital Pediatrics-1
TLR = Toll-Like Receptor
TNF = Tumor Necrosis Factor
TNF-RI = TNF receptor I

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Chapter 1. Introduction

Macrophages play a central and complex role in coordinating the immune response from start to finish. Their functions range from pathogen clearance to tissue repair and dysregulation can lead to severe complications, illustrating the importance of understanding their modulation. The goal of this project is to create an *in vitro* model of M1- and M2-like macrophages using the THP-1 cell line to measure the effects tumor necrosis factor (TNF) and interleukin-10 (IL-10) cytokine production after treatment with β-adrenergic receptor (β-AR) agonists and antagonists. Currently, there is little research exploring β-AR expression in polarized M1- and M2- like macrophages and the subsequent effects of activating these receptors on macrophage function. By investigating the immunomodulatory effects of β-AR signaling in macrophages, this research aims to fill gaps in our knowledge of the interactions between the sympathetic nervous system (SNS) and the immune system and potentially pave the way for novel therapeutic strategies for managing inflammatory and autoimmune diseases.

1.1. Monocytes and macrophage cells and their roles in immunity

Monocytes are white blood cells that are derived from the bone marrow. Monocytes, which are characterized by a single nucleus, primarily will circulate through blood and lymphatics scanning the body for inflammation or infections. If they enter an inflamed tissue, they can differentiate into macrophages to help maintain homeostasis during infection. (Espinoza & Emmady, 2024) A macrophage is specialized to consume pathogens by a process called phagocytosis (engulfment), and to alert other immune cells. Macrophages will contain an infection by engulfing and killing pathogens, as well as removing any dead cells and activating other immune cells during infection. (Lendeckel et al., 2022) Together, monocytes and macrophages cells bridge the innate and adaptive immune systems. The innate immune response is the first line of defence against infection and the response itself is less flexible towards pathogens, meaning it will only recognize general patterns, known as pathogen-associated molecular patterns (PAMPs). PAMPs are distinct structural components of microbes that distinguish them from self-tissues. The adaptive immune response would occur if the innate immune response failed to clear the pathogen and it is much more flexible and specific. This response is slower than the innate response but can store long-term “memory” after infection and

can use this memory to initiate a faster response if the same pathogen is encountered again. Macrophages are one of the key elements for the switch between innate to adaptive due to their roles in both responses. During the innate response, macrophages first encounter and recognize the pathogen via pattern recognition receptors (PRRs) present on the surface of the cells. These receptors are responsible for recognizing the PAMPs that are frequently found in pathogens. After the pathogen's pattern is recognized by these receptors, the cells will secrete cytokines and chemokines to recruit other immune cells to the site of infection. (Punt et al., 2019) Cytokines are small proteins secreted by cells that act as signalling molecules with a wide range of functions such as regulation of inflammation, modulation of cell growth, survival, and differentiation. Chemokines are proteins within the cytokine family and function mainly to regulate cell migration. (Ramesh et al., 2013) Cytokines and chemokines will activate the nearby macrophages to undergo phagocytosis (engulfing) of the pathogen to digest and, during the adaptive immune response, present fragmented peptides to T cells via their major histocompatibility complex II (MHC II) molecules on their membrane surface. By presenting antigen to T cells, the macrophages are bridging the innate and adaptive immune systems. Also, during the adaptive response, macrophages will present co-stimulatory molecules to activate T helper (Th) cells. (Punt et al., 2019) The secretion of cytokines by macrophages, which is the focus of this thesis project, is arguably the most crucial step in initiating the immune response upon infection.

Monocytes can be distinguished into classical, intermediate, and non-classical subsets based on their surface expression of the cluster of differentiation (CD) markers. Immunologists use CD as a nomenclature for proteins on the surface of immune cells that define their key characteristics. There are CD markers specific to each subtype of monocytes and the type of immune response. (Espinoza & Emmady, 2024) CD14 and CD16 are used to distinguish between classical, intermediate, and non-classical subsets. The + notation indicates the presence and intensity of the receptor, whereas the - indicates the absence. (Ziegler-Heitbrock et al., 2010) The classical monocytes ($CD14^{++}$ $CD16^{-}$) make up 85% of the monocytes in the body and are mainly responsible for entering infected tissue, transforming to macrophages, performing phagocytosis and promoting the inflammatory response. These macrophages are important phagocytes that contribute to ridding the tissue of the pathogen and acting as antigen presenting cells (APCs) for T lymphocytes. (Mussbacher et al., 2023) Intermediate monocytes ($CD14^{++}$

$CD16^+$) make up 5% of monocytes, highly express MHC II, and are involved in angiogenesis. (Wong et al., 2011) Lastly, the non-classical monocytes ($CD14^+$ $CD16^{++}$) make up 10% of monocytes and they patrol and monitor the blood vessels to promote wound healing and aid in resolving inflammation. (Thomas et al., 2015)

Most macrophages are derived from monocytes that enter inflamed tissues and these monocyte-derived macrophages have several subsets depending on the type of inflammation and pathogen that is present in the tissue. The first type is considered as naïve M0 macrophages that are capable of phagocytic activity and pathogen detection, however, they are not in their full effector state. (Chaintreuil et al., 2023) Depending on the type of stimulation, M0 macrophages can polarize towards M1 or M2 phenotypes, which in themselves can be divided into further subtypes. (Mill et al., 2000; Shapouri-Moghaddam et al., 2018) Generally, M1-like macrophages are responsible for defense against pathogens by secreting pro-inflammatory cytokines and ridding the pathogen through phagocytosis. Whereas M2-like macrophages have similar roles to non-classical monocytes like resolving inflammation, phagocytosis of cell debris and tissue reparation. (Mussbacher et al., 2023; Chaintreuil et al., 2023)

I am most interested in studying macrophages since they orchestrate the inflammation response and are responsible for maintaining homeostasis in health and disease. (Yáñez et al., 2017) The balance between M1 and M2 macrophages has implications in understanding the balance between pro- and anti-inflammatory states. M1 and M2 cells correspond with the two major subsets of T cells called Th1 cells and Th2 cells. If M1 macrophages present antigen to T cells they promote the Th1 phenotype, while M2 macrophages will promote a Th2 phenotype. Generally, the M1 and Th1 cells are considered to be pro-inflammatory, while the M2 and Th2 cells are anti-inflammatory. Inflammation has many benefits that protect the host, however, too much inflammation can lead to excessive or even chronic tissue damage. Thus, understanding the factors which control the polarization of macrophages to M1 versus M2 will teach us more about how to intervene in people who might have imbalances in their inflammatory state.

1.2. THP-1 cell line and polarization of M0-, M1-, M2-like macrophages.

For the purpose of this thesis, I used a reductionist approach by creating an *in vitro* model of M1- and M2- like macrophages using the Tohoku Hospital Pediatrics-1 (THP-1) cell line. This cell line is a monocytic precursor cell line derived from peripheral blood of a child with acute monocytic leukemia. (Bosshart & Heinzelmann, 2016) It is a commonly used cell line for modeling monocyte and macrophage function and is suitable for exploring topics in fundamental immunology. The main caveats of using a cell line are that they are cancerous, and that they only represent a sample size of one which means the data can not be generalized to the population. However, the THP-1 cell line is advantageous for this project due to its ease of handling, unlimited supply, its reproducibility, and a sizeable literature to draw from.

Monocytes are first differentiated into naïve M0-like macrophages before they can polarize into effector M1- or M2-like macrophages. The most widely used protocol for differentiating THP-1 monocytes to M0-like macrophages includes treatment with phorbol 12-myristate 13-acetate (PMA). (Mohd Yasin et al., 2022) PMA is a protein kinase C activator that initiated downstream signaling pathways related to increased adherence and phagocytic activity, priming the M0-like macrophages before they polarize into effector cells. (Schwende et al., 1996)

M1-like macrophages are activated by Th1-cell derived cytokines (eg.: tumor necrosis factor or interferon-γ) and/or pro-inflammatory compounds including the common PAMP lipopolysaccharide (LPS). M1-like macrophages will thus secrete tumor necrosis factor (TNF) as a result. Interferon-γ (IFNγ) is a cytokine that binds to its receptor (IFNγR) present on the surface of macrophage cells and promotes activation of the M1-like macrophages by enhancing their response to Toll-like receptor (TLR) ligands while also initiating feedback inhibitory loops to avoid toxicity from over-activation. (Hu et al., 2008) TLR is a PRR that is able to immediately sense the presence of LPS. LPS is an endotoxin embedded in the outer membrane of gram-negative bacteria and, if released into circulation, can cause fever, diarrhea, and can lead to septic shock. (Farhana & Khan, 2022) LPS is a TLR ligand and is detected at picomolar levels by the TLR4, found on the membrane surface or in the endosomes/lysosomes. It therefore initiates different severities of immune responses depending on the strain of bacteria. (Farhana & Khan, 2022) Recognition of LPS will result in activation of several downstream signaling

pathways, including the nuclear factor-κB (NF-κB) pathway, causing the secretion proinflammatory cytokines including TNF to initiate a pro-inflammatory response. (Chen et al., 2020)

M2-like macrophages are activated by Th2-cell derived cytokines (eg.: interleukin-4 (IL-4), IL-10, and IL-13) and will secrete IL-10. (Mussbacher et al., 2023) IL-4 and IL-13 cytokines both share similar functions and have shown to activate host defence and tissue repair responses in M2-like macrophages, making them important mediators for homeostasis in inflammation. (Bosurgi et al., 2017) IL-4 is known for inducting the "alternative" macrophage pathway, resulting in tissue repair and homeostasis and binds to the IL-4Rα receptor. (Gadani et al., 2012) Whereas IL-13 can inhibit inflammatory cytokine production in Th1 cells and binds to the IL-13Rα1 receptor. Nonetheless, both cytokines will result in induction of signalling pathways that activate STAT6, a transcription factor that initiates M2 polarization and cause subsequent production of IL-10. (Scott et al., 2023)

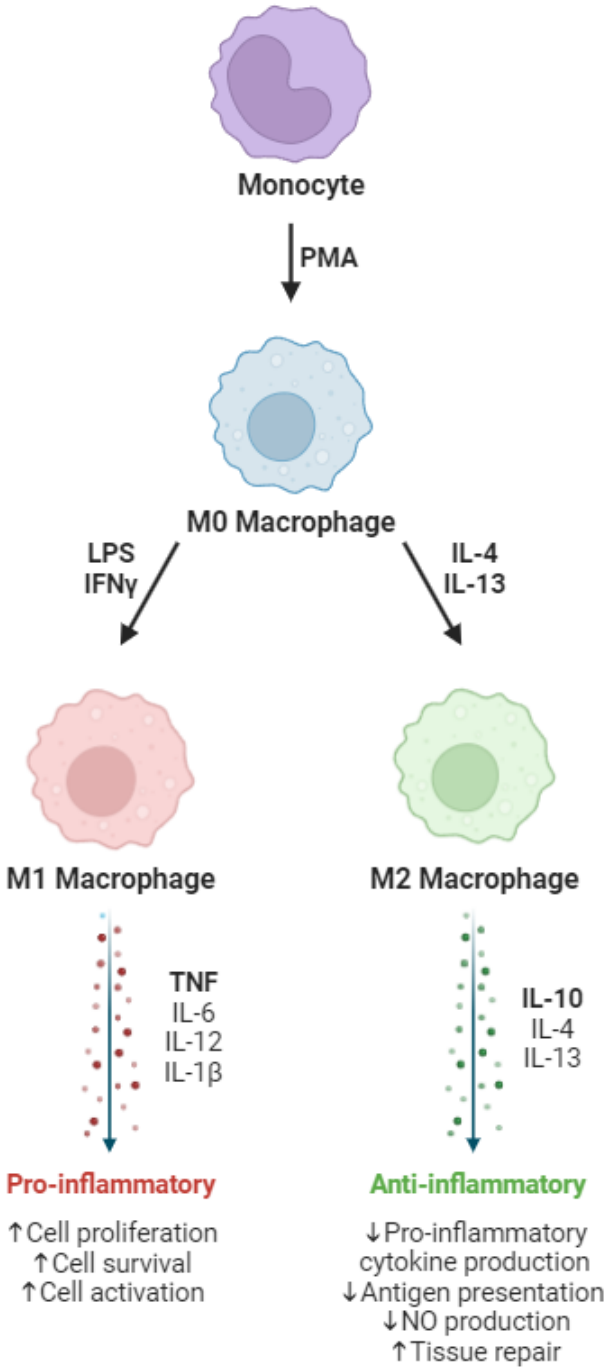


Figure 1. M1 and M2 macrophage polarization from monocytes. Monocytes can be primed with PMA towards naïve M0 macrophage cells which can further be polarized into M1 and M2 effector macrophage cells. M1 polarization requires LPS and IFNγ and M2 polarization requires IL-4 and IL-13. M1-like macrophages will produce pro-inflammatory cytokines (TNF, IL-6, IL-12, and IL1β) and M2-like macrophages will produce anti-inflammatory cytokines (IL-10, IL-4, and IL-13). Figure was created using BioRender.

1.3 Role of TNF and IL-10 in inflammation.

TNF is primarily produced by M1-like macrophages and is responsible for amplifying the immune response, hence why it is considered a pro-inflammatory cytokine. (Aggarwal et al., 2012) It does so by binding to TNF receptor I (TNF-RI) on the surface of macrophages and further promoting NF-κB activation, cell survival, differentiation, and proliferation; acting in positive feedback loops. (Peschon et al., 1998; Newton & Dixit, 2012) Thus, the secretion of TNF is highly indicative of the severity of an inflammatory response initiated by a stimulus.

IL-10 is produced by M2-like macrophages and is integral for maintaining homeostasis during and after an inflammatory response and is considered to be an anti-inflammatory cytokine. It binds to the IL-10 receptor (IL-10R) resulting in phosphorylation and activation of the transcription factor, STAT3, which is responsible for regulating further IL-10 production. IL-10 is capable of promoting tissue-healing caused by injury or inflammation and can counteract pro-inflammatory responses. It is therefore crucial in the coordination of an anti-inflammatory response and its role aims to minimize tissue damage caused by inflammation. (Ouyang et al., 2011)

LPS-induced macrophage activation and subsequent TNF and IL-10 measurement is commonly used in clinical research as a means to measure the effects of drugs on pro- and anti-inflammatory immune responses. Of interest, is how adrenergic drugs will affect the secretion of TNF and IL-10 in M1- and M2-like macrophages respectively.

1.4. Sympathetic nervous system and immune system crosstalk by adrenergic signaling in macrophages.

Over the last several decades there have been increased evidence of communication between the SNS and the immune system. The SNS represents the “fight or flight response” and regulates the metabolism, energetics and cardiovascular responses of most body tissues. The nerve terminals of the sympathetic neurons also innervate with the primary and secondary lymphoid organs where the immune cells are found. (Felten et al., 1985) Activation of the SNS results in the production of stress hormones like glucocorticoids and catecholamines that have shown to impact immune function depending on its nature (physical or psychological), duration,

and intensity. (Kolmus et al., 2015) Upon stimulation by either antigen or cytokines, the sympathetic nerve terminals that innervate the lymphoid tissues will release catecholamines hormones such as norepinephrine and epinephrine into the microenvironment and result in a targeted effect on the immune cells in proximity. (Kohm et al., 2000) The norepinephrine and epinephrine neurotransmitters are recognized by adrenergic receptors, which have been discovered on the membrane surface of immune cells. (Motulsky et al., 1986)

Adrenergic receptors (ARs) are transmembrane proteins that belong to the G protein–coupled receptor (GPCR) superfamily that can be divided into three major types (α_1, α_2, and β), each containing three subtypes (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3). (Kobilka, 2011) Although they are all activated by catecholamines, they regulate several different cellular and physiological processes depending on the subtype and density of the receptor found in the tissue-specific cell. Of interest for this project, it has been shown that human peripheral blood monocytes and monocyte-derived macrophages express the β_1-, β_2- and β_3-ARs. (Speidl et al., 2004; Talmadge et al., 1993; Hadi et al., 2017) The β_1 AR is mostly expressed in the heart to regulate ventricular contractility and heart rate, and β_2 ARs are found in all cells of the body but are mostly expressed in bronchial smooth muscle cells to dilate blood vessels and bronchioles. (Motiejunaite et al., 2021) Lastly, β_3 ARs are expressed in white and brown adipose tissue and activation will increase fat oxidation, energy expenditure, and glucose uptake. (Motiejunaite et al., 2021) For the purpose of this project, I will explore the immunomodulatory effects of β-AR agonists or antagonists on monocytes.

1.5. β-adrenergic receptor agonists and antagonists.

An agonist drug will bind to its target receptor and change the receptor activity in order to initiate a response. However, an antagonist drug will bind to its receptor and will not cause a response and thus, may block ligands from binding to the receptor. (Berg & Clarke, 2018)

The β-AR agonists of interest for this project are isoproterenol, a non-selective β_1- and β_2-AR agonist used to treat bradycardia conditions (Szymanski & Singh, 2023), and terbutaline, a β_2-AR specific agonist used a bronchodilator in asthmatic patients (National Center for Biotechnology Information, Terbutaline, 2024). Both β_1- and β_2-AR have similar signaling

pathways once activated: an increase in intracellular cyclic adenosine monophosphate (cAMP) which will activate protein kinase A (PKA) leading to phosphorylation and inactivation of myosin light chain kinase (MLCK). MLCK is responsible for phosphorylation of myosin which normally results in muscle contraction; however, inactivation causes smooth muscle relaxation, bronchial dilation, and peripheral vasodilation. (Szymanski & Singh, 2023)

β-AR agonists display anti-inflammatory properties in macrophages by reducing production of TNF and increasing production of IL-10. (Kast, 2000; Szelenyi et al., 2000) Isoproterenol can cause anti-inflammation (Ernst et al., 2019; Haskó et al., 1998), however, since isoproterenol is non-specific towards the β_1- and β_2-AR, it was of interest to explore a different and more selective β-AR agonist drug such as terbutaline. Terbutaline had anti-inflammatory effects in macrophages in a few studies, notably, only one study was done on human lung macrophages. (Gill et al., 2016; Keränen et al., 2016; Keränen et al., 2017) Hence, there remains a knowledge gap in the effects of β-AR drugs on polarized groups of macrophages.

The β-AR antagonists used in this project were bupranolol, a non-selective β_1- and β_2-AR antagonist used for cardiac arrhythmia, angina pectoris, hypertension, glaucoma, and as an antithrombotic (National Center for Biotechnology Information, Bupranolol, 2024), and ICI 118,551, a selective β_2-AR antagonist that is not currently approved for human use as a therapeutic agent. (O'Donnell & Wanstall, 1980) These antagonists serve to block the effects of the β-AR agonists in polarized macrophage cells and to confirm selectivity of the β-AR so that there are no off-target effects. An off-target effect is when a drug that is supposed to be specific for a receptor also works on other receptors. It is expected that the β-AR antagonists will reverse or block the effects of the agonists by bringing the concentration of cytokine back to baseline.

1.6. Rationale for thesis project.

There remain several knowledge gaps regarding the effects of β-AR drugs on polarized groups of macrophages. The literature showed that β-agonists affect macrophages, but whether those effects were attributable to β1 or β2 was not known, and the impact on M1 and M2 macrophage types was not known. The first aim for this project was to establish a M1- and M2-like model from THP-1 cells to measure TNF and IL-10 production respectively. Although the

mechanism of M1 and M2 polarization is well established in literature, there is no set protocol that has been agreed upon. Rather, there exists a systemic review by Mohd Yasin et al. (2022) from which the procedures for this project were based upon.

The second aim is to use the model to measure the effects of the β-AR drugs on TNF and IL-10 secretion in M1- and M2-like macrophage cells. It has been shown that monocytes and monocyte-derived macrophages express the β_1- and β_2-ARs. (Speidl et al., 2004; Talmadge et al., 1993; Hadi et al., 2017) However, since the β_1-ARs are mostly expressed in the heart and the β_2-ARs are mostly expressed in bronchial smooth muscle cells, there is little research exploring the subsequent immunomodulatory effects of activating these receptors in macrophages. Furthermore, there is less research examining specifically the effects of isoproterenol and terbutaline on cytokine secretion in M1 and M2 macrophages.

My thesis advanced our understanding of immune regulation and the therapeutic potential of β-AR modulation in macrophages. By exploring the effects of β-AR agonists and antagonists on TNF and IL-10 production in M1- and M2-like macrophages, this project seeks to provide valuable insights into the crosstalk between the nervous and immune systems, ultimately contributing to the development of novel treatments for inflammatory and autoimmune diseases.

Chapter 2. Materials and Methods

2.1. THP-1 cell culture.

THP-1 cells (Sigma Aldrich, Mississauga, Ontario, CA) were thawed according to the manufacturer's protocol. Importantly, the cells had to be centrifuged with low settings throughout the procedures (100 x g). The cells were seeded at a density of 0.3 – 0.5 x 10^6 cells/mL in Roswell Park Memorial Institute (RPMI) 1640 medium (Wisent Inc., Montreal, Quebec, CA) containing 20 % heat-inactivated Fetal Bovine Serum (FBS; Wisent Inc.), 1 % Gluta-plus (Wisent Inc.), 1 % penicillin/streptomycin (Wisent Inc.) in a 24-well culture plate and placed in an incubator (37 °C; 5 % CO_2). They were monitored daily for 7 days and were transferred to a T25 flask and kept at a cell density of 0.3 – 0.8 x 10^6 cells/mL to maintain exponential growth. Once the culture was established, the concentration of FBS was reduced to 10 %. After two weeks, the cells were aliquoted in cryotubes at 5 x 10^6 cells in 5 % dimethyl-sulfoxide (DMSO; Sigma Aldrich) and 95 % FBS to be stored in liquid nitrogen (-196 °C) for further use. Cells were counted and their viability was monitored by taking aliquots from the T25 flask and using Trypan Blue 0.4 % (Gibco™, Waltham, Massachusetts, US) to stain for dead cells.

For experimental conditions, THP-1 cells were constantly kept at a cell density of 0.3 – 0.8 x 10^6 cells/mL in RPMI 1640 medium containing 10 % FBS, 1 % Gluta-plus, 1 % penicillin/streptomycin in a T25 flask in an incubator (37 °C; 5 % CO_2). The cells were discarded once they reached passage 20 and a new frozen aliquot of THP-1 cells was thawed according to the manufacturer's protocol mentioned above.

2.2 THP-1 differentiation to M0/M1/M2, and adrenergic drug treatment.

Each experiment contained three types of cells that were being examined: 1) non-differentiated THP-1 cells, 2) M0-like cells, and 3) either M1-like (with or without IFNγ) or M2-like cells. In all experiments, the THP-1 cells were obtained from the stock T25 flask and were transferred into a round bottom 96-well culture plate at a cell density of 0.25 x 10^6 cells/mL for ELISA or into a flat bottom 24-well culture plate at 0.138 x 10^6 cells/mL for RT-qPCR. The cells were counted, and their viability was checked using Trypan Blue 0.4 % (Gibco™). Non-

differentiated THP-1 cells were incubated in fresh media for 48 hours. All other conditions received 15.4 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich; dissolved in DMSO) and were placed in the incubator (37 °C; 5 % CO_2). After 48 hours, the cell culture plate was centrifuged (5 mins, 100 x g, at room temperature), the supernatants were discarded and replaced with fresh medium. The cell culture plate was placed in the incubator to rest for 24 hours. After the rest period, the β-AR drugs, cytokines, and LPS were added to their respective conditions to obtain certain cell types. The THP-1 cells served as a "non-activated" control (labelled as: No Act), and the M0-like cells served as a PMA-treated control (labelled as: M0). The M1-like (with and without IFNγ) and the M2-like cells served as the "activated" conditions (labelled as: M1 or M2) and were treated with their respective cytokines and β-AR drugs at the same time. To obtain activated M1-like cells either 1) 1 ng/mL LPS (Sigma Aldrich) alone or 2) 1 ng/mL of LPS and 20 ng/mL IFNγ (GenScript Biotech, Piscataway, New Jersey, US) were added. Activated M2-like cells were obtained by adding 1 ng/mL of LPS, 20 ng/mL IL-4 (Cedarlane, Burlington, Ontario, CA), and 20 ng/mL IL-13 (Cedarlane).

The β-AR drugs and their concentrations used for *in vitro* cell culture treatment included: 10^{-5} M terbutaline (Sigma Aldrich) dissolved in MilliQ double distilled H_2O (ddH_2O), 10^{-5} M isoproterenol (Cayman Chemical Co., Ann Arbor, Michigan, US) dissolved in DMSO, 10^{-5} M bupranolol (Cayman Chemical Co.) dissolved in DMSO, 10^{-7} M ICI 118,551 (Cayman Chemical Co.) dissolved in MilliQ ddH_2O, and 10^{-5} M DMSO used as a solvent control group for isoproterenol, bupranolol, and PMA. First, the appropriate β-AR drug(s) were added to the PMA-treated THP-1 cells, followed immediately by LPS and cytokines. The conditions with the β-AR drugs consisted of either β-AR agonist alone, β-AR antagonist alone, or both β-AR agonist and antagonist. β-AR agonist alone served as the experimental condition, β-AR antagonist alone served as a control to confirm that the blocker did not have any independent effects, and the combination of both served also as a confirmation that the antagonist was able to block the effects. The treatments with the β-AR drugs were added immediately after one another and were added right after the LPS and cytokine treatments.

After drug, cytokine, and LPS treatment, the M1-like cells were placed in the incubator for 4 or 24 hours; and the M2-like cells for 4, 24, 48, 72, or 120 hours. The supernatants were collected and stored at -20 °C for future enzyme-linked immunosorbent assay (ELISA)

experiments. For quantitative real-time polymerase chain reaction (RT-qPCR) experiments, the cells for the THP-1, M0-like, M1-like (with and without IFNγ), and M2-like conditions were collected at the end of their respective incubation period for messenger RNA (mRNA) extraction.

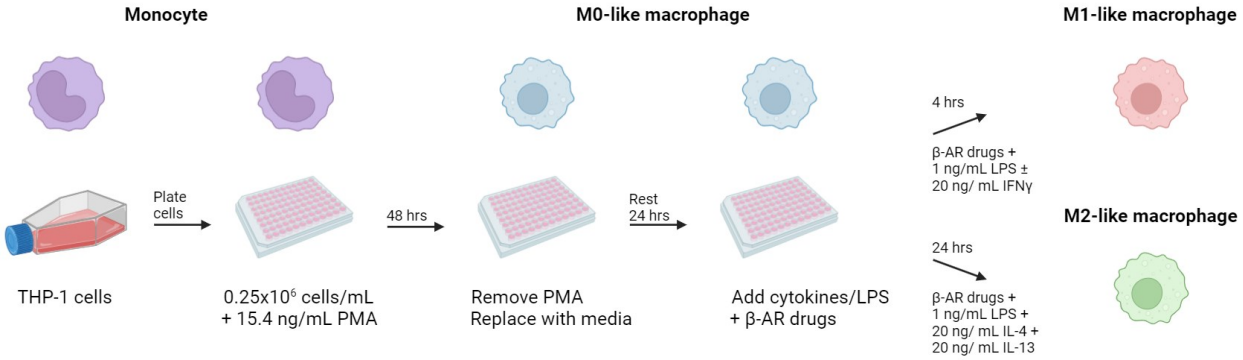


Figure 2. Schematic of THP-1 differentiation to M0/M1/M2 and adrenergic drug treatment. THP-1 monocytes were primed to M0-like macrophages using PMA; the M0-like cells were then polarized to M1- and M2-like macrophages using the appropriate cytokines/LPS and/or β-AR drugs. Figure was created using BioRender.

2.3. Enzyme-linked Immunosorbent Assay (ELISA)

The supernatants collected from cell culture were thawed at room temperature to measure cytokine concentrations for each condition. TNF, IL-10 and IFNγ cytokine concentrations were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (BD Bioscience, San Jose, CA, USA) as per manufacturer's protocol. Absorbance was measured using the BioTek Synergy H1 microplate reader (Agilent Technologies, Santa Clara, CA, United States) and concentrations in pg/mL were calculated and obtained through the Gen5 (Version 2.09) software based on the standard curve. A R^2 value of ≥0.98 for the standard curve was required, otherwise, the data was disregarded, and the ELISA was repeated.

2.4. Quantitative real-time polymerase chain reaction (RT-qPCR)

The expression of the β1-, β2-, and β3-adrenergic receptors in THP-1, M0-like, M1-like (with and without IFNγ), and M2-like cells was measured by quantitative real-time polymerase chain reaction (RT-qPCR). These receptors are encoded by the genes *ADRB1, ADRB2,* and *ADRB3*, respectively. To obtain the appropriate cell types, the THP-1 cells were cultured as described in section 2.2 and they were plated in a 24-well flat-bottom plate at a cell density of 0.138 x 10^6 cells/mL. THP-1 cells are non-adherent, but M0-, M1-, and M2-like cells will adhere to the wells. To detach the cells, the plate was centrifuged (5 mins, 100 x g, 9 ACC, 9 DEC, at room temperature) and the supernatant was removed to add 2 mL of cold phosphate buffered saline (PBS) with 0.025 M ethylenediaminetetraacetic acid (EDTA) for 10 minutes. Approximately 1 x 10^6 cells from each condition were collected to perform mRNA extraction using the PureLink™ RNA Mini Kit (Invitrogen™, Carlsbad, CA, USA) as per manufacturer's protocol. The concentration and purity of extracted RNA was measured using the NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the RNA was stored at -80 °C. A A_{260}/A_{280} ratio of approximately 2.0 for RNA indicates a pure RNA sample. The RNA was then converted into a cDNA library using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacture's protocol and stored at -80 °C. RT-qPCR was performed using the CFX96 Real Time System C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the respective TaqMan™ gene expression assays for each β-AR, as per manufacturer's protocol. Gene ID's for the β-ARs include: ADRB1 Hs02330048_s1 (β1-adrenergic receptor), ADRB2 Hs00240532_s1 (β2-adrenergic receptor), ADRB3 Hs00609046_m1 (β3-adrenergic receptor) (Thermo Fisher Scientific Inc.). The house keeping gene, Peptidylprolyl Isomerase A (PPIA; Gene ID: PPIA Hs99999904_m1), served as a control that had consistent expression levels. The Bio-Rad CFX Maestro Software (Version 1.1) was used to analyze the gene expression of each β-AR.

2.5. Statistical analysis

In every cell culture experiment, biological replicates were done in quintuplicate for each condition and each cell type experiment with AR drug treatment was repeated three times. The

ELISA samples were plated in duplicate technical replicates for each biological condition, and the concentration of cytokine for the duplicates were averaged. From the averaged duplicates, the standard deviation (SD) and average were calculated, and an upper and lower threshold was determined by adding the value of the SD to the average and subtracting the SD from the average, respectively. Any outliers that fell outside of the determined range, by calculating the upper and lower threshold, were removed. This process was performed using Microsoft® Excel® for Microsoft 365 MSO (Version 2403). The ELISA data was presented as either a compiled figure or an individual figure and were analyzed and plotted using GraphPad Prism (Version 10.0.2). The individual figures included the averaged biological quintuplicate data without outliers, and the error bars were expressed at the SD. The compiled figure demonstrated the pooled data from the three repeated cell type experiments with AR drug treatment. The data from the three individual experiments were normalized so that the "M1" or "M2" condition would equal 1 and the remaining conditions would show as a fold change in relation to the "M1" or "M2" condition. This was done by calculating the average cytokine concentration of the quintuplicate "M1" or "M2" sample, then dividing all values for each condition by the "M1" or "M2" average value. The compiled figures then included all averaged biological quintuplicate data without outliers from three experiments, and the error bars were expressed as the SD. The data for the individual and compiled figures was then analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test, with $P < 0.05$ considered as significant ($P>0.05$ (ns), ≤ 0.0332 (*), ≤ 0.0021 (**), ≤ 0.0002 (***), <0.0001 (****)). The RT-qPCR data was presented as individual figures and were analyzed using Microsoft® Excel® for Microsoft 365 MSO (Version 2403) and plotted using GraphPad Prism (Version 10.0.2). The RT-qPCR samples were plated in duplicate as technical replicates, and the Cq values for the duplicates were averaged. The expression of the β-AR was normalized to the housekeeping gene (PPIA) by calculating ΔCt, where $\Delta Ct = Cq_{\text{target gene}} - Cq_{\text{housekeeping gene}}$ and the Cq values were the averages of the duplicates. The "No Act" condition was then used to determine the fold increase in relative expression by first calculating ΔΔCt, then $2^{-\Delta\Delta Ct}$. $\Delta\Delta Ct = \Delta Ct_{\text{M0-like or M1/M2 condition}} - \Delta Ct_{\text{No Act condition}}$, then this value is inserted into the equation $2^{-\Delta\Delta Ct}$ to obtain the normalized relative expression of the gene in comparison to the "No Act" condition.

Chapter 3. Results

Macrophages play a critical role in the adaptive immune response, balancing inflammation to eliminate pathogens while minimizing tissue damage. Disruptions in this balance can lead to autoimmune diseases, severe infections, and other inflammatory disorders. Furthermore, studies show that monocyte-derived macrophages express β-adrenergic receptors on the surface of the cell, which are receptors of the sympathetic nervous system (SNS) that respond to catecholamines hormones such as norepinephrine and epinephrine. This expression of β-ARs in immune cells sheds light on a connection between the SNS and the immune system. The goal of my thesis is to explore the interactions between this connection by treating a monocyte-like cell line with β-adrenergic receptor (β-AR) agonist and antagonist drugs. Previous studies have shown that β2-AR drugs possess anti-inflammatory properties in macrophages, suggesting their potential as therapeutic agents. However, there is limited research on how these drugs specifically affect M1 and M2 macrophage phenotypes and their interaction with the SNS. Exploring this topic could enhance our understanding of how these drugs can be used to treat inflammatory and autoimmune conditions where macrophage imbalance plays a role in disease progression.

The first aim of this project was to establish an *in vitro* M1- and M2-like model to measure TNF and IL-10 secretion in polarized macrophages. In order to accomplish this, the proper doses of reagents and timepoints were determined for polarization based on TNF and IL-10 secretion in M1- and M2-like cells, respectively, using ELISA. M0-like macrophages were differentiated from THP-1 cells using 15.4 ng/mL of PMA for 24 hours with a 48-hour rest time based on the systematic review by Mohd Yasin et al. (2022). M1-like cells required a dose response for LPS, and both M1- and M2-like cells required a time-point experiment. The concentration of 20 ng/mL of IFNγ (for M1-like cells) and 20 ng/mL of IL-4 and IL-13 (for M2-like cells) were acquired from the systematic review by Mohd Yasin et al. (2022). |The expression of the *ADRB1*, *ADRB2,* and *ADRB3* genes was determined in all monocyte-derived macrophages using RT-qPCR to verify the presence of the three β-AR subtypes.

The second aim was to measure the immunomodulatory effects of β-AR drugs on TNF and IL-10 secretion in M1- and M2-like macrophage cells, using the M1/M2 model that was established in the first aim. Experiments were performed using the M1/M2 model with different combinations of β-agonist and antagonist drugs to observe any effect on TNF and IL-10 secretion.

3.1. 1 ng/mL of LPS is the appropriate dosage for M1-like cells to produce sufficient TNF.

An LPS dose response was performed to determine the appropriate concentration of LPS needed for polarization from M0- to M1-like cells for the M1 model based on TNF secretion by ELISA measurement. A ten-fold dosage range of 0.001 – 10 ng/mL of LPS was determined based on research articles (Mohd Yasin et al., 2022). The "No Act" and M0-like cells showed no production of TNF, and neither did the vehicle control of 15.4 ng/mL of DMSO. Production of TNF began at 0.1 ng/mL of LPS and showed a significant ($P<0.0001$) increase between 0.1 to 1 ng/mL, and 1 to 10 ng/mL of LPS (Figure 3). At 10 ng/mL, the TNF production was above the threshold concentration and therefore deemed as an inappropriate concentration since it would require several dilutions to fall below the threshold. 1 ng/mL of LPS was chosen as an appropriate dosage since it produced ~10 000 pg/mL of TNF.

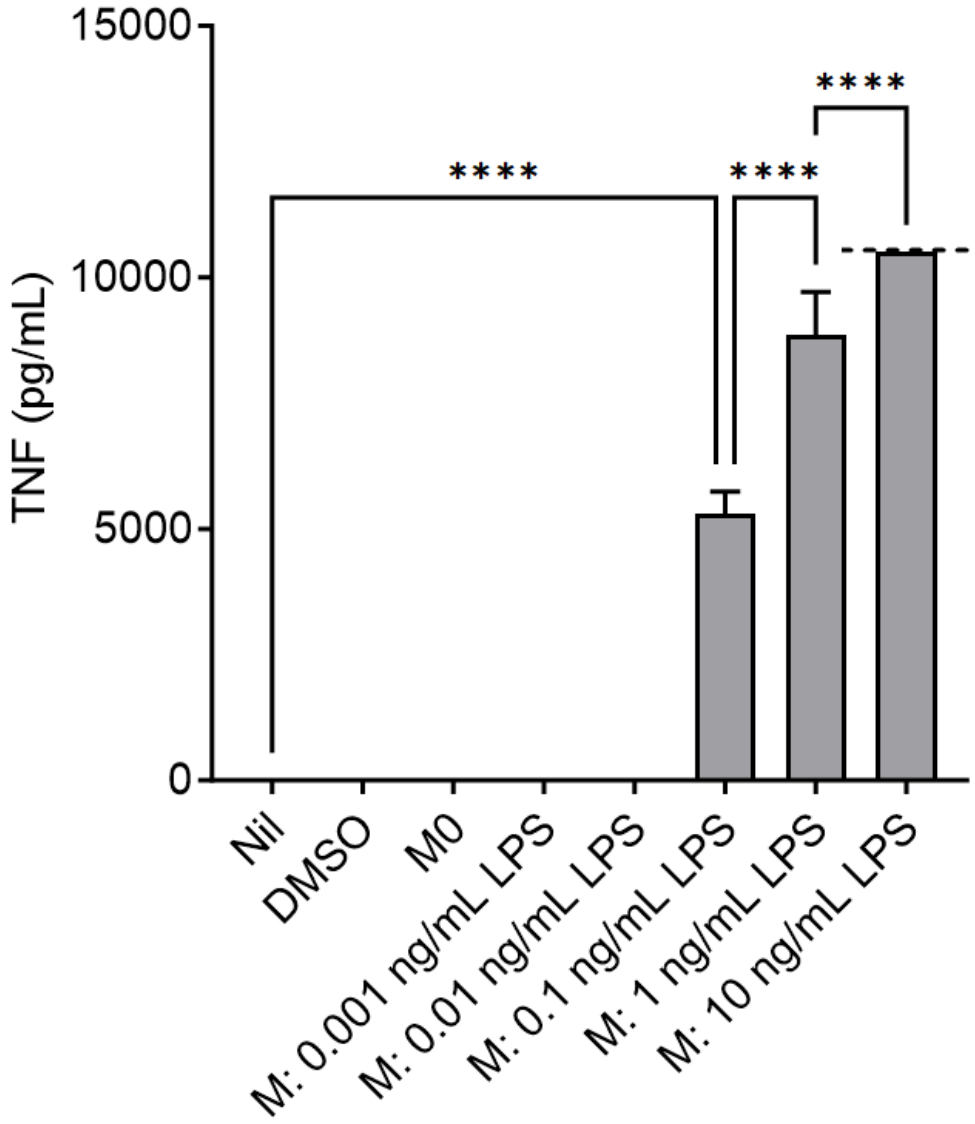


Figure 3. TNF cytokine production resulting from LPS dose response in M1-like cells (without IFNγ) at 4 hours. TNF concentration was measured in pg/mL (y-axis) for each condition. Nil: THP-1 cells; DMSO: THP-1 cells treated with 15.4 ng/mL of DMSO, served as a solvent control for PMA; M0: M0-like cells (15.4 ng/mL of PMA); and M: M1-like cells without IFNγ with varying concentrations of LPS. The dotted line represents a threshold in which the samples were too concentrated to measure.

3.2. The 4-hour time-point is favorable for TNF production in M1-like cells.

To determine the appropriate time-point for polarization of M1-like cells in the M1 model, the M0-like cells were polarized to M1-like cells using 1 ng/mL LPS (with and without 20 ng/mL of IFNγ) for either 4 or 24 hours and the TNF cytokine production was measured using ELISA. These time-points were chosen based on research articles presented in the systematic review by Mohd Yasin et al. (2022). Both the M1-like cells with and without IFNγ showed significant ($P<0.0001$) TNF cytokine production at 4 hours compared to 24 hours (Figure 4). Therefore, the 4-hour time-point was selected for differentiation of M1-like cells.

To ensure that the cells were truly M1-like, IL-10 cytokine concentration was measured in M1-like cells (with and without IFNγ) at 4 and 24 hours to determine if they would produce IL-10, the cytokine usually produced by M2-like cells. At 4 hours, none of the THP-1, M0-like, and M1-like cells (with and without IFNγ) produced IL-10. The positive control condition containing M2-like cells produced ~50 pg/mL of IL-10 (Figure 5). At 24 hours, the M1-like cells began to produce IL-10; the M1-like cells without IFNγ produced a significantly ($P<0.0001$) higher amount compared to the M1-like cells with IFNγ. However, these values are significantly ($P<0.0001$) less than the positive control of M2-like cells at 24 hours (Figure 5). This further solidified the reasoning behind selecting the 4-hour time-point for the M1-like cells, since they do not produce IL-10, and will therefore define a true M1-like cell.

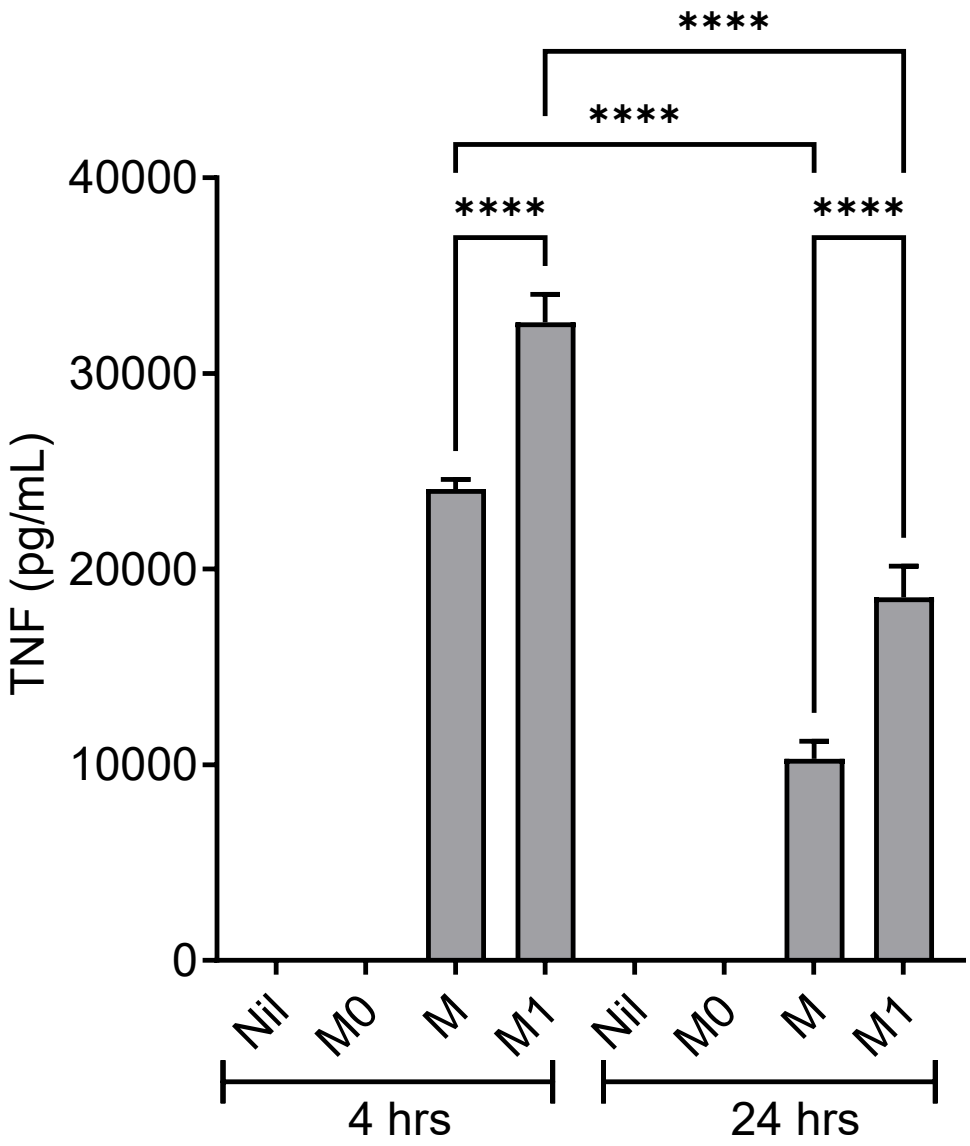


Figure 4. TNF cytokine production in M1-like cells (with and without IFNγ) at 4-hour and 24-hour time-points. TNF concentration was measured in pg/mL (y-axis) for each condition. Nil: THP-1 cells; M0: M0-like cells (15.4 ng/mL PMA); M: M1-like cells (1 ng/mL LPS); and M1: M1-like cells (1 ng/mL LPS + 20 ng/mL IFNγ). The time-point is denoted as either "4 hrs" for 4 hours or "24 hrs" for 24 hours.

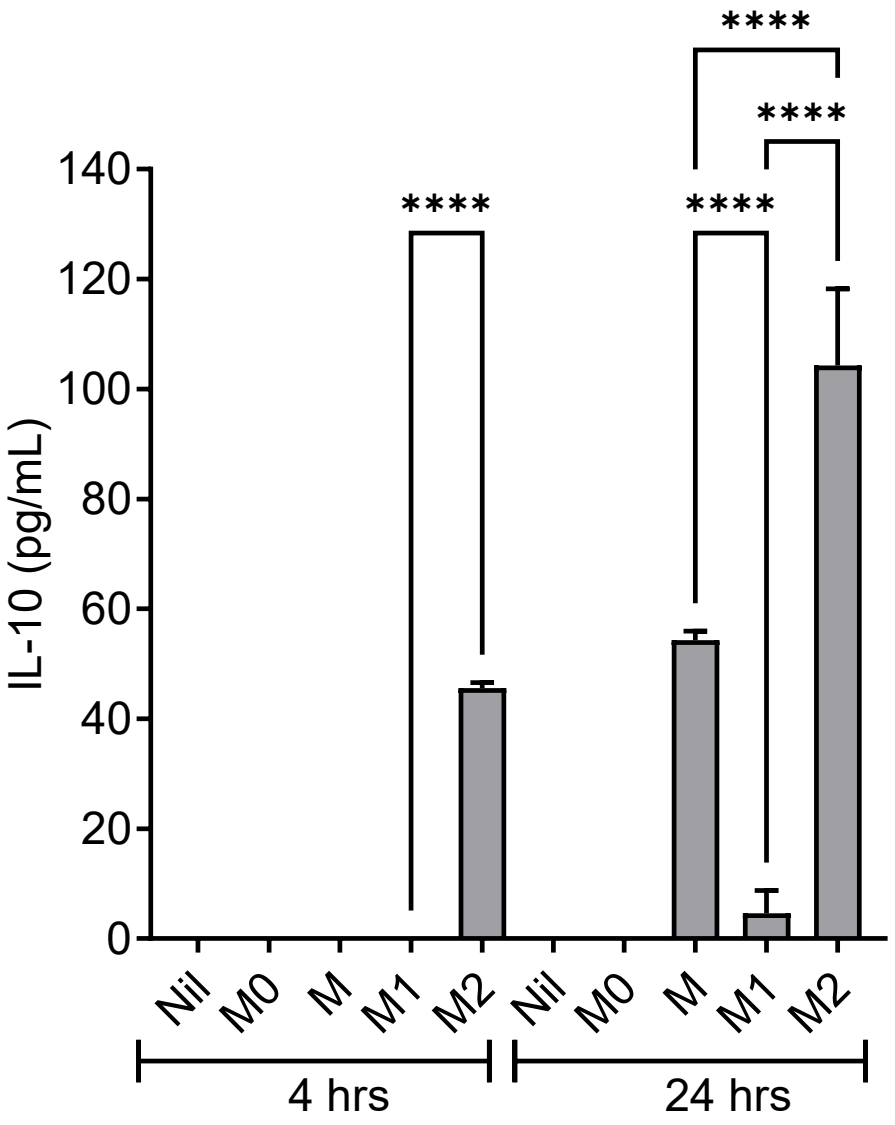


Figure 5. IL-10 cytokine production in M1-like cells (with and without IFNγ) at 4-hour and 24-hour time-points. IL-10 concentration was measured in pg/mL (y-axis) for each condition. Nil: THP-1 cells; M0: M0-like cells (15.4 ng/mL PMA); M: M1-like cells (1 ng/mL LPS); M1: M1-like cells (1 ng/mL LPS + 20 ng/mL IFNγ); and M2: M2-like cells (1 ng/mL LPS + 20 ng/mL IL-4 + 20 ng/mL IL-13). The time-point is denoted as either "4 hrs" for 4 hours or "24 hrs" for 24 hours.

3.3. The 24-hour time-point is favorable for IL-10 production in M2-like cells.

To determine the appropriate time-point for polarization of M2-like cells in the M2 model, the M0-like cells were polarized into M2-like cells for either 4, 24, 48, 72, or 120 hours and the IL-10 cytokine production was measured using ELISA. The time-points were determined based on research articles presented in the systematic review (Mohd Yasin et al., 2022). The M2-like cells required LPS to produce significant ($P<0.0001$) levels of IL-10 for all time points compared to M2-like cells that were treated with only 20 ng/mL IL-4 and 20 ng/mL IL-13 (Figure S1). The production of IL-10 for M2-like cells treated with 1 ng/mL of LPS increased significantly ($P\leq0.0002$) between 4 and 24 hours (Figure 6). However, the IL-10 production begins to plateau after 24 hours of treatment, with no significant difference between 24 to 48 hours, 48 to 72 hours, and 72 to 120 hours (Figure 6). There was significance ($P\leq0.0332$) comparing the 24- and 72-hour timepoint and significance ($P\leq0.0002$) comparing 24 versus 120 hours. As a result, the 24-hour time-point was chosen for differentiation of the M2-like cells for reasons of efficiency, since incubation for longer periods resulted in only a slight improvement of IL-10 production.

To ensure that the cells were truly M2-like, TNF cytokine concentration was measured at 4 and 24 hours to determine if they would produce TNF, the cytokine usually produced by M1-like cells. Since 1 ng/mL of LPS is required to differentiate the M0-like cells to M2-like cells, it is expected that some TNF will be produced. At 4 hours, ~20 000 pg/mL of TNF was produced by M2-like cells, but this is significantly less ($P<0.0001$) than the M1-like cells (with IFNγ) (Figure 7). At 24 hours, the level of TNF produced by M2-like cells decreases significantly ($P<0.0001$) compared to 4 hours, and the level is also significantly less ($P\leq0.0002$) than the M1-like cells (with IFNγ) (Figure 7). Consequently, the 24-hour time-point had further proven to be the appropriate choice since it adequately demonstrated an M2-like cell profile.

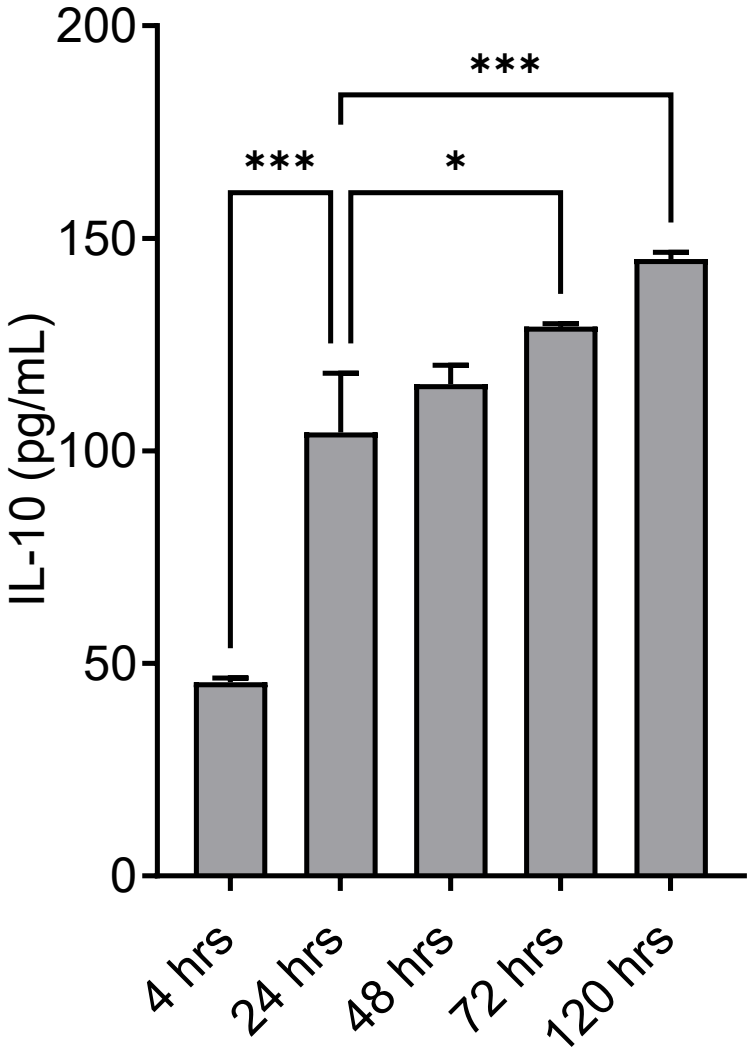


Figure 6. IL-10 cytokine production in M2-like cells at 4-, 24-, 48-, 72-, and 120-hour time-points. All conditions contain THP-1 cells primed with 15.4 ng/mL PMA to differentiate into M0-like cells, which were then treated with 1 ng/mL of LPS, 20 ng/mL IL-4, and 20 ng/mL IL-13 to obtain M2-like cells. IL-10 concentration was measured in pg/mL (y-axis) for each condition. The time-point is denoted as "4 hrs" for 4 hours, "24 hrs" for 24 hours, and so on. The "Nil" (THP-1 cells), M0-like cells (15.4 ng/mL of PMA) and M2-like cells (without LPS) control conditions for each timepoint can be found in the Supplementary Figures (Supplementary Figure S1).

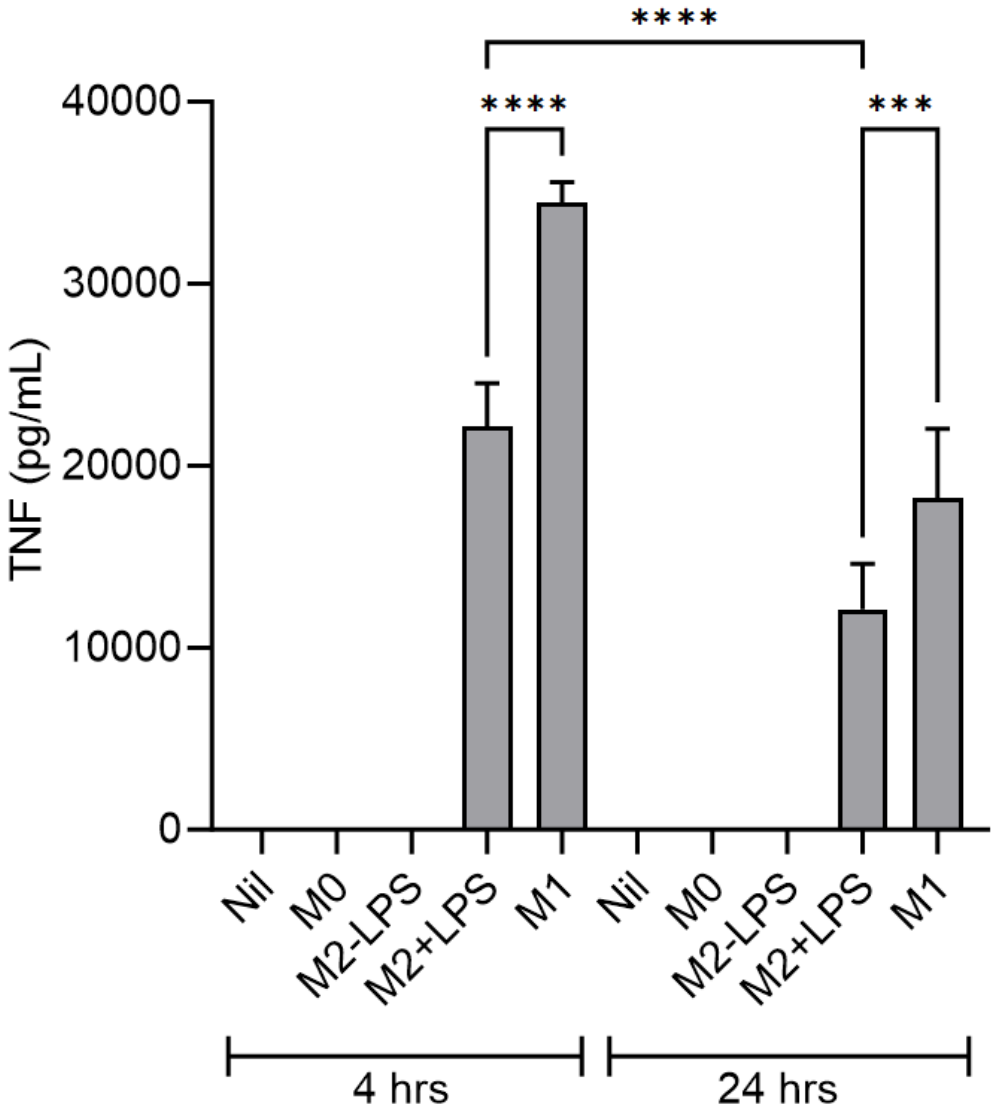


Figure 7. TNF cytokine production in M2-like cells at 4-hour and 24-hour time-points. TNF concentration was measured in pg/mL (y-axis) for each condition. Nil: THP-1 cells; M0: M0-like cells (15.4 ng/mL PMA); M2-LPS: M2-like cells (20 ng/mL IL-4 + 20 ng/mL IL-13); M2+LPS: M2-like cells (1 ng/mL LPS + 20 ng/mL IL-4 + 20 ng/mL IL-13); M1: M1-like cells (1 ng/mL LPS + 20 ng/mL IFNγ) served as a positive control. The time-point is denoted as either "4 hrs" for 4 hours or "24 hrs" for 24 hours.

3.4. The ADRB1 gene is up-regulated in M1-like cells with IFNγ and down-regulated in M2-like cells.

Gene expression of the β1-adrenergic receptor in THP-1, M0-like, M1-like (with and without IFNγ), and M2-like cells was measured using RT-qPCR to determine the presence and observe any up- or down-regulation of the receptor in different phenotypes. The gene corresponding to the β1-adrenergic receptor is the *ADRB1* gene. *ADRB1* mRNA was measured at 4 hours for M1-like cells (with and without IFNγ) and 24 hours for M2-like cells. The data in each graph in Figure 8 is normalized to the "No Act" (THP-1 cells) condition.

M0-like cells and M1-like cells without IFNγ showed no significant difference in *ADRB1* mRNA compared to the non-activated THP-1 cells (Figure 8a). Notably, M1-like cells without IFNγ still showed a small trend in up-regulation of the *ADRB1* mRNA. M1-like cells with IFNγ greatly increased *ADRB1* mRNA ($P \leq 0.0002$) in comparison with THP-1, M0-like, and M1-like cells without IFNγ (Figure 8a).

Relative to THP-1 cells, the *ADRB1* gene expression significantly ($P < 0.0001$) decreased in M0-like cells and M2-like cells (Figure 8b). Remarkably, the M0-like cells showed no significant change in *ADRB1* gene expression at the 4-hour time-point, but the 24-hour time-point showed a significant ($P < 0.0001$) decrease. The *ADRB1* mRNA was expressed slightly less in M2-like cells ($P \leq 0.0021$) compared to M0-like cells (Figure 8b).

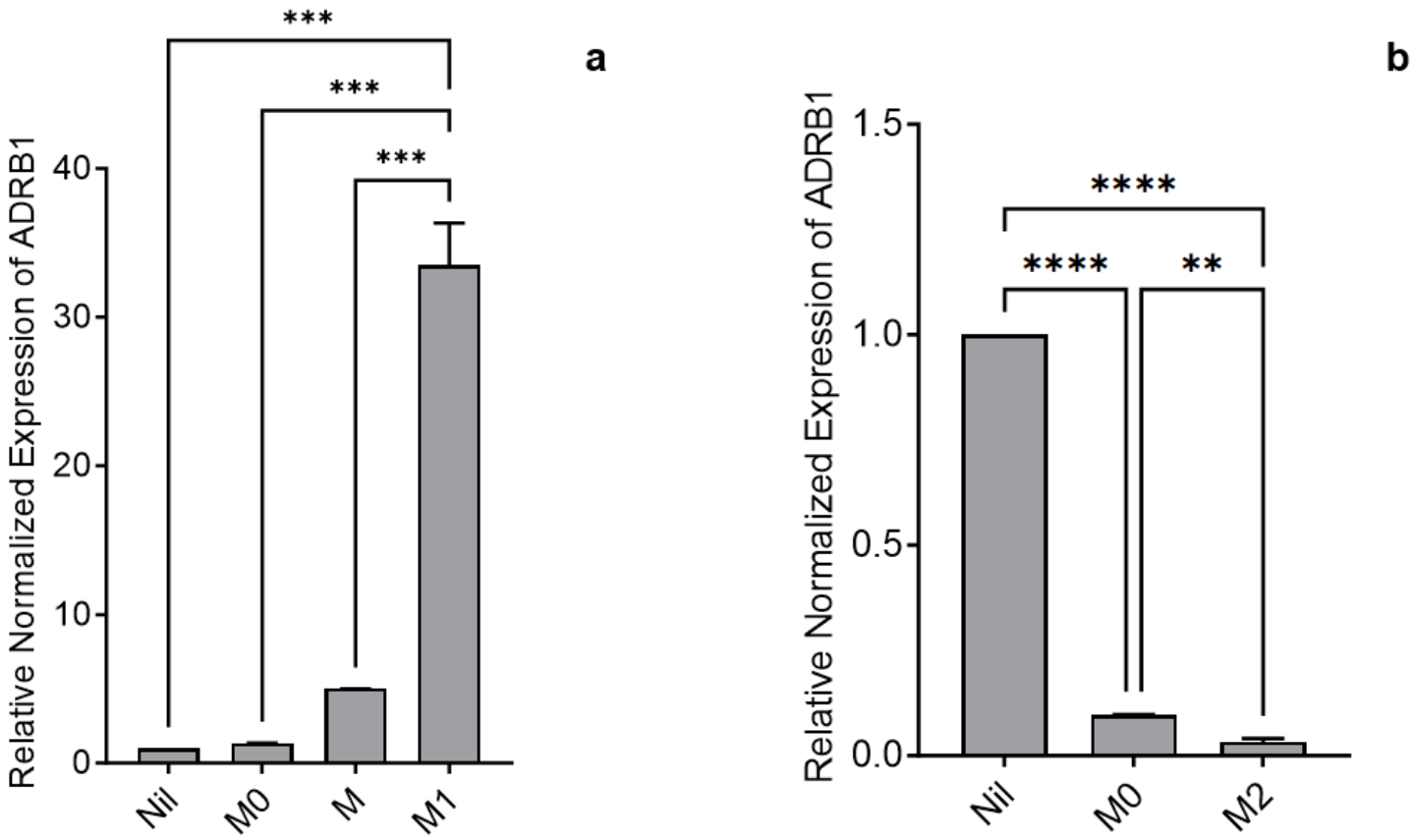


Figure 8. *ADRB1* gene expression in THP-1, M0-like, (a) M1-like (with and without IFNγ) cells at 4 hours, and (b) M2-like cells at 24 hours. Relative normalized expression of *ADBR1* is plotted on the y-axis and depicts gene expression that was calculated relative to the house keeping gene, *PPIA*, and normalized to the "Nil" condition. In both panel **(a)** and **(b)**, Nil: THP-1 cells and M0: M0-like cells (15.4 ng/mL PMA). In panel **(a)**, M: M1-like cells (1 ng/mL LPS) and M1: M1-like cells (1 ng/mL LPS + 20 ng/mL IFNγ) at the 4-hour time-point. In panel **(b)**, M2: M2-like cells (1 ng/mL LPS + 20 ng/mL IL-4 + 20 ng/mL IL-13) at the 24-hour time-point. Note the differences in scale bars between the panels.

3.5. The ADRB2 gene is upregulated in M1-like cells with IFNγ and downregulated in M2-like cells.

Gene expression of the β2-adrenergic receptor in THP-1, M0-like, M1-like (with and without IFNγ), and M2-like cells was measured using RT-qPCR to determine the presence and observe any up- or down-regulation of the receptor. The gene corresponding to the β2-adrenergic receptor is the *ADRB2* gene. *ADRB2* mRNA was measured at 4 hours for M1-like cells (with and without IFNγ) and 24 hours for M2-like cells. The data in each graph in Figure 9 is normalized to the "No Act" (THP-1 cells) condition.

There was no significant change in expression of the *ADRB2* gene between the THP-1 cells and the M0-like cells or M1-like cells without IFNγ (Figure 9a). Like the *ADRB1* mRNA levels, although the M1-like cells without IFNγ show no significant difference in *ADRB2* mRNA compared to the M0-like cells, we observe a slight trend in up-regulation. *ADRB2* mRNA increased in M1-like cells with IFNγ in comparison to the non-activated THP-1 cells ($P \leq 0.0002$), M0-like cells ($P \leq 0.0002$), and the M1-like cells without IFNγ ($P \leq 0.0021$) (Figure 9a).

M0-like cells and M2-like cells showed a significant ($P < 0.0001$) decrease in *ADRB2* mRNA relative to the THP-1 cells (Figure 9b). Again, mRNA levels in M0-like cells showed no significant difference at the 4-hour time-point but decreased ($P < 0.0001$) significantly at the 24-hour time-point. In comparison to the M0-like cells, the mRNA decreased significantly ($P \leq 0.0002$) for the M2-like cell.

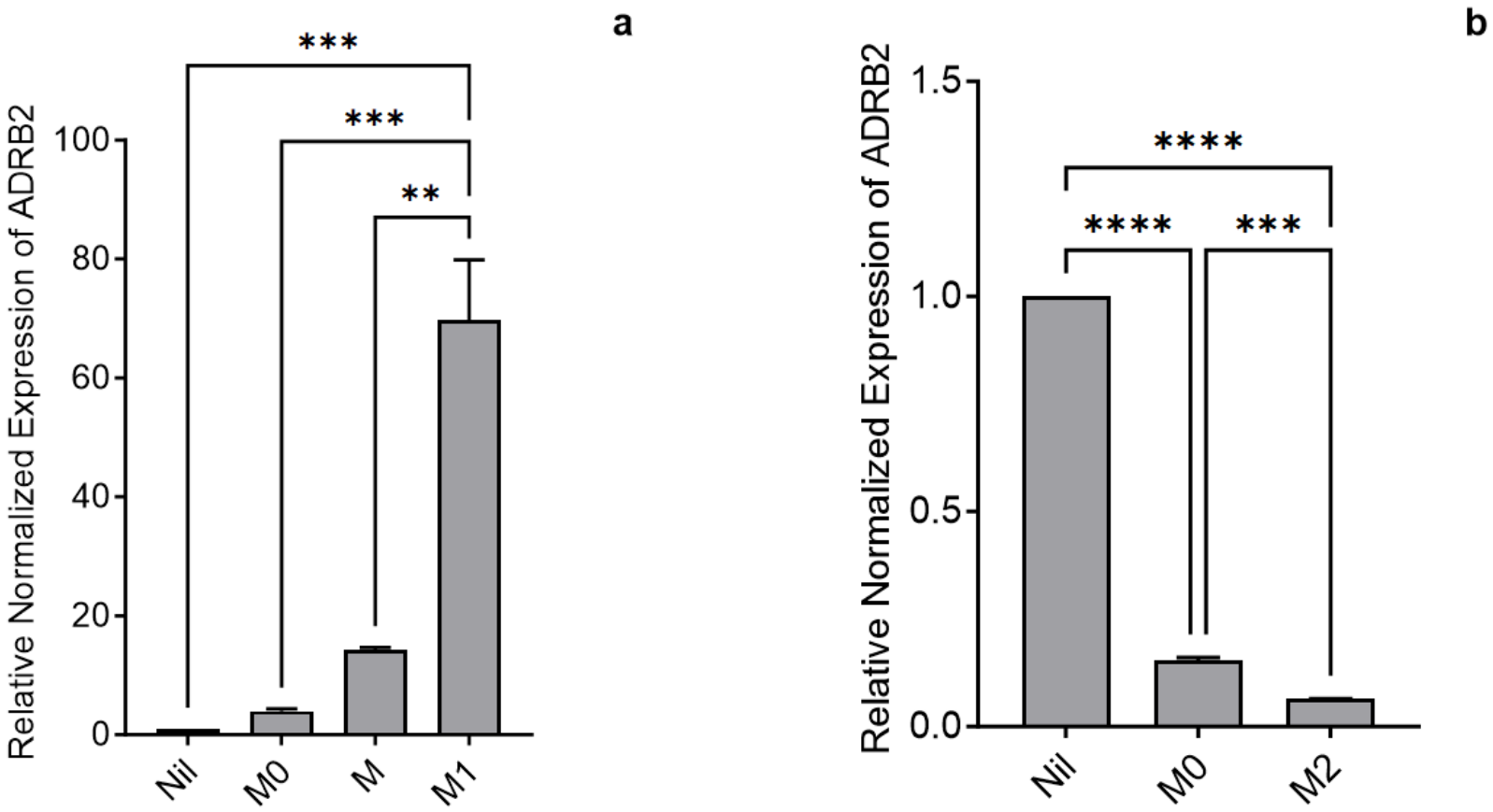


Figure 9. *ADRB2* gene expression in THP-1, M0-like, (a) M1-like (with and without IFNγ) cells at 4 hours, and (b) M2-like cells at 24 hours. Relative normalized expression of *ADBR2* is plotted on the y-axis and depicts gene expression that was calculated relative to the house keeping gene, *PPIA*, and normalized to the "Nil" condition. In both panel **(a)** and **(b)**, Nil: THP-1 cells and M0: M0-like cells (15.4 ng/mL PMA). In panel **(a)**, M: M1-like cells (1 ng/mL LPS) and M1: M1-like cells (1 ng/mL LPS + 20 ng/mL IFNγ) at the 4-hour time-point. In panel **(b)**, M2: M2-like cells (1 ng/mL LPS + 20 ng/mL IL-4 + 20 ng/mL IL-13) at the 24-hour time-point.

3.6. The ADRB3 gene is not expressed in M1-like cells (with and without IFNγ) and M2-like cells.

Gene expression of the β3-adrenergic receptor in THP-1, M0-like, M1-like (with and without IFNγ), and M2-like cells was measured to determine the presence and observe any up- or down-regulation of the receptor. The gene corresponding to the β3-adrenergic receptor is the *ADRB3* gene. *ADRB3* mRNA was measured at 4 hours for M1-like cells (with and without IFNγ) and 24 hours for M2-like cells.

Raw data showed no *ADRB3* mRNA in THP-1, M0-like, M1-like (with and without IFNγ), and M2-like cells. Currently, only two studies show that macrophages express the β3-adrenergic receptor (Hadi et al., 2017; Zheng et al., 2020), therefore, further exploration may be required.

3.7. Effects of terbutaline and isoproterenol with non-selective β-blocker bupranolol on M1-like macrophages (with and without IFNγ) and M2-like macrophages

Having established the *in vitro* model for polarization of M1- and M2-like cells, I next tested the effect of β-AR drugs on TNF and IL-10 production in M1/M2 phenotypes. ELISA was used to measure the change in TNF or IL-10 production after treatment with β-AR agonists and antagonists. M1-like cells (with and without IFNγ) and M2-like cells were treated with either bupranolol, terbutaline, or isoproterenol, or a combination of β-AR antagonist and agonist. DMSO was used as a vehicle control since the PMA, bupranolol, and isoproterenol drugs were dissolved in DMSO. All conditions were normalized to the "Act" condition to observe the fold change in TNF or IL-10 concentration compared to the M1-like cells (with and without IFNγ) or M2-like cells respectively.

3.7.1. Terbutaline and isoproterenol decreased TNF production in M1-like cells without IFNγ and were blocked by bupranolol.

Both terbutaline and isoproterenol decreased TNF production with significance ($P<0.0001$). Whereas terbutaline or isoproterenol combined with bupranolol showed a significant ($P<0.0001$) increase in TNF back to the same level as the "Act" control. Bupranolol therefore reversed the effect of the β-AR agonists by increasing the TNF production so that there is no significance compared to the "Act" condition (Figure 10). Bupranolol alone showed a significant ($P\leq0.0332$) increase in TNF production in the compiled data which was not expected since it is a blocker (Figure 10). However, two individual experiments showed that bupranolol does not increase TNF concentration (Figure S2). There was no significant difference in TNF production for the DMSO vehicle control compared to the "Act" condition.

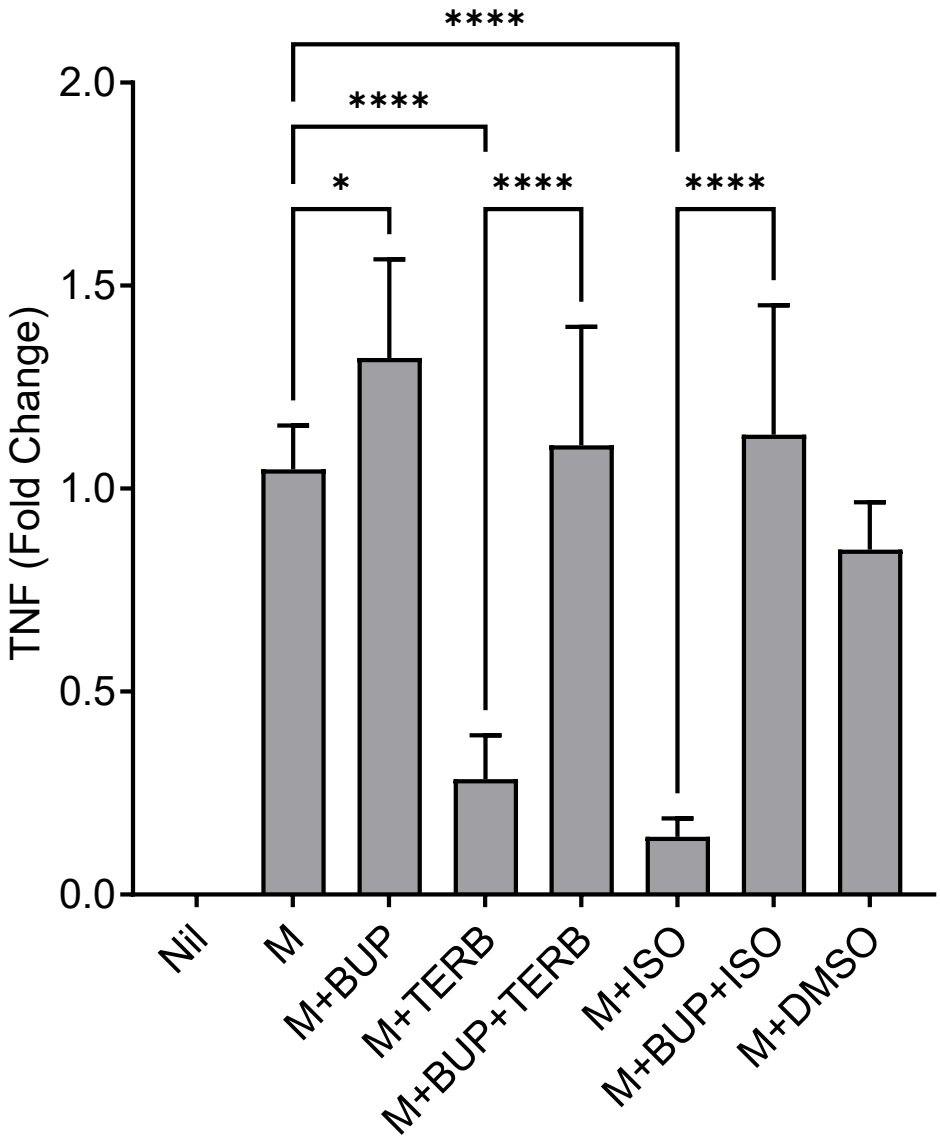


Figure 10. TNF cytokine production in M1-like cells without IFNγ treated terbutaline, isoproterenol and/or bupranolol at 4 hours. The fold change for each condition was normalized to the "M" condition. Nil: THP-1 cells and M: M1-like cells (1 ng/mL LPS). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and BUP: 10^{-5} M bupranolol. DMSO: 10^{-5} M DMSO solvent control. Data is compiled from three experiments.

3.7.2. Terbutaline and isoproterenol decreased TNF production in M1-like cells with IFNγ and were blocked by bupranolol.

Terbutaline and isoproterenol inhibited TNF production ($P \leq 0.0002$, $P < 0.0001$) in M1-like cells with IFNγ. Thus, the effects of isoproterenol are β-AR dependent. The addition of bupranolol to either the terbutaline- or isoproterenol-treated M1 like cells resulted in increase of TNF production. This increase showed no significant difference compared to the "Act" condition, and therefore bupranolol blocked the effects of terbutaline and isoproterenol (Figure 11). Bupranolol alone and the DMSO vehicle control had no effect on TNF production compared to the "Act" condition, which as expected since there were no agonists present to block and the blocker alone should not have an effect (Figure 11).

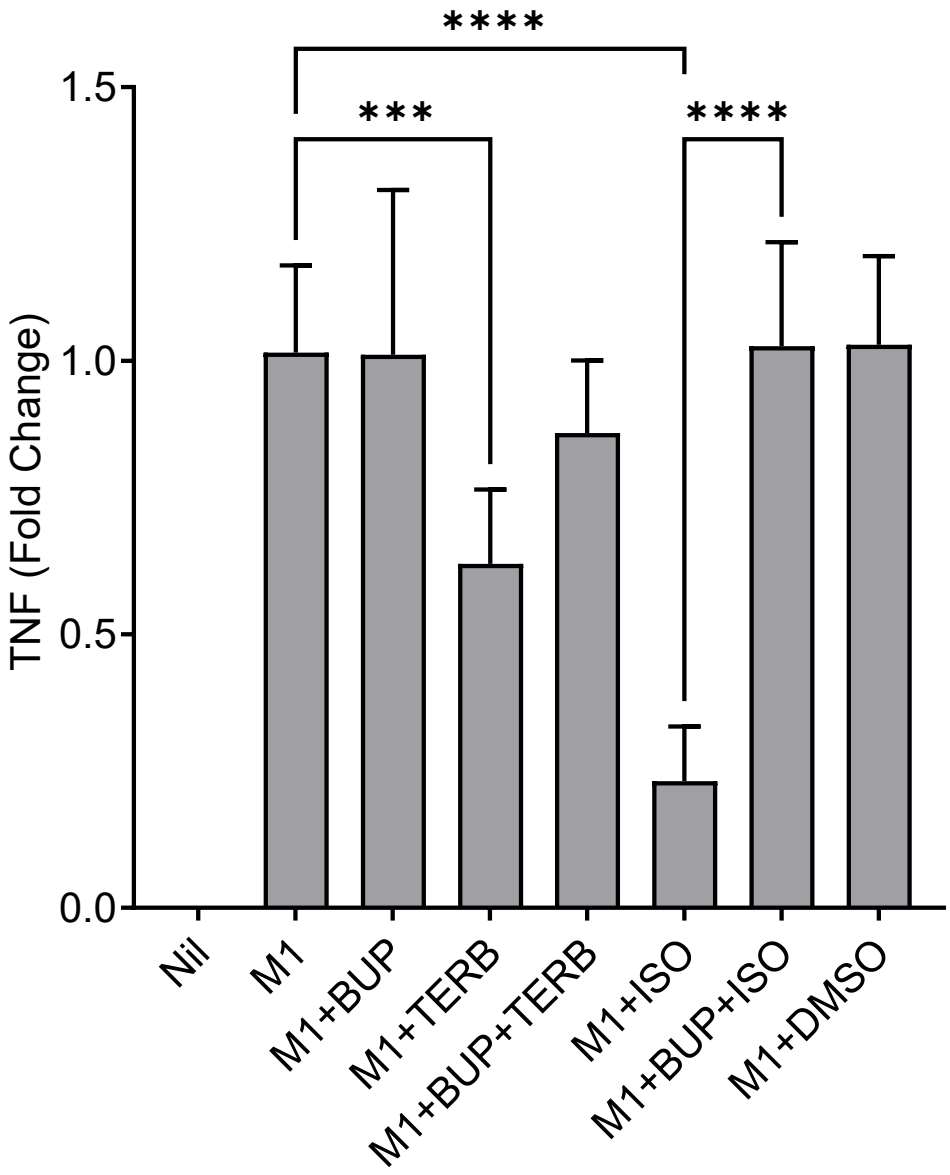


Figure 11. TNF cytokine production in M1-like cells with IFNγ treated with terbutaline, isoproterenol and/or bupranolol at 4 hours. The fold change for each condition was normalized to the "M1" condition. Nil: THP-1 cells and M1: M1-like cells (1 ng/mL LPS + 20 ng/mL IFNγ). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and BUP: 10^{-5} M bupranolol. DMSO: 10^{-5} M DMSO solvent control. Data is compiled from three experiments.

3.7.3. Terbutaline and isoproterenol increased IL-10 production in M2-like cells and were blocked by bupranolol.

Terbutaline and isoproterenol increased IL-10 cytokine production significantly ($P<0.0001$), which was opposite to what was observed with TNF in M1-like cells (with and without IFNγ). Bupranolol significantly ($P<0.0001$) blocked the effects of terbutaline and isoproterenol relative to terbutaline and isoproterenol alone (Figure 12). Therefore, bupranolol reversed the effects of terbutaline and isoproterenol since the IL-10 concentration became nonsignificant in comparison to the "Act" control. Bupranolol alone and the DMSO vehicle control showed no significant change in IL-10 production compared to M2-like cells (Figure 12).

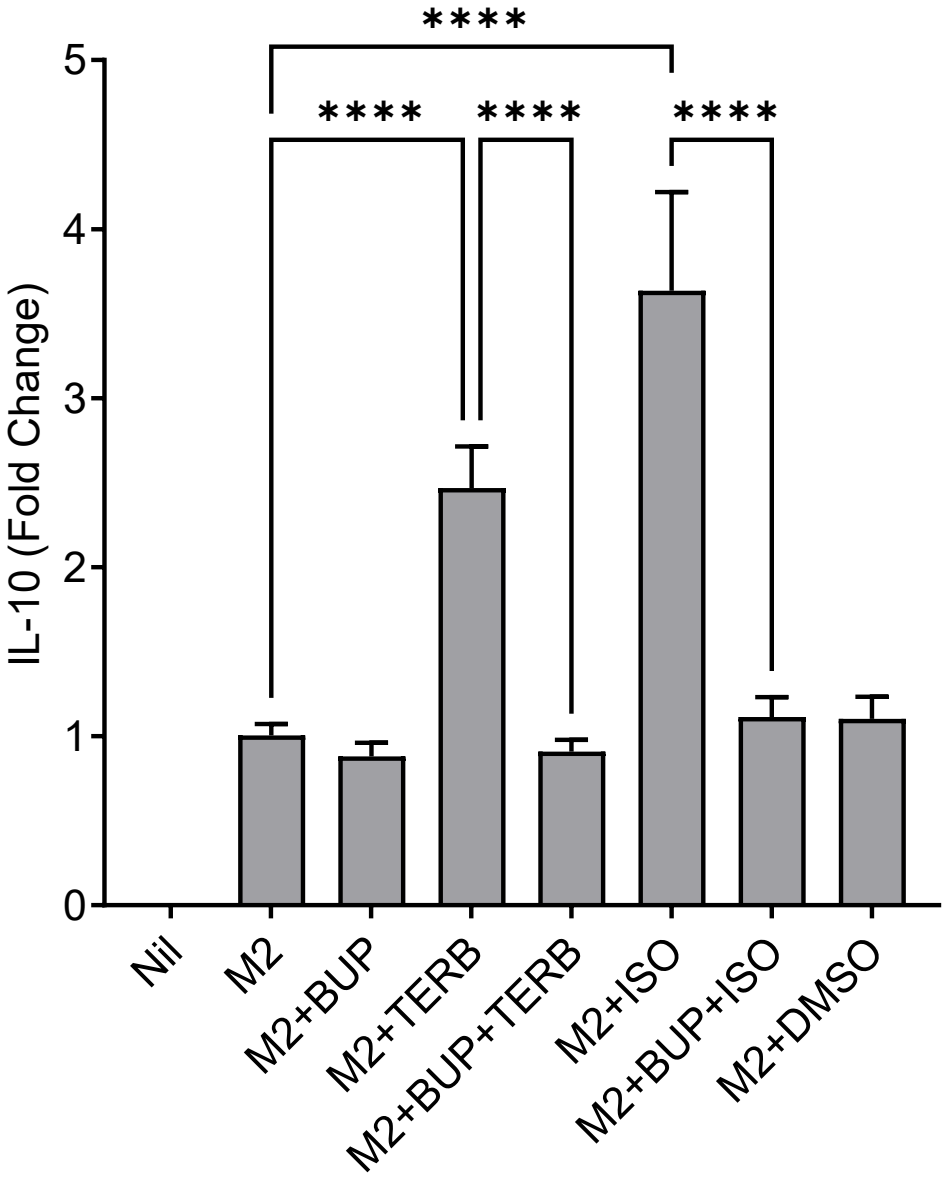


Figure 12. IL-10 cytokine production in M2-like cells treated with terbutaline, isoproterenol and/or bupranolol at 24 hours. The fold change for each condition was normalized to the "M2" condition. Nil: THP-1 cells and M2: M2-like cells (1 ng/mL LPS + 20 ng/mL IL-4 + 20 ng/mL IL-13). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and BUP: 10^{-5} M bupranolol. DMSO: 10^{-5} M DMSO solvent control. Data is compiled from three experiments.

3.8. Effects of terbutaline and isoproterenol with β2-selective blocker ICI 118,551 on M1-like macrophages (with and without IFNγ) and M2-like macrophages.

After testing the non-selective β-AR antagonist, I then tested the β2-selective antagonist ICI 118,551 to observe any differences in TNF or IL-10 production in M1/M2 phenotypes when receptor specificity is changed. ELISA was used to measure the change in TNF or IL-10 production after treatment with β-AR agonists and antagonists. M1-like cells (with and without IFNγ) and M2-like cells were treated with either ICI 118,551, terbutaline, or isoproterenol, or a combination of β-AR antagonist and agonist. DMSO was used as a vehicle control since the PMA and isoproterenol were dissolved in DMSO. All conditions were normalized to the "Act" condition to observe the fold change in TNF or IL-10 concentration compared to the M1-like cells (with and without IFNγ) or M2-like cells respectively.

3.8.1. Terbutaline and isoproterenol decreased TNF production in M1-like cells without IFNγ and terbutaline was blocked by ICI 118,551, but not isoproterenol.

Both terbutaline and isoproterenol alone decreased TNF production with significance ($P<0.0001$), however, ICI 118,551 only reversed the effect of terbutaline. ICI 118,551 combined with terbutaline had no significant difference compared to the "Act" control and therefore ICI 118,551 blocked terbutaline. (Figure 13) However, ICI 118,551 did not block isoproterenol since TNF production only increased a slight amount and is still lower ($P<0.0001$) compared to the "Act" condition (Figure 13). ICI 118,551 and the DMSO vehicle control had no effect on TNF production compared to the "Act" condition (Figure 13).

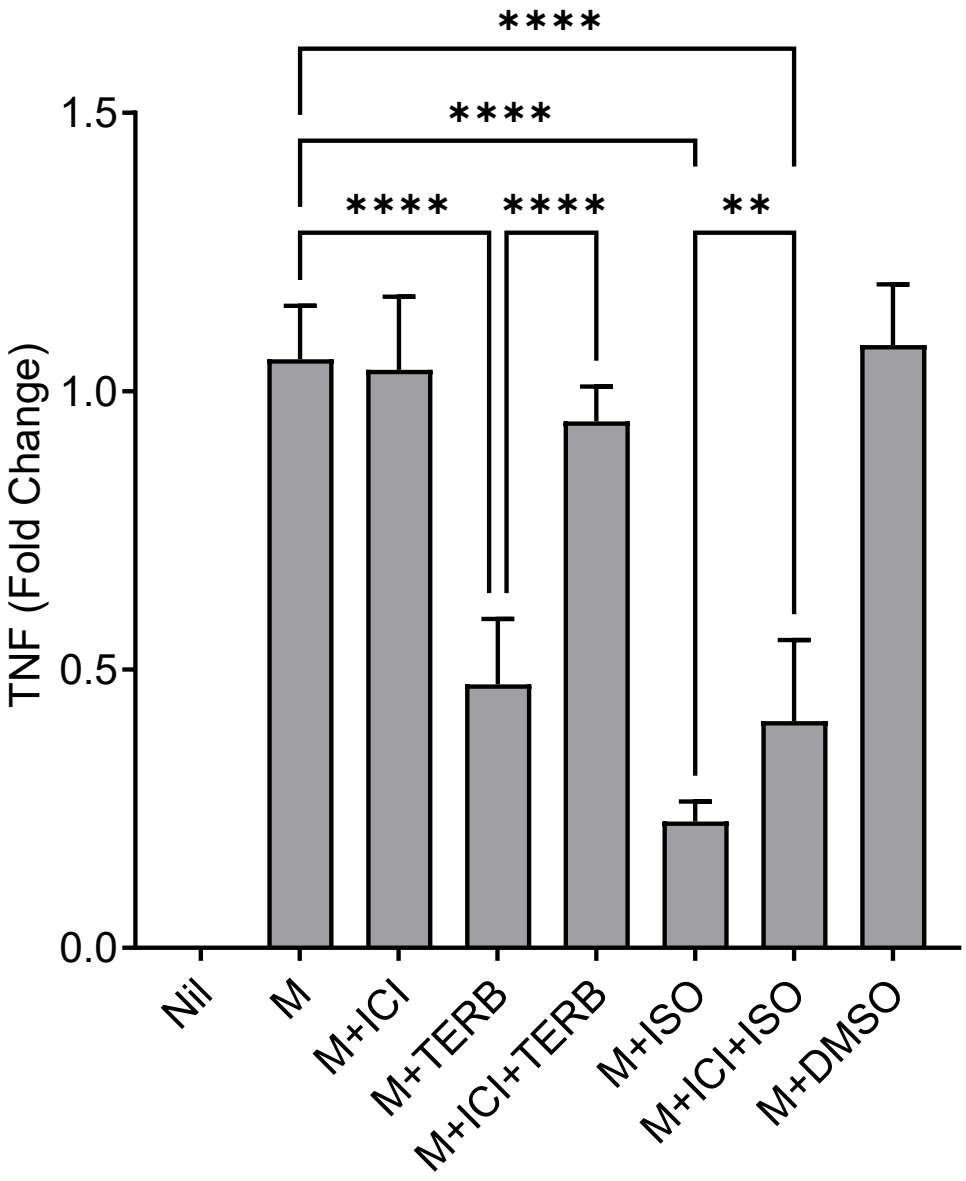


Figure 13. TNF cytokine production in M1-like cells without IFNγ treated with terbutaline, isoproterenol and/or ICI 118,551 at 4 hours. The fold change for each condition was normalized to the "M" condition. Nil: THP-1 cells and M: M1-like cells (1 ng/mL LPS). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and ICI: 10^{-7} M ICI 118,551. DMSO: 10^{-5} M DMSO solvent control. Data is compiled from three experiments.

3.8.2. Terbutaline and isoproterenol decreased TNF production in M1-like cells with IFNγ and terbutaline was blocked by ICI 118,551, but not isoproterenol.

TNF production decreased significantly ($P<0.0001$) in M1-like cells (with IFNγ) treated with terbutaline and isoproterenol. Similarly to M1-like cells without IFNγ, ICI 118,551 only reversed the effect of terbutaline and not isoproterenol. ICI 118,551 combined with terbutaline resulted in TNF levels that were not significantly different form the "Act" control, meaning that terbutaline was successfully blocked by ICI 118,551 (Figure 14). Notably, ICI 118,551 combined with isoproterenol did increase TNF levels significantly ($P\leq0.0002$) in comparison with isoproterenol alone. However, ICI 118,551 with isoproterenol still showed a significant ($P<0.0001$) decrease in TNF compared to the "Act" condition (Figure 14). Therefore, the effects of isoproterenol were not reversed by ICI 118,551, suggesting that isoproterenol is β2-AR independent. The DMSO vehicle control showed a significant ($P\leq0.0021$) increase in TNF. Although there is a change in TNF production in the compiled graph, it is important to note that two out of the three individual experiments showed no significant change (Figure S6). ICI 118,551 alone had no significant effect on TNF production compared to the "Act" condition (Figure 14).

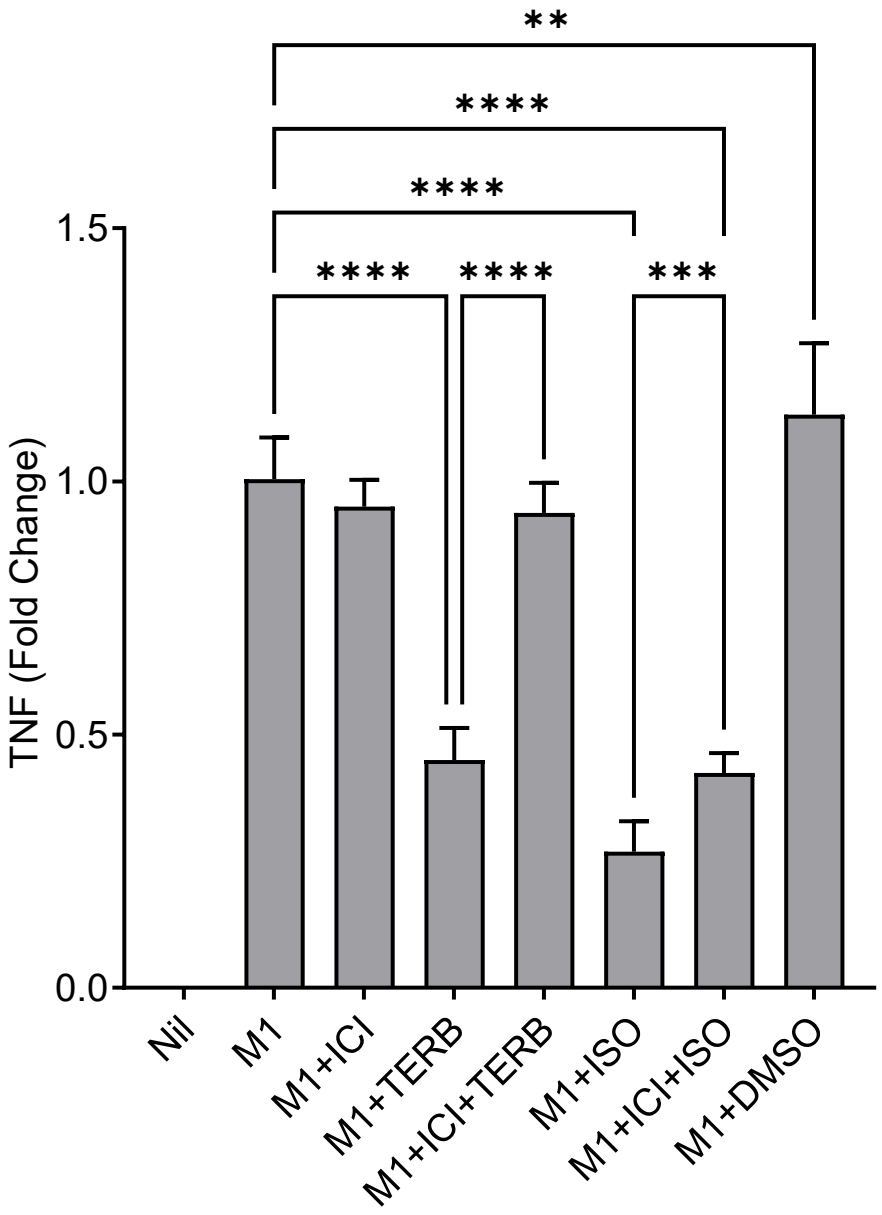


Figure 14. TNF cytokine production in M1-like cells with IFNγ treated with terbutaline, isoproterenol and/or ICI 118,551 at 4 hours. The fold change for each condition was normalized to the "M1" condition. Nil: THP-1 cells and M1: M1-like cells (1 ng/mL LPS + 20 ng/mL IFNγ). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and ICI: 10^{-7} M ICI 118,551. DMSO: 10^{-5} M DMSO solvent control. Data is compiled from three experiments.

3.8.3. Terbutaline and isoproterenol increased IL-10 production in M2-like cells and terbutaline was blocked by ICI 118,551, but not isoproterenol.

As what was previously seen, terbutaline and isoproterenol significantly ($P<0.0001$) increased the IL-10 production in M2-like cells. Similarly to the trends seen in M1-like cells (with and without IFNγ), ICI 118,551 reversed the effects of terbutaline but not isoproterenol (Figure 15). ICI 118,551 with terbutaline showed a significant ($P<0.0001$) decrease in IL-10 compared to terbutaline alone, and showed no significant difference compared to the "Act" condition. ICI 118,551 was therefore able to block the effects of terbutaline on IL-10 production. However, ICI 118,551 with isoproterenol only showed a slight decrease ($P\leq0.0332$) in IL-10 relative to isoproterenol alone, and still showed a significant ($P<0.0001$) increase compared to the "Act" condition (Figure 15). Therefore ICI 118,551 was not successful at blocking isoproterenol. No significant change was observed when M2-like cells were treated with ICI 118,551 alone and DMSO vehicle control (Figure 15).

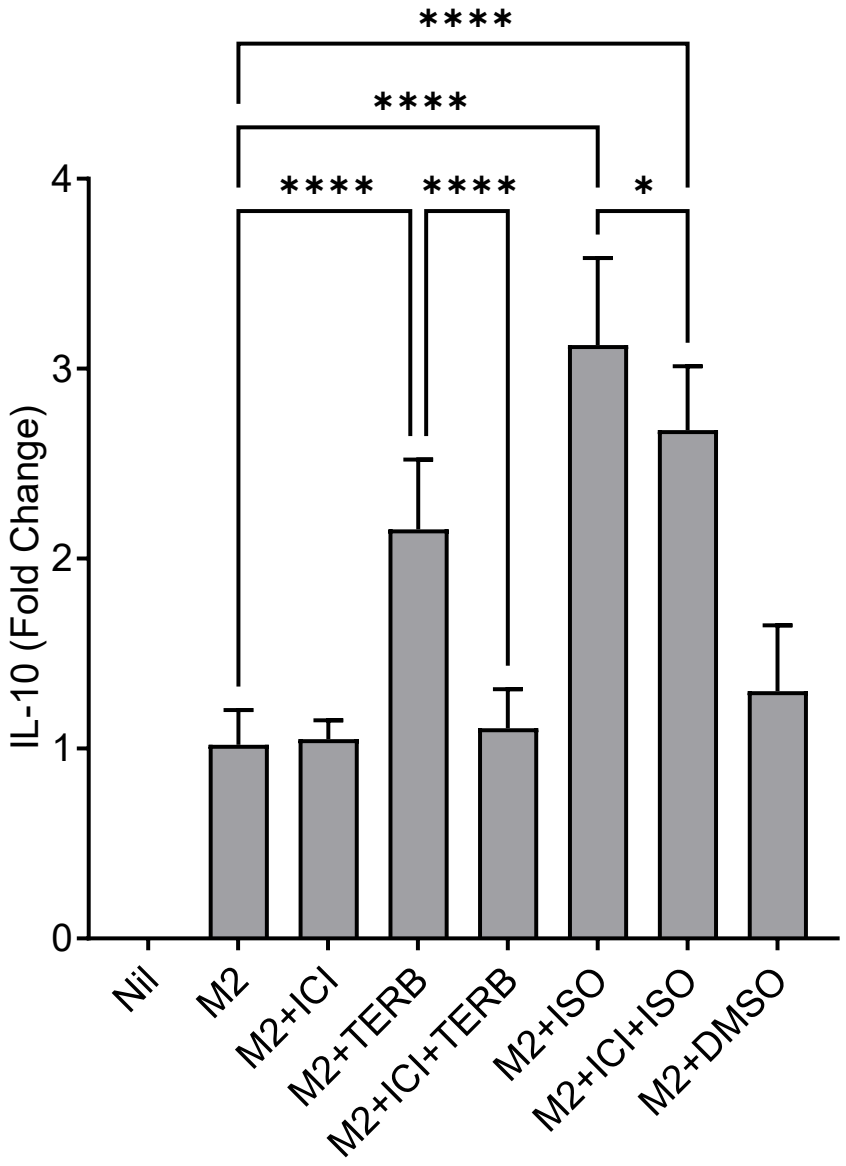


Figure 15. IL-10 cytokine production in M2-like cells treated with terbutaline, isoproterenol and/or ICI 118,551 at 24 hours. The fold change for each condition was normalized to the "M2" condition. Nil: THP-1 cells and M2: M2-like cells (1 ng/mL LPS + 20 ng/mL IL-4 + 20 ng/mL IL-13). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and ICI: 10^{-7} M ICI 118,551. DMSO: 10^{-5} M DMSO solvent control. Data is compiled from three experiments.

Chapter 4. Discussion

Macrophages play a crucial role in coordinating the adaptive immune response and regulating inflammatory balance. The M1 and M2 macrophage phenotypes each have their responsibilities for ensuring an effective response to rid the body of pathogens, while also protecting the body from any further damage. The M1-like macrophages are considered to be the pro-inflammatory phenotype since they secrete pro-inflammatory cytokines like TNF that amplify the immune response. Whereas the M2-like macrophages are anti-inflammatory in that they help resolve inflammation and promote tissue repair by secreting anti-inflammatory cytokines, such as IL-10. These phenotypes determine the body's ability to properly fight infection, therefore, any disruption in this balance can lead to autoimmune diseases, severe infections, and other inflammatory disorders. Understanding the modulation of this balance is essential for developing effective therapies to help treat these conditions according to the patient's needs.

In my thesis, I have explored the interactions between the SNS and the immune system by treating a monocyte-like cell line with β-AR agonist and antagonist drugs. Medications such as β2-AR drugs showed potential as therapeutics since they have displayed anti-inflammatory properties in macrophages in the past. (Kast, 2000; Szelenyi et al., 2000). However, there was still a gap in the literature bridging the immune system and the SNS and, furthermore, even less knowledge exploring β2-adrenergic receptors on modulation of macrophage phenotypes. Some studies on β-AR agonist drugs isoproterenol and terbutaline showed evidence of anti-inflammation (Ernst et al., 2019; Haskó et al., 1998; Gill et al., 2016; Keränen et al., 2016; Keränen et al., 2017), yet there is a lack in detailed research on their specific effects on M1 and M2 macrophages. By exploring the immunomodulatory effects of β-AR drugs on M1 and M2 macrophages, we can better understand how we can use these medications to treat a range of inflammatory and autoimmune conditions where an imbalance in macrophage activity contributes to disease progression.

The first aim for this project was to establish an *in vitro* model of M1- and M2-like macrophages from THP-1 cells to measure TNF and IL-10 production. This was accomplished by testing several LPS dosages and time-points inferred from the systemic review by Mohd Yasin et al. (2022). The review stated that the best protocol for differentiation of the THP-1 cells

into M0-like cells required treatment with 15.4 ng/mL of PMA for 48 hours followed by 24 hours rest without PMA, and therefore, this protocol was not changed. However, there was many conflicting protocols regarding the polarization of M0-like cells to M1- and M2-like cells; I therefore needed to establish an appropriate *in vitro* model for this project. To produce M1-like cells, I determined that 1 ng/mL of LPS (Figure 3) with a 4-hour treatment time (Figure 4) was adequate to produce high levels of TNF. LPS concentrations higher than 1 ng/mL produced unnecessarily high concentrations of TNF (Figure 3) and polarization of M0-like to M1-like cells (with and without IFNγ) was more robust at 4 hours versus 24 hours of treatment (Figure 4). Furthermore, IL-10 concentration was also measured to ensure that the M1-like cells were strictly M1-like. At the 4-hour time-point, there was no IL-10 production in M1-like cells, however, there was slight production at 24 hours (Figure 5). This further confirmed the choice for a 4-hour treatment. Notably, some protocols only required LPS to produce M1-like cells, but based on literature we know that IFNγ is also needed. Hence, I also decided to create M1-like cells using IFNγ and chose the most popular concentration of 20 ng/mL. (Mohd Yasin et al., 2022)

To produce M2-like cells, the 24-hour time-point was the most suitable for production of IL-10 (Figure 6) and, based on the systemic review, the most common concentrations used for IL-4 and IL-13 treatment was 20 ng/mL. (Mohd Yasin et al., 2022) However, in the review, other research groups were able to polarize M0-like cells to M2-like cells using only IL-4 and IL-13 cytokines, but I was not successful in doing this. Treatment of M0-like cells with 20 ng/mL of IL-4 and 20 ng/mL of IL-13 showed no significant production of IL-10 until 72 and 120 hours of treatment (Figure S1). I therefore added 1 ng/mL LPS to the treatment with 20 ng/mL of IL-4 and 20 ng/mL of IL-13 and IL-10 was produced at high levels at each time-point (Figure 6). IL-10 was produced at higher levels at 24 hours compared to 4 hours and did not increase until 120 hours (Figure 6). The 24-hour time-point was chosen since the 120-hour time-point would double the length of the experiment form start to finish and the IL-10 production was quite sufficient. Similar to the optimization of the M1-like protocol, TNF concentration was measured to ensure that the M2-like cells were strictly M2-like. In this case, LPS was added to the culture so it is expected that TNF will be produced. However, the 24-hour time-point showed less TNF production compared to 4 hours, and also less production compared to the M1-like control at 24 hours (Figure 7).

β-AR expression in THP-1, M0-like, M1-like (with and without IFNγ) and M2-like cells was measured to confirm its presence on the surface of monocyte and macrophage cells. For this project, the gene expression was normalized to the THP-1 cells since we are more interested in the gene expression of β-ARs on macrophage cells. The *ADRB1* and *ADRB2* gene was detected in all cells, however, *ADRB3* was not expressed in any. The lack of expression of the β3-AR in all monocyte-derived macrophage cells was puzzling since two studies detected its presence (Hadi et al., 2017; Zheng et al., 2020). This could be due to human error while performing the experiment (forgetting to add probe, adding the incorrect amount, etc.) or could have been caused by a faulty reagent (received incorrect probe, probe was denatured, expired reagent, etc.) Further exploration may be required to confirm the presence of this receptor in monocyte derived macrophages by using a positive control such as white and brown adipose tissue cells. M0-like cells showed no significant changes in gene expression of the *ADRB1* or *ADRB2* gene at 4 hours (Figure 8a & 9a) but showed a decrease at 24 hours (Figure 8b & 9b). The M0-like macrophage cells are considered the naïve non-effector macrophage cells that may be polarized towards the M1 or M2-like state, so plasticity in its receptor expression is to be expected. In M1-like cells without IFNγ, statistical analysis showed that there was no change in *ADRB1* and *ADRB2* gene expression, however, visually we can see that there is a slight trend of an increase in expression (Figure 8a & 9a). Whereas M1-like cells treated with IFNγ showed an increase in expression of the *ADRB1* and *ADRB2* gene (Figure 8a & 9a). This suggests that when M0 progenitors are polarized towards the M1 effector cells due to inflammation, the β1- and β2-ARs are upregulated on the surface of macrophage cells. In contrast, expression of the *ADRB1* and *ADRB2* gene in M2-like cells had decreased as compared to the non-activated THP-1 cells (Figure 8b & 9b). This implies that when inflammation is being resolved and M2 polarization is occurring, the β1- and β2-ARs are downregulated. The up- and downregulation of β-ARs depending on the state of inflammation suggests that the SNS is playing a role in regulating the immune response, and thus requires more exploration to understand this process.

The second aim of this project was to use the established *in vitro* model to measure the effects of the β-AR drugs on TNF and IL-10 secretion in M1- and M2-like macrophage cells. Isoproterenol is a non-selective β-AR agonist which will activate the β-AR signaling pathway upon binding. In M1-like cells (with and without IFNγ) isoproterenol decreased TNF production (Figure 10, 11, 13, & 14) and significantly increased IL-10 production in M2-like cells (Figure

12 & 15). This was to be expected since some studies have shown evidence of anti-inflammation properties regarding isoproterenol. (Ernst et al., 2019; Haskó et al., 1998) However, Ernst et al. (2019) studied only IL-10 expression in mouse macrophages and Haskó et al. (1998) had conflicting evidence of a decrease in IL-10 and TNF secretion. The effects of isoproterenol were reversed in both M1- and M2-like cells by the non-selective β-AR antagonist bupranolol (Figure 10-12), but not by the β2-specific antagonist ICI 118,551 (Figure 13-15), since TNF and IL-10 concentrations returned to base level. This is in agreeance with the literature since the antagonist will bind to the receptor and block the agonist from binding, preventing activation of the β-AR and downstream signalling. Bupranolol is non-selective and will therefore bind to both β1- and β2-ARs, preventing isoproterenol from binding to both receptors. In contrast, ICI 118,551 is only β2-selective and will not block the β1-ARs present on the surface of the M1- and M2-like cells, thus we only observe a partial reversal effect.

Terbutaline is the second β-AR agonist chosen for this project and is selective towards the β2-AR. It was of interest since there are no studies examining β-AR specificity in M1 and M2 phenotypes. Terbutaline showed a similar trend to isoproterenol in that TNF production was decreased in M1-like cells (with and without IFNγ) (Figure 10, 11, 13, & 14) and increased IL-10 production in M2-like cells (Figure 12 & 15). One study done on human lung macrophages (Gill et al., 2016) and two studies done on mouse macrophages (Keränen et al., 2016; Keränen et al., 2017) showed that terbutaline had an anti-inflammatory effect, therefore these results were to be expected. The effects of terbutaline were reversed in M1-like (with and without IFNγ) and M2-like cells by both β-AR antagonists (Figure 10-15) seeing that TNF and IL-10 levels returned to baseline. Because terbutaline is β2-selective, we expected that both bupranolol (non-selective) and ICI 118,551 (β2-selective) would reverse the effects since in both instances, the β2-AR is being targeted.

Based on the results collected from this project, it is evident that there is significant crosstalk between the immune system and SNS and this communication aids in avoiding chronic inflammation and helps regulate the immune response overall. Activation of the immune response will upregulate the β-ARs and activation of the SNS will cause an anti-inflammatory state by the β-ARs. In the M1-like state, specifically with IFNγ present, both the *ADRB1* and *ADRB2* genes are upregulated, and thus more receptors will be present on the surface during

inflammation. However, activating these receptors, as we have seen through the β-AR agonist drugs, will cause secretion of TNF to decrease, IL-10 to increase, and therefore exhibit an anti-inflammatory effect. The presence of IFNγ is key for polarization from M0-like to M1-like cells but also seems to prime the M1 cells for "deactivation" when the SNS is active by upregulating more β-ARs compared to M1-like cells without IFNγ. Once the β-AR agonists are present after SNS activation, the inflammatory state of the M1-like cells begins to reverse itself to return to homeostasis. This resembles negative regulatory looping in that first the presence of IFNγ and then β-AR agonists will negatively regulate the M1-like state and reduce inflammation. The M2-like cells exhibited a decrease in *ADRB1* and *ADRB2* gene expression, causing less receptors on the surface to be present during the resolution phase of inflammation. This seems logical since the M2-like state is already considered anti-inflammatory, therefore there is no need for an upregulation of β-ARs that, upon activation, will cause an anti-inflammatory response. However, there are still β-ARs present on the surface and as seen in the results, activating these receptors using an agonist resulted in further anti-inflammatory reactions by increasing IL-10 secretion.

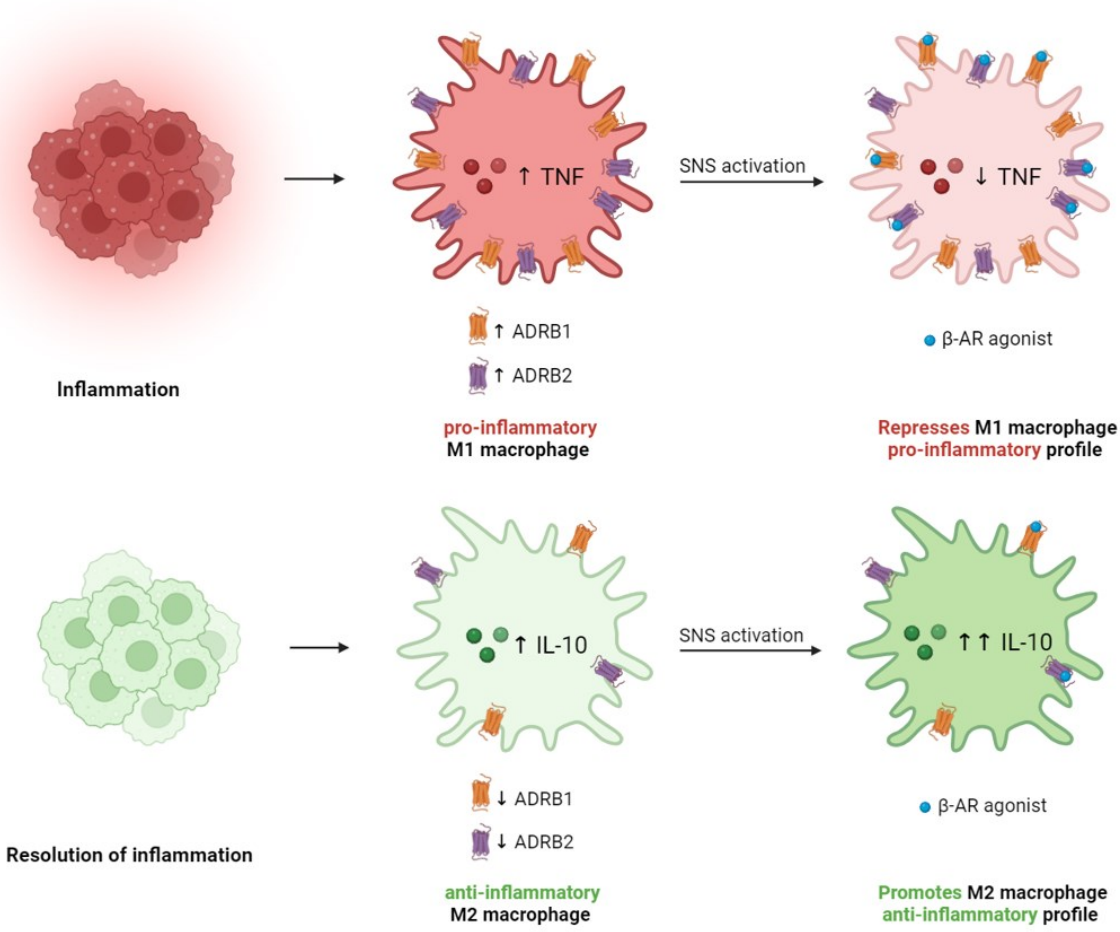
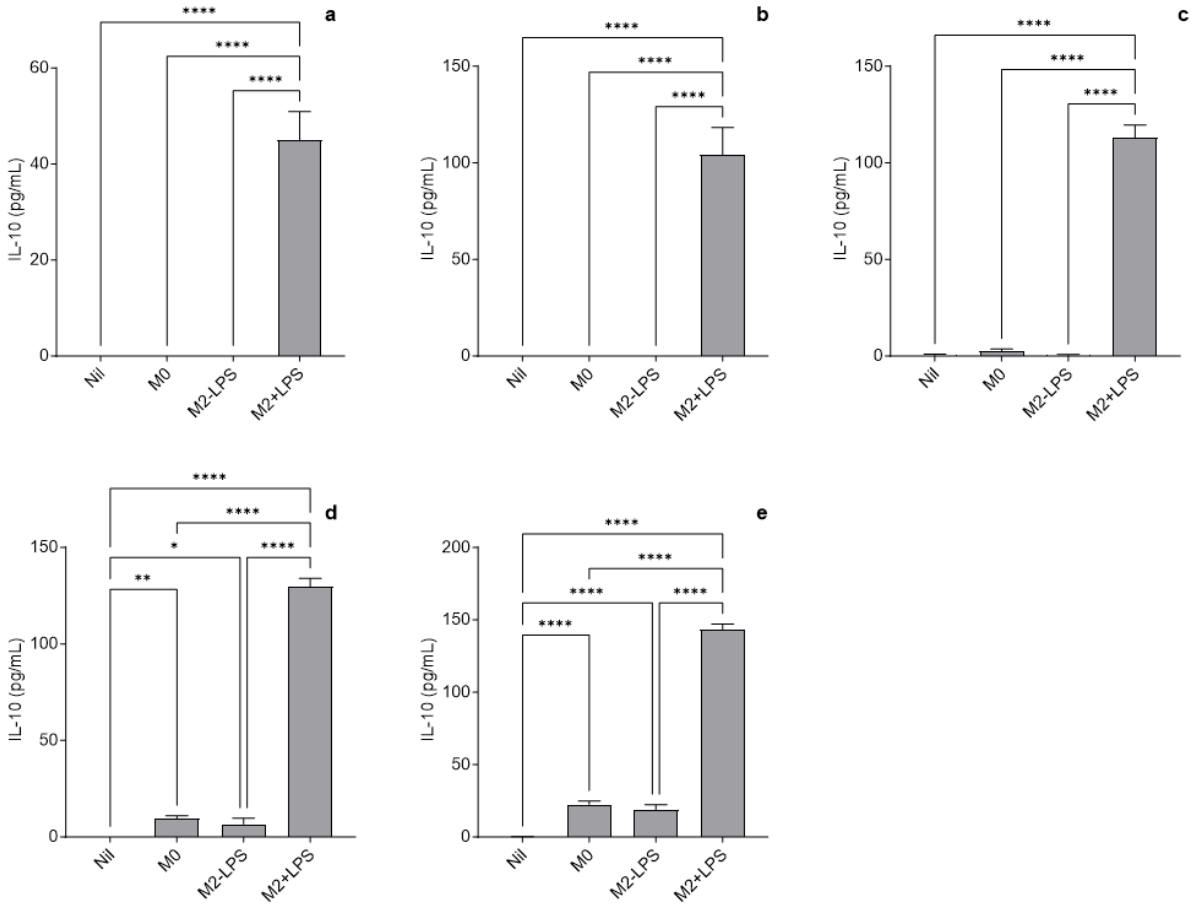


Figure 16. Schematic of M1/M2 phenotype and SNS crosstalk. Figure illustrates the influence of polarization to M1/M2 phenotypes on gene expression of β1- and β2-AR and effects of β-AR agonist on TNF and IL-10 cytokine secretion. Inflammation will cause an increase of β1- and β2-AR expression in M1-like cells, which primes them to be more susceptible to activation by a β-AR agonist after SNS activation. β-AR activation in M1-like cells causes a decrease in TNF and thus represses the M1 pro-inflammatory state. M2-like cells have lower expression of the β1- and β2-ARs, however, they are still present. Activation of the SNS and, thus, the β-ARs causes an increased IL-10 secretion and promotes the M2 anti-inflammatory state. Figure was created using BioRender.

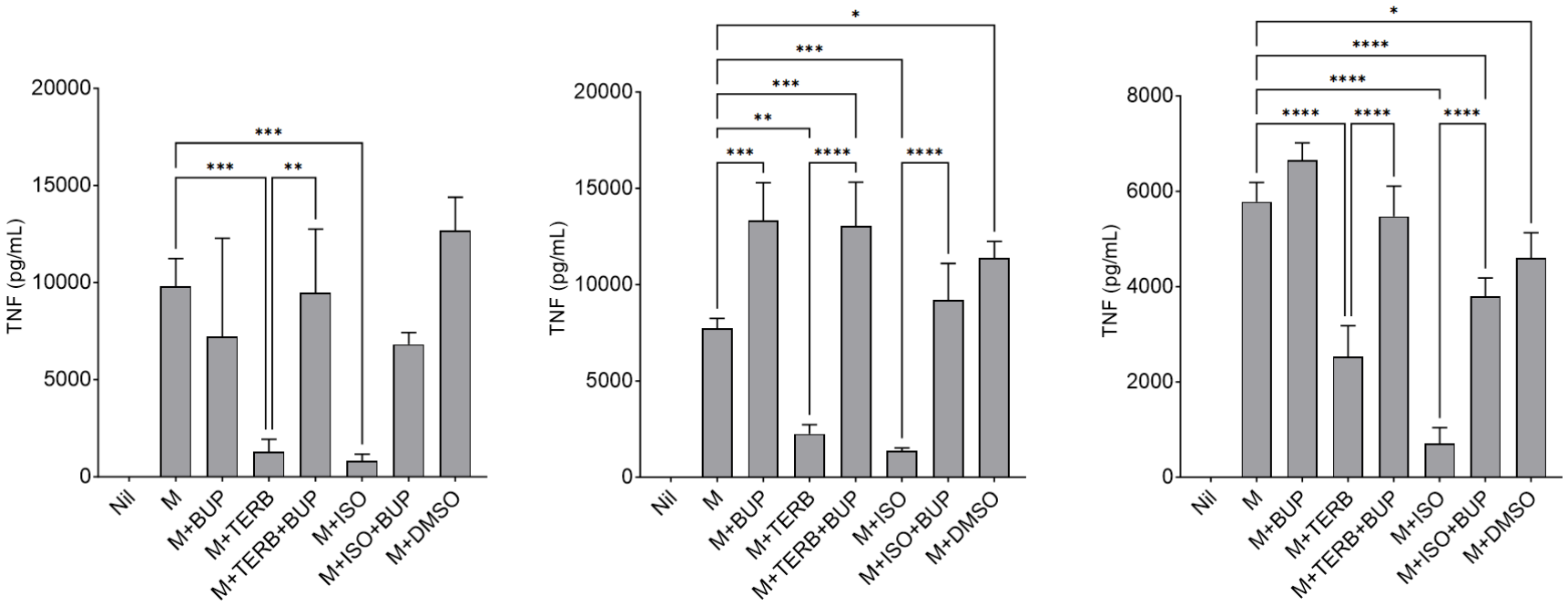
Some limitations of this project include the use of the THP-1 cells, measuring the cytokine profile only through ELISA experiments to confirm the M1/M2 phenotypes, and confirming the presence of the β-ARs with only gene expression. Although the use of cancer cell lines for exploratory and fundamental immunology research is well-established, it is always best to also examine these effects in human macrophages isolated from peripheral blood in healthy and diseased participants. This would give a clearer depiction of the immunomodulatory effects of the β-AR drugs in humans. Furthermore, the M1-like and M2-like macrophage phenotypes were determined by their cytokine profile, examining only the TNF and IL-10 cytokines by ELISA. However, as mentioned previously, the M1 and M2 phenotypes secrete more types of cytokines and have distinct surface receptors that classify them into their respective phenotype. A more accurate method to determine if the M1 and M2 phenotypes were truly replicated *in vitro* would be to use flow cytometry for measuring the presence of the respective surface receptors and perform a more extensive measurement of their cytokine profiles. Furthermore, flow cytometry could be used to measure the intracellular cytokine concentrations upon treatment with the β-AR drugs, as opposed to measuring only the secreted levels with ELISA. Lastly, the presence of the β-ARs were only detected through gene expression, but this excludes the receptors that are already expressed and present on the surface of the cell. A western blot to measure the concentration of receptor in M1 and M2-like cells would complete the picture and may shed light on the lack of β3-AR gene expression.

In summary, this project explored the immunomodulatory effects of β-AR drugs on THP-1-derived M1-like and M2-like macrophage phenotypes and demonstrated that 1) β-AR expression varies with macrophage polarization states and 2) β-adrenergic signalling by the SNS and subsequent activation of β-ARs promotes an M2 anti-inflammatory profile and supresses the M1 pro-inflammatory profile. The novelty of this project lies in the fact that we are the first to 1) explore these effects in THP-1 derived macrophage cells that are 2) polarized into M1 and M2 phenotypes, 3) measure β-adrenergic receptor expression in THP-1 derived M1 and M2 cells, and 4) test β-adrenergic receptor specificity. Altogether, this research brings us one step closer to understanding the crosstalk between the SNS and the immune system, providing opportunities to explore potential therapies for inflammatory and autoimmune conditions.

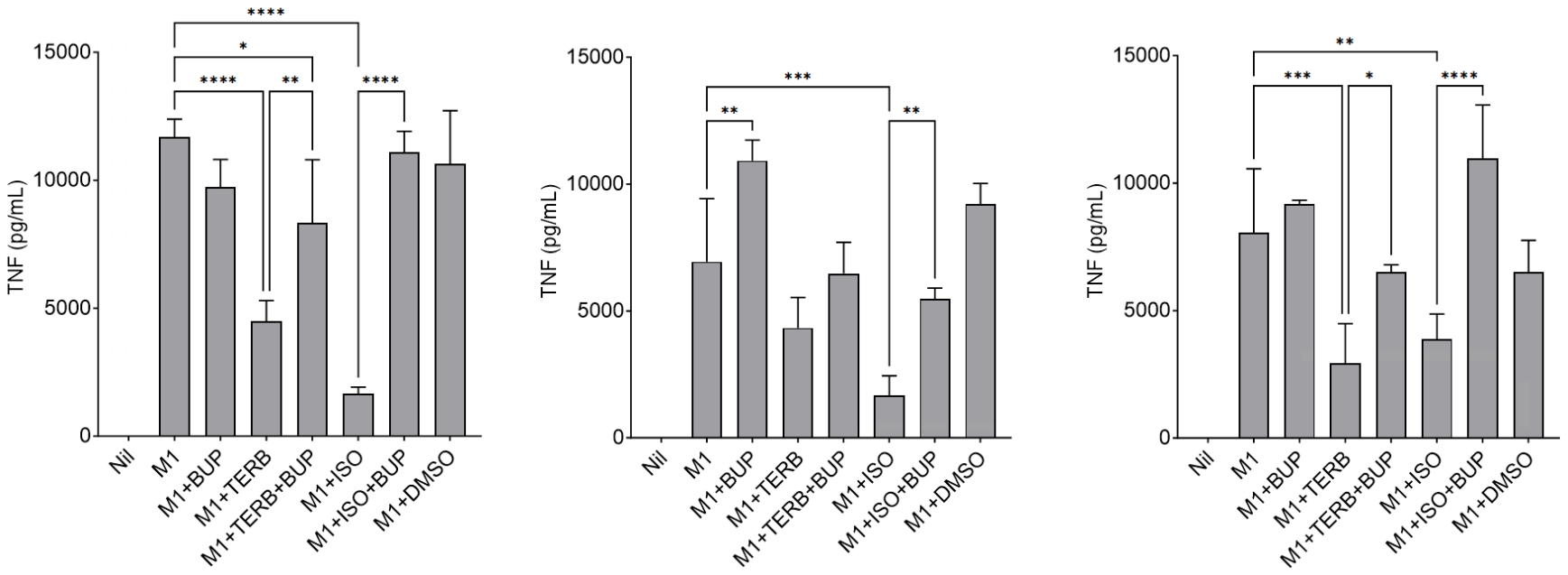
Supplementary Figures



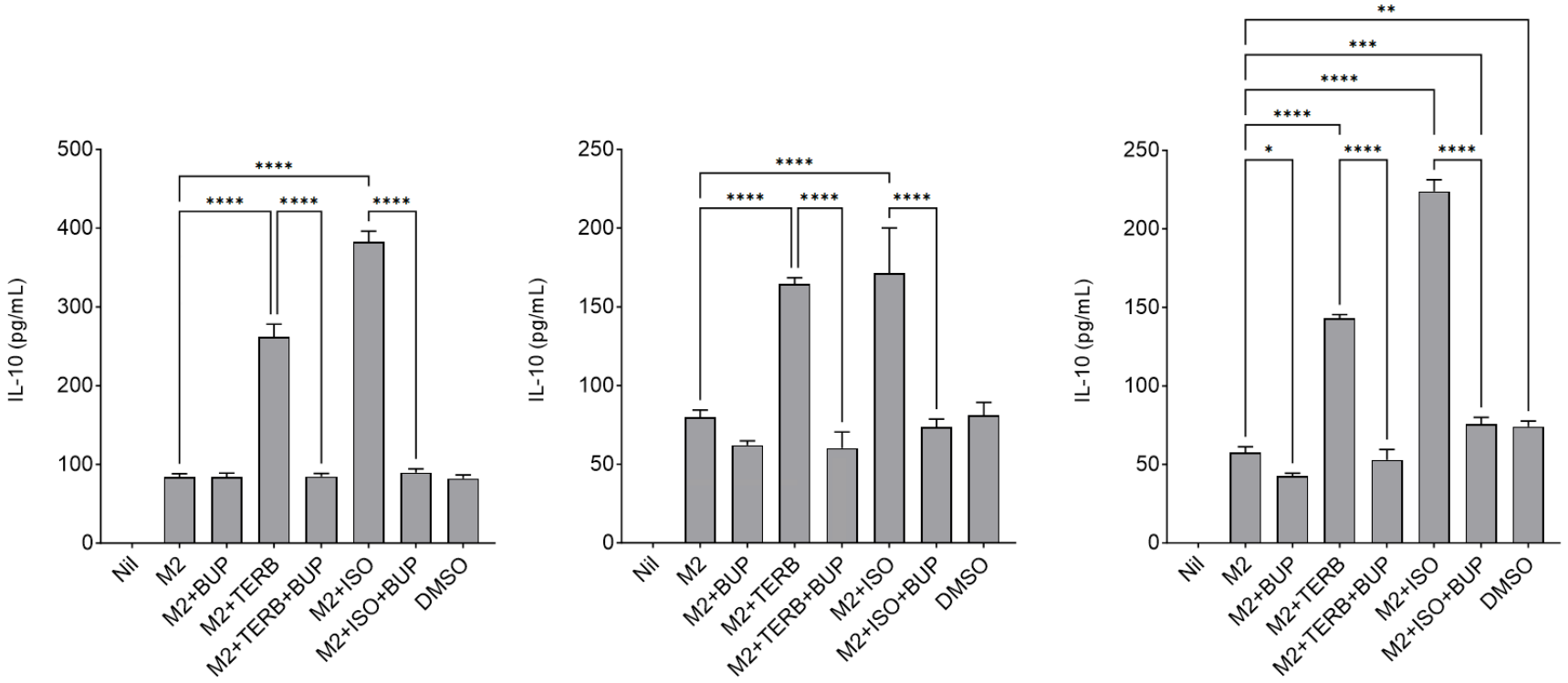
Supplementary Figure S1. IL-10 cytokine production in M2-like cells with controls at (a) 4-, (b) 24-, (c) 48-, (d) 72-, and (e) 120-hour time-points. IL-10 concentration was measured in pg/mL (y-axis) for each condition. Nil: THP-1 cells; M0: M0-like cells (15.4 ng/mL PMA); M2-LPS: M2-like cells (20 ng/mL IL-4 + 20 ng/mL IL-13); and M2+LPS: M2-like cells (1 ng/mL LPS + 20 ng/mL IL-4 + 20 ng/mL IL-13).



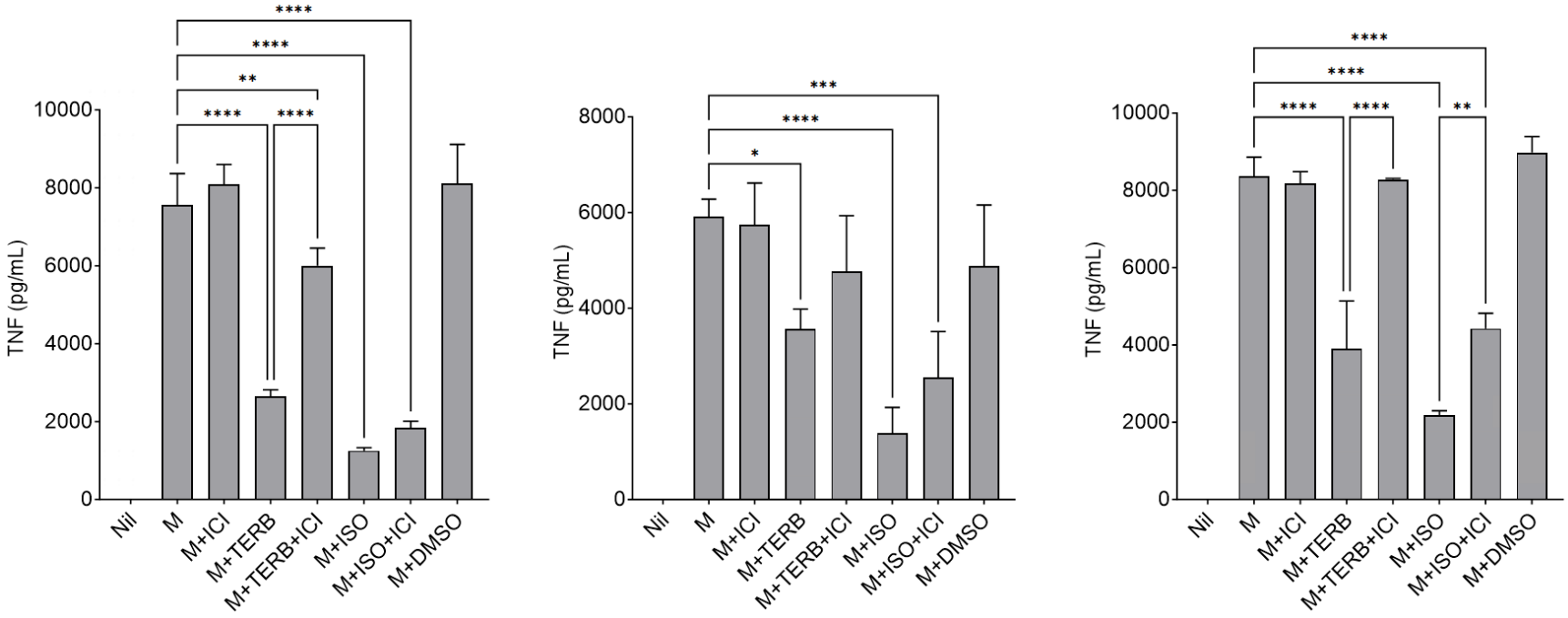
Supplementary Figure S2. Individual experiments showing TNF cytokine production in M1-like cells without IFNγ treated with bupranolol, terbutaline, or isoproterenol at 4 hours. TNF concentration was measured in pg/mL (y-axis) for each condition. Nil: THP-1 cells and M: M1-like cells (1 ng/mL LPS). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and BUP: 10^{-5} M bupranolol. DMSO: 10^{-5} M DMSO solvent control.



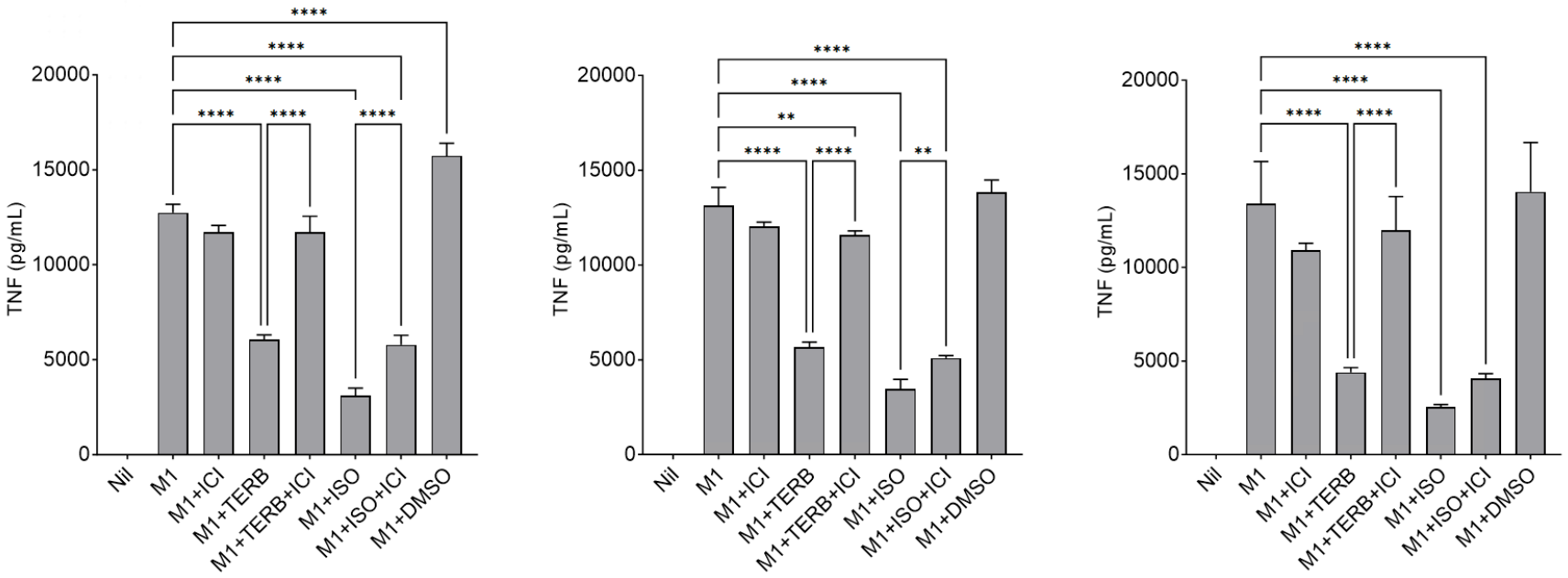
Supplementary Figure S3. Individual experiments showing TNF cytokine production in M1-like cells with IFNγ treated with bupranolol, terbutaline, or isoproterenol at 4 hours. TNF concentration was measured in pg/mL (y-axis) for each condition. Nil: THP-1 cells and M1: M1-like cells (1 ng/mL LPS + 20 ng/mL IFNγ). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and BUP: 10^{-5} M bupranolol. DMSO: 10^{-5} M DMSO solvent control.



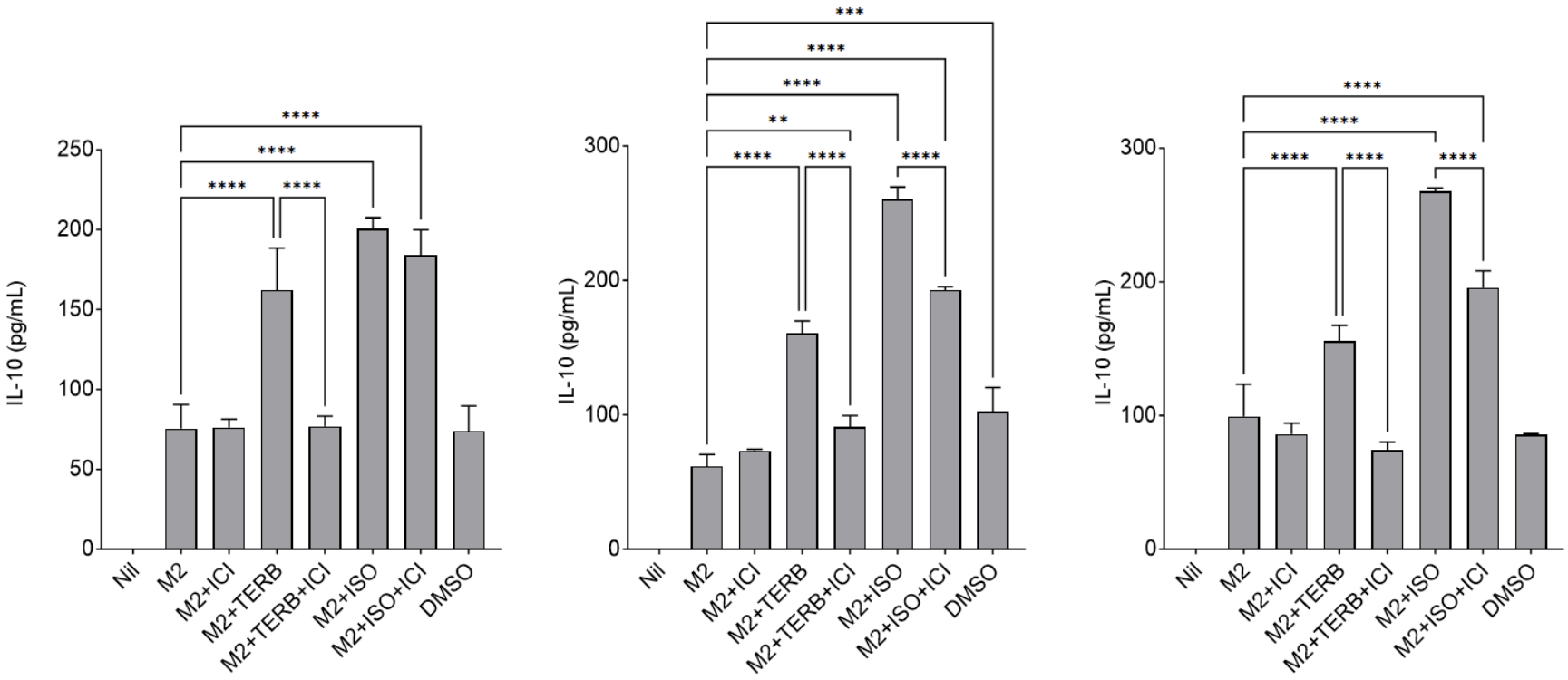
Supplementary Figure S4. Individual experiments showing IL-10 cytokine production in M2-like cells treated with bupranolol, terbutaline, or isoproterenol at 24 hours. IL-10 concentration was measured in pg/mL (y-axis) for each condition. Nil: THP-1 cells and M2: M2-like cells (1 ng/mL LPS + 20 ng/mL IL-4 + 20 ng/mL IL-13). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and BUP: 10^{-5} M bupranolol. DMSO: 10^{-5} M DMSO solvent control.



Supplementary Figure S5. Individual experiments showing TNF cytokine production in M1-like cells without IFNγ treated with ICI 118,551, terbutaline, or isoproterenol at 4 hours. TNF concentration was measured in pg/mL (y-axis) for each condition. Nil: THP-1 cells and M: M1-like cells (1 ng/mL LPS). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and ICI: 10^{-7} M ICI 118,551. DMSO: 10^{-5} M DMSO solvent control.



Supplementary Figure S6. Individual experiments showing TNF cytokine production in M1-like cells with IFNγ treated with ICI 118,551, terbutaline, or isoproterenol at 4 hours. TNF concentration was measured in pg/mL (y-axis) for each condition. Nil: THP-1 cells and M1: M1-like cells (1 ng/mL LPS + 20 ng/mL IFNγ). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and ICI: 10^{-7} M ICI 118,551. DMSO: 10^{-5} M DMSO solvent control.



Supplementary Figure S7. Individual experiments showing IL-10 cytokine production in M2-like cells treated with ICI 118,551, terbutaline, or isoproterenol at 24 hours. IL-10 concentration was measured in pg/mL (y-axis) for each condition. Nil: THP-1 cells and M2: M2-like cells (1 ng/mL LPS + 20 ng/mL IL-4 + 20 ng/mL IL-13). TERB: 10^{-5} M terbutaline, ISO: 10^{-5} M isoproterenol, and ICI: 10^{-7} M ICI 118,551. DMSO: 10^{-5} M DMSO solvent control.

References

Aggarwal, B. B., Gupta, S. C., & Kim, J. H. (2012). Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. *Blood*, *119*(3), 651–665. https://doi.org/10.1182/blood-2011-04-325225

Berg, K. A., & Clarke, W. P. (2018). Making Sense of Pharmacology: Inverse Agonism and Functional Selectivity. *The international journal of neuropsychopharmacology*, *21*(10), 962–977. https://doi.org/10.1093/ijnp/pyy071

Bos, J. L. (2003). Epac: a new cAMP target and new avenues in cAMP research. *Nature reviews Molecular cell biology*, *4*(9), 733-738.

Bosshart, H., & Heinzelmann, M. (2016). THP-1 cells as a model for human monocytes. *Annals of translational medicine*, *4*(21), 438. https://doi.org/10.21037/atm.2016.08.53

Bosurgi, L., Cao, Y. G., Cabeza-Cabrerizo, M., Tucci, A., Hughes, L. D., Kong, Y., Weinstein, J. S., Licona-Limon, P., Schmid, E. T., Pelorosso, F., Gagliani, N., Craft, J. E., Flavell, R. A., Ghosh, S., & Rothlin, C. V. (2017). Macrophage function in tissue repair and remodeling requires IL-4 or IL-13 with apoptotic cells. *Science (New York, N.Y.)*, *356*(6342), 1072–1076. https://doi.org/10.1126/science.aai8132

Chaintreuil, P., Kerreneur, E., Bourgoin, M., Savy, C., Favreau, C., Robert, G., Jacquel, A., & Auberger, P. (2023). The generation, activation, and polarization of monocyte-derived macrophages in human malignancies. *Frontiers in immunology*, *14*, 1178337. https://doi.org/10.3389/fimmu.2023.1178337

Chen, T., Huang, W., Qian, J., Luo, W., Shan, P., Cai, Y., Lin, K., Wu, G., & Liang, G. (2020). Macrophage-derived myeloid differentiation protein 2 plays an essential role in ox-LDL-induced inflammation and atherosclerosis. *EBioMedicine*, *53*, 102706. https://doi.org/10.1016/j.ebiom.2020.102706

De Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S., Wittinghofer, A., & Bos, J. L. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*, *396*(6710), 474-477.

Eichel, K., Jullié, D., & von Zastrow, M. (2016). β-Arrestin drives MAP kinase signalling from clathrin-coated structures after GPCR dissociation. *Nature cell biology*, *18*(3), 303–310. https://doi.org/10.1038/ncb3307

Ernst, O., Glucksam-Galnoy, Y., Bhatta, B., Athamna, M., Ben-Dror, I., Glick, Y., Gerber, D., & Zor, T. (2019). Exclusive Temporal Stimulation of IL-10 Expression in LPS-Stimulated Mouse Macrophages by cAMP Inducers and Type I Interferons. *Frontiers in immunology*, *10*, 1788. https://doi.org/10.3389/fimmu.2019.01788

Espinoza VE, Emmady PD. Histology, Monocytes. [Updated 2023 Apr 24]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www-ncbi-nlm-nih-gov.lib-ezproxy.concordia.ca/books/NBK557618/

Farhana A, Khan YS. Biochemistry, Lipopolysaccharide. [Updated 2022 Apr 21]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK554414/?report=classic

Felten, D. L., Felten, S. Y., Carlson, S. L., Olschowka, J. A., & Livnat, S. (1985). Noradrenergic and peptidergic innervation of lymphoid tissue. *Journal of immunology (Baltimore, Md. : 1950)*, *135*(2 Suppl), 755s–765s.

Ferguson, S. S., Downey, W. E., 3rd, Colapietro, A. M., Barak, L. S., Ménard, L., & Caron, M. G. (1996). Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science (New York, N.Y.)*, *271*(5247), 363–366. https://doi.org/10.1126/science.271.5247.363

Gadani, S. P., Cronk, J. C., Norris, G. T., & Kipnis, J. (2012). IL-4 in the brain: a cytokine to remember. *Journal of immunology (Baltimore, Md. : 1950)*, *189*(9), 4213–4219. https://doi.org/10.4049/jimmunol.1202246

Gill, S. K., Marriott, H. M., Suvarna, S. K., & Peachell, P. T. (2016). Evaluation of the anti-inflammatory effects of β-adrenoceptor agonists on human lung macrophages. *European journal of pharmacology*, *793*, 49–55. https://doi.org/10.1016/j.ejphar.2016.11.005

Hadi, T., Douhard, R., Dias, A. M. M., Wendremaire, M., Pezzè, M., Bardou, M., Sagot, P., Garrido, C., & Lirussi, F. (2017). Beta3 adrenergic receptor stimulation in human macrophages inhibits NADPHoxidase activity and induces catalase expression via PPARγ activation. *Biochimica et biophysica acta. Molecular cell research*, *1864*(10), 1769–1784. https://doi.org/10.1016/j.bbamcr.2017.07.003

Haskó, G., Németh, Z. H., Szabó, C., Zsilla, G., Salzman, A. L., & Vizi, E. S. (1998). Isoproterenol inhibits Il-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages. *Brain research bulletin*, *45*(2), 183–187. https://doi.org/10.1016/s0361-9230(97)00337-7

Hu, X., Chakravarty, S. D., & Ivashkiv, L. B. (2008). Regulation of interferon and Toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. *Immunological reviews*, *226*, 41–56. https://doi.org/10.1111/j.1600-065X.2008.00707.x

Ippolito, M., & Benovic, J. L. (2021). Biased agonism at β-adrenergic receptors. *Cellular signalling*, *80*, 109905. https://doi.org/10.1016/j.cellsig.2020.109905

Kast R. E. (2000). Tumor necrosis factor has positive and negative self regulatory feed back cycles centered around cAMP. *International journal of immunopharmacology*, *22*(11), 1001–1006. https://doi.org/10.1016/s0192-0561(00)00046-1

Kawamoto, H., & Minato, N. (2004). Myeloid cells. *The international journal of biochemistry & cell biology*, *36*(8), 1374–1379. https://doi.org/10.1016/j.biocel.2004.01.020

Keränen, T., Hömmö, T., Hämäläinen, M., Moilanen, E., & Korhonen, R. (2016). Anti-Inflammatory Effects of β2-Receptor Agonists Salbutamol and Terbutaline Are Mediated by MKP-1. *PloS one*, *11*(2), e0148144. https://doi.org/10.1371/journal.pone.0148144

Keränen, T., Hömmö, T., Moilanen, E., & Korhonen, R. (2017). β_2-receptor agonists salbutamol and terbutaline attenuated cytokine production by suppressing ERK pathway through cAMP in macrophages. *Cytokine*, *94*, 1–7. https://doi.org/10.1016/j.cyto.2016.07.016

Kobilka B. K. (2011). Structural insights into adrenergic receptor function and pharmacology. *Trends in pharmacological sciences*, *32*(4), 213–218. https://doi.org/10.1016/j.tips.2011.02.005

Kohm, A. P., Tang2, Y., Sanders, V. M., & Jones3, S. B. (2000). Activation of antigen-specific CD4+ Th2 cells and B cells in vivo increases norepinephrine release in the spleen and bone marrow. *Journal of immunology (Baltimore, Md. : 1950)*, *165*(2), 725–733. https://doi.org/10.4049/jimmunol.165.2.725

Kolmus, K., Tavernier, J., & Gerlo, S. (2015). β2-Adrenergic receptors in immunity and inflammation: stressing NF-κB. *Brain, behavior, and immunity*, *45*, 297–310. https://doi.org/10.1016/j.bbi.2014.10.007

Lendeckel, U., Venz, S., & Wolke, C. (2022). Macrophages: shapes and functions. *Chemtexts*, *8*(2), 12. https://doi-org.lib-ezproxy.concordia.ca/10.1007/s40828-022-00163-4

Maestroni G. J. (2000). Dendritic cell migration controlled by alpha 1b-adrenergic receptors. *Journal of immunology (Baltimore, Md. : 1950)*, *165*(12), 6743–6747. https://doi.org/10.4049/jimmunol.165.12.6743

Mayr, B., & Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nature reviews Molecular cell biology*, *2*(8), 599-609.

Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J., & Hill, A. M. (2000). M-1/M-2 macrophages and the Th1/Th2 paradigm. *Journal of immunology (Baltimore, Md. : 1950)*, *164*(12), 6166–6173. https://doi.org/10.4049/jimmunol.164.12.6166

Mohd Yasin, Z. N., Mohd Idrus, F. N., Hoe, C. H., & Yvonne-Tee, G. B. (2022). Macrophage polarization in THP-1 cell line and primary monocytes: A systematic review. *Differentiation; research in biological diversity*, *128*, 67–82. https://doi.org/10.1016/j.diff.2022.10.001

Motiejunaite, J., Amar, L., & Vidal-Petiot, E. (2021). Adrenergic receptors and cardiovascular effects of catecholamines. *Annales d'endocrinologie*, *82*(3-4), 193–197. https://doi.org/10.1016/j.ando.2020.03.012

Motulsky, H. J., Cunningham, E. M., DeBlasi, A., & Insel, P. A. (1986). Desensitization and redistribution of beta-adrenergic receptors on human mononuclear leukocytes. *The American journal of physiology*, *250*(5 Pt 1), E583–E590. https://doi.org/10.1152/ajpendo.1986.250.5.E583

Mussbacher, M., Derler, M., Basílio, J., & Schmid, J. A. (2023). NF-κB in monocytes and macrophages - an inflammatory master regulator in multitalented immune cells. *Frontiers in immunology*, *14*, 1134661. https://doi.org/10.3389/fimmu.2023.1134661

National Center for Biotechnology Information (2024). PubChem Compound Summary for CID 2475, Bupranolol. Retrieved June 5, 2024 from https://pubchem.ncbi.nlm.nih.gov/compound/Bupranolol.

National Center for Biotechnology Information (2024). PubChem Compound Summary for CID 5403, Terbutaline. Retrieved June 5, 2024 from https://pubchem.ncbi.nlm.nih.gov/compound/Terbutaline

Newton, K., & Dixit, V. M. (2012). Signaling in innate immunity and inflammation. *Cold Spring Harbor perspectives in biology*, *4*(3), a006049. https://doi.org/10.1101/cshperspect.a006049

Nobles, K. N., Xiao, K., Ahn, S., Shukla, A. K., Lam, C. M., Rajagopal, S., Strachan, R. T., Huang, T. Y., Bressler, E. A., Hara, M. R., Shenoy, S. K., Gygi, S. P., & Lefkowitz, R. J. (2011). Distinct phosphorylation sites on the β(2)-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Science signaling*, *4*(185), ra51. https://doi.org/10.1126/scisignal.2001707

O'Donnell, S. R., & Wanstall, J. C. (1980). Evidence that ICI 118, 551 is a potent, highly Beta 2-selective adrenoceptor antagonist and can be used to characterize Beta-adrenoceptor populations in tissues. *Life sciences*, *27*(8), 671–677. https://doi.org/10.1016/0024-3205(80)90008-9

Ouyang, W., Rutz, S., Crellin, N. K., Valdez, P. A., & Hymowitz, S. G. (2011). Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annual review of immunology*, *29*, 71–109. https://doi.org/10.1146/annurev-immunol-031210-101312

Peschon, J. J., Torrance, D. S., Stocking, K. L., Glaccum, M. B., Otten, C., Willis, C. R., Charrier, K., Morrissey, P. J., Ware, C. B., & Mohler, K. M. (1998). TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *Journal of immunology (Baltimore, Md. : 1950)*, *160*(2), 943–952.

Punt, Owen, J. A., Stranford, S. A., Jones, P. P., & Kuby, J. (2019). Kuby immunology (Eighth edition.). W.H. Freeman/Macmillan Learning.

Ramesh, G., MacLean, A. G., & Philipp, M. T. (2013). Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain. *Mediators of inflammation*, *2013*, 480739. https://doi.org/10.1155/2013/480739

Schwende, H., Fitzke, E., Ambs, P., & Dieter, P. (1996). Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *Journal of leukocyte biology*, *59*(4), 555–561.

Scott, T.E., Lewis, C.V., Zhu, M. *et al.* (2023). IL-4 and IL-13 induce equivalent expression of traditional M2 markers and modulation of reactive oxygen species in human macrophages. *Sci Rep* 13, 19589. https://doi.org/10.1038/s41598-023-46237-2

Shapouri-Moghaddam, A., Mohammadian, S., Vazini, H., Taghadosi, M., Esmaeili, S. A., Mardani, F., Seifi, B., Mohammadi, A., Afshari, J. T., & Sahebkar, A. (2018). Macrophage plasticity, polarization, and function in health and disease. *Journal of cellular physiology*, *233*(9), 6425–6440. https://doi.org/10.1002/jcp.26429

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., & Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *The Journal of experimental medicine*, *189*(11), 1777-1782.

Speidl, W. S., Toller, W. G., Kaun, C., Weiss, T. W., Pfaffenberger, S., Kastl, S. P., Furnkranz, A., Maurer, G., Huber, K., Metzler, H., & Wojta, J. (2004). Catecholamines potentiate LPS-induced expression of MMP-1 and MMP-9 in human monocytes and in the human monocytic cell line U937: possible implications for peri-operative plaque instability. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *18*(3), 603–605. https://doi.org/10.1096/fj.03-0454fje

Szelenyi, J., Kiss, J. P., Puskas, E., Szelenyi, M., & Vizi, E. S. (2000). Contribution of Differently Localized α2-and β-Adrenoceptors in the Modulation of TNF-α and IL-10 Production in Endotoxemic Mice. *Annals of the New York Academy of Sciences*, *917*(1), 145-153.

Szymanski MW, Singh DP. Isoproterenol. [Updated 2023 May 1]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www-ncbi-nlm-nih-gov.lib-ezproxy.concordia.ca/books/NBK526042/

Talmadge, J., Scott, R., Castelli, P., Newman-Tarr, T., & Lee, J. (1993). Molecular pharmacology of the beta-adrenergic receptor on THP-1 cells. *International journal of immunopharmacology*, *15*(2), 219–228. https://doi.org/10.1016/0192-0561(93)90098-j

Tobias, P. S., Soldau, K. A. T. R. I. N., & Ulevitch, R. J. (1986). Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *The Journal of experimental medicine*, *164*(3), 777-793.

Thomas, G., Tacke, R., Hedrick, C. C., & Hanna, R. N. (2015). Nonclassical patrolling monocyte function in the vasculature. *Arteriosclerosis, thrombosis, and vascular biology*, *35*(6), 1306–1316. https://doi.org/10.1161/ATVBAHA.114.304650

Walsh, D. A., Perkins, J. P., & Krebs, E. G. (1968). An adenosine 3′, 5′-monophosphate-dependant protein kinase from rabbit skeletal muscle. *Journal of Biological Chemistry*, *243*(13), 3763-3765.

Wong, K. L., Tai, J. J., Wong, W. C., Han, H., Sem, X., Yeap, W. H., Kourilsky, P., & Wong, S. C. (2011). Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood*, *118*(5), e16–e31. https://doi.org/10.1182/blood-2010-12-326355

Wright, S. D., Tobias, P. S., Ulevitch, R. J., & Ramos, R. A. (1989). Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. *The Journal of experimental medicine*, *170*(4), 1231-1241.

Yáñez, A., Coetzee, S. G., Olsson, A., Muench, D. E., Berman, B. P., Hazelett, D. J., Salomonis, N., Grimes, H. L., & Goodridge, H. S. (2017). Granulocyte-Monocyte Progenitors and Monocyte-Dendritic Cell Progenitors Independently Produce Functionally Distinct Monocytes. *Immunity*, *47*(5), 890–902.e4. https://doi.org/10.1016/j.immuni.2017.10.021

Zheng, M., Zhang, S. J., Zhu, W. Z., Ziman, B., Kobilka, B. K., & Xiao, R. P. (2000). β2-adrenergic receptor-induced p38 MAPK activation is mediated by protein kinase A rather than by Gi or Gβγ in adult mouse cardiomyocytes. *Journal of Biological Chemistry*, *275*(51), 40635-40640.

Zheng, M., Zhou, Z., Tian, X., Xiao, D., Hou, X., Xie, Z., Liang, H., & Lin, S. (2020). ADRB3 expression in tumor cells is a poor prognostic factor and promotes proliferation in non-small cell lung carcinoma. *Cancer* immunology, immunotherapy: *CII, 69*(11), 2345–2355. https://doi.org/10.1007/s00262-020-02627-3

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D. N., Leenen, P. J., Liu, Y. J., MacPherson, G., Randolph, G. J., Scherberich, J., Schmitz, J., Shortman, K., Sozzani, S., Strobl, H., Zembala, M., Austyn, J. M., & Lutz, M. B. (2010). Nomenclature of monocytes and dendritic cells in blood. *Blood*, *116*(16), e74–e80.